



REQUEST FOR INTRATHECAL DELIVERY OF HPBCD FOR NIEMANN PICK TYPE C PATIENTS

ADDISON HEMPEL Compassionate Use IND: 104,114

CASSIDY HEMPEL Compassionate Use IND: 104,116

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Submission Date to FDA: August 13, 2010

INVESTIGATOR'S ORIGINAL

August 13, 2010

Ruyi He, M.D.

Acting Deputy Director

U.S. Food & Drug Administration

Center for Drug Evaluation and Research

Division of Gastroenterology Products

5901-B Ammendale Road

Beltsville, MD 20705-1266

RE INDs: 104,114 and 104,116 new protocol

Dear Dr. He:

On July 15, 2010, we submitted documentation proposing intrathecal administration of hydroxy-propyl-beta-cyclodextrin (HPBCD) for the above INDs. Subsequent to that submission, a teleconference with the Division of Gastroenterology Products on August 4, 2010 provided additional guidance. Accordingly, we are now submitting a revised protocol for the intrathecal administration of HPBCD under the above INDs.

Specific areas of change to the protocol include:

- Changing the drug diluent from Elliott's B to sterile saline, consistent with the formulation used in the current INDs.
- Additional chemistry data concerning the formulation, including pH and osmolarity.
- Indicating that saline was used in the animal studies of HPBCD.
- Data demonstrating rapid clearance of HPBCD from the CSF of animals following intrathecal administration.

We will not proceed with the intrathecal delivery until an agreement has been reached with the FDA on the feasibility, safety monitoring and dosage regimen to be used for intrathecal delivery of HPBCD. As documented more completely in our 2010 Annual Reports and the July 15, 2010 submission the rationale for implementing intrathecal administration of HPBCD in the Hempel twins is predicated upon:

- The Hempel twins continue to show disease progression as reflected in recent PET imaging (hypometabolism) and neurological testing (seizures). Whether these findings are a concomitant of disease progression and lack of efficacy of HPBCD administered by intravenous infusion have yet to be determined
- Research shows that HPBCD either does not cross the blood brain barrier or only to a very limited extent
- Intrathecal administration of HPBCD in a feline model of NPC has been shown to produce striking reductions in the onset of disease-related ataxia
- Very high systemic doses and chronic administration of HPBCD appears to cause pulmonary and hearing toxicity in animals, yet extremely high systemic doses appear necessary to obtain neurological benefits in NPC animals
- The Hempel twins have tolerated high dose intravenous HPBCD for over one year, with no discernable side effects, although the dose level achieved has been lower than that used in animal studies
- There are no other alternatives to deliver HPBCD into the CNS
- NPC is a fatal disease. The twins continue to decline neurologically and there are no other treatment options available to us at this time

We believe the enclosed intrathecal protocol can safely deliver HPBCD to the Hempel twins and offers a potentially lifesaving treatment given the promising animal data.

The parents of the Hempel twins, Hugh and Christine Hempel, are fully aware of the risks associated with intrathecal administration of HPBCD. The parents have provided a consent

letter to the FDA acknowledging the risks of this treatment and their desire to move forward as soon as possible despite the potential risks.¹

I look forward to your response as we move forward in this promising new treatment direction. I appreciate your assistance and guidance to help us achieve safe intrathecal administration of HPBCD to the Hempel twins.

Sincerely,

A handwritten signature in black ink that reads "Caroline Hastings MD". The signature is written in a cursive, flowing style.

Caroline Hastings, M.D.

Cc:

Chantal Phillips, M.S.H.S.

LCDR, U.S. Public Health - Regulatory Health Project Manager

U.S. Food & Drug Administration - Division of Gastroenterology Products

White Oak, Bldg. 22, Room 5128, 10903 New Hampshire Ave.

Silver Spring, MD 20993

¹ Letter from Mr. and Mrs. Hugh Hempel, parents and legal guardians of Addison and Cassidy Hempel

Hydroxy-Propyl-Beta-Cyclodextrin (HPBCD) Intrathecal Protocol for the Treatment of Niemann Pick Type C

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DATE OF SUBMISSION:

- August 13, 2010

SPONSOR:

- Caroline Hastings, M.D.

TITLE:

- A study of intrathecal cyclodextrin (HPBCD) treatment for cognitive decline in Niemann Pick Type C disease

PROPOSED TREATMENT SITES:

- Children's Hospital & Research Center Oakland (CHRCO), Oakland, California

- Renown Regional Medical Center (RRMC), Reno, Nevada

NUMBER OF PATIENTS (TWO, IDENTICAL TWINS):

- Addison Ruth Hempel: Current IND #: 104,114
- Cassidy Helene Hempel: Current IND #: 104,116

GENERAL BACKGROUND AND TREATMENT HISTORY:

Niemann-Pick Type C disease (NPC) is an autosomal recessive neurodegenerative disorder characterized by defective intracellular cholesterol transport. Niemann-Pick Type C disease classically presents in infancy or early childhood with insidious onset of progressive ataxia, vertical supranuclear ophthalmoplegia and dementia. Other disabling features include dystonia, dysarthria, cataplexy, dysphagia and frequently seizures.

Currently there are no FDA approved treatments for NPC. The condition is always fatal and death commonly occurs in the first two decades of life. Approximately 95% of patients diagnosed with NPC have mutations within the NPC1 gene and 5% have mutations within the NPC2 gene. For expert review of Niemann Pick Type C disease, see the following papers.^{2, 3}

Addison and Cassidy Hempel (DOB 1/23/2004), identical twin Caucasian females, received a definitive diagnosis of Niemann Pick Type C disease in October 2007 through genetic testing conducted at the Mayo Clinic and Stanford University. Mayo Clinic identified the Hempel twins' genetic mutations (1920delG in exon 12 and IVS9-1009G>A a missense mutation) located on the NPC1 gene on Chromosome 18.⁴ The Hempel twins have been receiving Miglustat (Zavesca®) 100 mg BID for more than two years, as well as nutritional supplements including curcumin, fish oil, B multivitamins, vitamin E, vitamin C and N-acetylcysteine (NAC). A complete summary of the Hempel twins' medical history can be reviewed here.⁵

On April 13, 2009, the Hempel twins were approved by the FDA and the IRB at Renown Regional Medical Center, Reno, Nevada, to receive intravenous infusions of hydroxy-propyl-beta-cyclodextrin (HPBCD) Trappsol™ brand under compassionate use, Individual Patient INDs (104,114 and 104,116).

² Marc C. Patterson, et al. Part 16:Lysosomal Disorders, Chapter 145: Niemann-Pick Disease Type C: A Lipid Trafficking Disorder, Scriver's OMMBD

³ Stephen L. Sturley, Marc C. Patterson, et al. The pathophysiology and mechanisms of NPC disease: *Biochimica et Biophysica Acta* 1685 (2004) 83– 87

⁴ D. Brian Dawson, Ph.D., Mayo Clinic, Discovery of a Deep Intronic Mutation in Intron 9 of the NPC1 gene in twin females with classic Niemann-Pick Type C

⁵ Medical summary overview compiled by Chris Hempel, mother

The dosing schedule for HPBCD treatment to date is attached and can also be referenced in numerous submissions we have made to the FDA since April 2009.⁶

On May 17, 2010, the FDA's Office of Orphan Product Development (OOPD) issued an orphan drug designation for hydroxy-propyl-beta-cyclodextrin (HPBCD) Trappsol™ brand for the treatment of Niemann Pick Type C disease.⁷

REPORTING INTRAVENOUS HPBCD TREATMENT RESULTS

As described in periodic and annual reports to the FDA, no significant changes in clinical laboratory findings or drug-related serious adverse events have been observed in the Hempel twins during the intravenous administration of HPBCD treatment. Noteworthy is the fact that the patients received HPBCD 2500 mg/kg, administered as an eight hour infusion, twice weekly for more than one year (November 2009) without adverse events. Prior to this, the patients had tolerated well escalating doses up to 2900 mg/kg since initiation of therapy in April 2009. In spite of this treatment, the patients continue to show neurological symptoms and disease progression, perhaps at a slower rate that would be expected without the HPBCD infusions.

ELEVATIONS IN PLASMA AND CSF BIOMARKERS

When HPBCD intravenous therapy was initiated on the Hempel twins in April 2009, biomarkers for Niemann Pick Type C disease were not readily available. Over the past year, a number of studies have been conducted on human specimens, including the Hempel twins, which provide a clearer picture of NPC disease and potential new plasma and CSF biomarkers.

One of the biomarkers discovered is 7-Ketocholesterol. 7-Ketocholesterol is a major oxidation product of cholesterol found in human atherosclerotic plaques and is more atherogenic than cholesterol in some animal studies.

A patent has been filed by Dr. Daniel Ory at Washington University St. Louis showing that 7-Ketocholesterol is one of the key plasma based oxysterol biomarkers in NPC.⁸ These findings were recently presented at the 6th World Symposium of the Lysosomal Disease Network.⁹ Preliminary analysis of biomarker data suggests that changes in the Hempel twins' biomarkers have been observed through interventions, including Miglustat® (2007 – 2009) and cyclodextrin (see Figure 1).

⁶ HPBCD Approved FDA Treatment Protocol

⁷ FDA letter from Dr. Timothy Cote and OOPD (Designation Request# 10-3039), May 17, 2010

⁸ United States Patent Application Publication, Ory et al. US 2009/0286272 A1, November, 19, 2009

⁹ D. Ory, F. Porter, D. Scherrer, et al. *Cholesterol Oxidation Products are Sensitive and Specific Blood-Based Biomarkers for Niemann-Pick C1 Disease*: 6th World Symposium 2010, page 35

In January 2010, plasma oxysterol measurements were also conducted by Dr. Ingemar Björkhem, Karolinska Institute, Stockholm, Sweden. This analysis clearly confirms the findings that the Hempel twins have higher levels of 7-ketocholesterol, 7-beta-hydroxycholesterol and triol than normal (see Figure 2).

The levels of 24S-hydroxycholesterol appear to be normal, given the age of the twins. In addition, the levels of 27-hydroxycholesterol are normal. Measurements of levels of 27-hydroxycholesterol from Dr. Ory were much lower than Dr. Björkhem's analysis. However, this could be as a result of a hydrolysis procedure that is not sufficient to cleave the esters.

FIGURE #1

Quantization of oxysterols in plasma: Dr. Daniel Ory, Washington University St. Louis, USA.

Plasma Collection Dates	7 Keto Cholesterol		Triol		24 Hydroxy Cholesterol		27 Hydroxy Cholesterol	
	Addi	Cassi	Addi	Cassi	Addi	Cassi	Addi	Cassi
November 2007 Baseline from NIH - Before interventions	536.25	560.1	117.73	159.24	83.41	94.37	n/a	n/a
April 2009 Baseline (pre-Cyclodextrin treatment)	234.55	318.22	99.22	120.55	57.17	65.66	57.34	68.54
July 2009 - 13 weeks into Cyclodextrin infusions	174.41	130.3	86.57	103.67	51.83	57.51	55.19	57.48

FIGURE #2

Quantification of oxysterols in plasma: Dr. Ingemar Björkhem, Karolinska Institute, Sweden.

Collection Date - January 22, 2010

Subject	7-beta	7-keto	Triol	24S-OH	25-OH	27-OH
			ng/ml			
Addison Hempel	65	80	208	141	7	215
Cassidy Hempel	36	43	152	127	7	179
Controls in parallel (n=4)	12	19	15	55	7	135
Reference levels#	(3-7*)	6-39*	**	30-127#	0-11#	89-243#

*Luliano et al. Anal. Biochem. 312(2003) 217-223. The levels of 7-beta in that investigation are somewhat lower than those obtained here in spite of the fact that the same method is used. In a normal Swedish population the levels are seldom above 20 ng/ml

#Dzeletovic et al. Anal. Biochem. 225 (1995) 73-80

**Accurate reference material for the triol is not available because of the considerable variations. It has not been demonstrated convincingly that this oxysterol is formed in vivo. In the original paper by Dzeletovic et al the levels were reported to be between 0 and 128 ng/ml but considerably lower values have been recorded later (in general below 20 ng/ml in our Swedish population).

CSF BIOMARKER DATA:

Cerebrospinal fluid measurements conducted by Alzheimer's researcher Dr. Kaj Blennow, Sahlgrenska University Hospital, Gothenburg, Sweden, confirm that the Hempel twins have CSF elevations of multiple markers including Abeta 38MS, Abeta 40MS and L-Ttau (see Figure 3).

CSF samples were drawn from the Hempel twins by the National Institutes of Health (NIH) in November 2007 (before medical intervention of any kind) and August 2008 (following Zavesca® and supplement intervention) when the Hempel twins were participating in a Natural History Study at the NIH.

This CSF biomarker data is currently unpublished, but was recently presented at the 11th International Symposium on Mucopolysaccharide and Related Diseases.¹⁰ Interestingly, the genetic link between cholesterol, Abeta and Alzheimer's disease suggests the possibility of cyclodextrin utility in other neurodegenerative diseases. Further, Simons et al.¹¹ showed that the combination of lovastatin and methyl-beta-cyclodextrin (5 mM) not only reduced hippocampal neuron cholesterol levels by 70% without affecting neuronal viability, but also completely inhibited the formation of Abeta.

¹⁰ N. Mattsson, H. Zetterberg, N. Yanjanin et.al. *Cerebrospinal fluid biomarkers and B-amyloid metabolism in Niemann-Pick Type C disease*, 11th International Symposium, June 23-26, 2010. Adelaide, Australia, page 51

¹¹ Simons, M. et al. *Cholesterol depletion inhibits the generation of B-amyloid in hippocampal neurons*. Proc Natl Acad Sci, 95: 6460-6464, May 1998

FIGURE #3

No	Notes	Sex	Age	Diagnosis	Abeta38MS	Abeta40MS	Abeta42MS	sAPPA	sAPPB	LAB1-42	L-Ttau	L-Ptau
Addison	11/1/2007 is when this sample was taken from Addison - baseline before treatment	F	3.75	NPC	674	8200	747	751	263	305	996	39
Addison	Miglustat Intervention and some natural supplements	F	4.50	NPC	711	6491	560	751	202	274	719	46
Cassidy	11/1/2007 is when this sample was taken from Cassidy - baseline before treatment	F	3.75	NPC	1370	10637	1045	971	282	353	1271	54
Cassidy	Miglustat Intervention and some natural supplements	F	4.50	NPC	1145	10226	942	950	290	315	736	51
Controls												
C572		F	8 yr 5 mo	Control	149	1439	46	405	107	67	70	31
C573		F	10 yr 7 mo	Control	364	3040	158	653	166	144	93	30
C574		F	4 yr	Control	127	1704	74	285	65	89	80	28
C582		M	3 yr 6 mo	Control	26	442	26	503	146	42	68	31
C587		M	9 yr 4 mo	Control	143	1719	82	376	116	128	54	10
C588		M	6 yr	Control	143	2040	91	247	67	119	84	19
C619		F	2 yr 9 mo	Control	131	1650	96	160	34	89	110	19
C692		F	1 yr 5 mo	Control	151	2007	131	293	91	148	116	22
C694		M	23 yrs	Control	19	16	35	15	0.8	13	10	7
Additional NPC Samples												
NPC22		M	10.67	NPC	1131	9078	812	801	264	309	460	26
NPC25		F	6.67	NPC	318	4221	307	422	109	211	563	21
NPC34		F	3.25	NPC	704	6614	559	335	87	270	627	43
NPC36		F	6.83	NPC	517	5222	398	238	53	296	471	44
NPC38		F	16.83	NPC	432	4616	413	442	148	276	124	25
NPC42		M	17.2	NPC	466	4486	372	672	200	264	146	34
NPC43		F	8.1	NPC	275	3892	308	308	74	244	143	22

CSF BIOMARKER TRACKING:

The cerebrospinal fluid is separated from the brain tissue by only a single cell layer, which allows a virtually unrestricted flow of molecules from the brain interstitial fluid into the CSF. Therefore, metabolism in the brain is reflected in the CSF, and biochemical effects of CNS active drugs such as HPBCD may be monitored using CSF analyses.

We propose in this protocol that Dr. Blennow, a leading authority on CSF based biomarkers in neurodegenerative conditions, continues the analysis of CSF samples from the Hempel twins to determine the effect of intrathecal delivery of HPBCD.

Following are the potential biomarkers Dr. Blennow and his team of experts will measure:

- *Amyloid metabolism:* Amyloid peptides have been extensively studied as key components of the pathogenesis in Alzheimer's disease (intracellular amyloid peptides accumulate in NPC neurons (Jin et al, Am J Pathol 2004; 164: 975-85). This disturbance of amyloid metabolism in NPC may be caused either by the impaired vesicular trafficking in NPC or by direct modulation by lipid alterations on amyloid processing enzymes, such as gamma-secretase. It is known that amyloid metabolism is linked to lipid homeostasis (Grimm et al. Trends Mol Med. 2007; 13: 337-44.). In a recent study on a large number of NPC patients it was shown that disturbed amyloid metabolism in NPC may be monitored in CSF (Mattsson et al, see Reference #10). In this particular study, CSF levels of amyloid peptides were significantly increased in NPC patients. This is a unique pattern, which has not been seen in any other neurodegenerative condition. With this background, CSF amyloid peptides are possible treatment markers in NPC and may be measured throughout the course of the HPBCD intrathecal treatment.
- *Axonal degeneration:* Two established CSF markers of axonal degeneration are available; total-tau and neurofilament light protein (NFL). CSF total-tau is increased in several conditions with cortical axonal degeneration, including Alzheimer's disease and Creutzfeld-Jakobs disease (Blennow and Hampel, Lancet Neurol. 2003; 2: 605–613, Blennow et al. Int. J. Mol. Med. 2005; 16: 1147–1149). The levels of CSF total-tau correlate with the ongoing degree of axonal degeneration. In a recent NPC study (Mattsson et al, see Reference #10), CSF total-tau was markedly increased in NPC patients. Furthermore, CSF total-tau decreased after initiation of treatment with Miglustat[®], indicating a possible decline of axonal degeneration as a result of treatment. Tau may be aberrantly phosphorylated and deposited in intraneuronal tangles, seen in for example Alzheimer's disease and NPC. Although NPC patients had normal CSF phospho-tau levels (outlined in Reference #10), this biomarker still needs to be evaluated as a treatment marker in NPC. CSF NFL is increased in conditions with damage to myelinated axons in white matter, including subcortical dementia and multiple sclerosis (Norgren et al. Neurology 2004; 63: 1586–1590, Rosengren et al. Neurology 1999; 52: 1090–1093). Since NPC presents with white matter damage (Walterfang M. Neurology, 2010; 75: 49-56), CSF NFL is a likely biomarker for white matter damage in NPC patients.
- *Astrogliosis:* GFAP is a protein produced by astrocytes. CSF GFAP is a marker of astrogliosis, and is used to monitor such pathology in multiple sclerosis (Malmeström et al. Neurology 2003; 61: 1720-1725). Astrogliosis is present in the NPC brain (Luan et al. J Neurol Sci 2008; 268: 108-116). CSF GFAP may be used in the conjunction with CSF tau

and CSF NFL to precisely characterize the nature of brain damage. Any treatment affecting gliosis, either primarily or secondarily, might be expected to also affect CSF GFAP levels.

- *Lipid profiles:* NPC is characterized by aberrant lipid homeostasis and accumulation of different lipid species in the CNS. Although the NPC1 and NPC2 proteins are primarily involved in cholesterol transport, the lipids that mostly accumulate in NPC brains are gangliosides. Cyclodextrin treatment might affect the lipid composition of neurons, which could be reflected in the CSF. A careful characterization of lipid profiles, including gangliosides and sterols in the CSF before and after treatment with HPBCB could result in the identification of useful treatment biomarkers.
- *Microglia activation:* Microglia cells are activated in NPC (German et al. Neuroscience 2002; 109: 437-450, Baudry et al. Exp Neurol 2003; 184: 887-903). Microglia are the resident macrophages of the CNS. There are well established markers of activation of peripheral macrophages, including chitotriosidase activity, CCL2 and CCL18 (Barone et al. Clin Lab 2007; 53: 321-333, Deshmane et al. J Interferon Cytokine Res 2009; 29: 313-326). It has recently been found that these markers also may be monitored in human CSF, where they likely represent activation of microglia in the CNS (unsubmitted data, personal communication with Drs. Niklas Mattsson, Henrik Zetterberg and Kaj Blennow). These variables may be measured in NPC CSF and evaluated as treatment markers.
- Additional biomarkers related to CNS function to assess possible efficacy of HPBCD will be added. For example, cholesterol, oxysterol, sphingosine, sphingosine-1-phosphate, gangliosides, as well as markers of neuronal degeneration (calbindin, Lamp-1) have been associated with Niemann Pick Type C disease and may provide CSF markers sensitive to HPBCDs effects.

PROPOSED HPBCD INTRATHECAL TREATMENT PROTOCOL

OBJECTIVES:

The primary objectives of this HPBCD intrathecal protocol are as follows:

- To evaluate the safety of intrathecal HPBCD administered bi-monthly for up to two months
- To determine the maximum tolerated dose of intrathecal HPBCD
- To determine whether intrathecal HPBCD can reduce or stabilize neurological and cognitive decline by measuring changes utilizing the National Institutes of Health NPC severity scale (see Appendix 3) including:
 - Eye movement
 - Ambulation
 - Fine motor skills
 - Speech
 - Swallowing
 - Cognition
 - Hearing
 - Memory
 - Seizures
 - Modifiers
- In addition, we will have assessments that will include:
 - General well being as assessed by parents
 - Detailed neurological exams
- To determine whether intrathecal HPBCD can induce changes in CNS and blood biomarkers shown to be altered in NPC. Note that although plasma markers have been shown to be modified with systemic therapy, it is unlikely that sufficient systemic concentrations will be achieved following direct intrathecal administration of HPBCD to result in alterations in the following biomarkers:
 - Plasma oxysterols
 - Blood Lysotracker
 - Plasma glutathionine and ergothionine

By contrast, the following potential CSF biomarkers, some which have been shown to be altered in NPC and were referenced above, may respond to intrathecal HPBCD administration:

- CSF T-Tau levels
- CSF Amyloid peptides
- CSF neurofilament light protein (NFL)
- CSF GFAP levels
- CSF sterols and oxysterols
- Chitotriosidase activity, CCL2 and CCL18
- Calbindin
- LAMP-1
- GM2 / GM3, gangliosides
- Sphingosine and Sphingosine-1-phosphate (S1P)

RATIONALE FOR INTRATHECAL ADMINISTRATION OF HPBCD

Although experiments in animal models of NPC have consistently demonstrated the therapeutic benefit of high doses of HPBCD administered systemically, Dr. David Begley of Kings College London reported at the 6th World Symposium Lysosomal Disease Network (February 2010), that HPBCD does not cross the blood-brain-barrier. A copy of Dr. Begley's data is included here.¹²

The mechanism of action of HPBCD in increasing longevity and retarding the onset of neurological symptoms in animal models remains to be elucidated; although the work of Dr. Begley indicates a high degree of binding of HPBCD to the blood brain barrier endothelium.

Evidence for Efficacy in animals following CNS administration

Throughout 2009 and early 2010, and in parallel to our intravenous treatment on the Hempel twins, further research has been conducted with HPBCD in animal models of NPC.

Dr. Charles Vite, School of Veterinary Medicine at the University of Pennsylvania, studies naturally-occurring feline model of human neurological diseases. Dr. Vite performed several studies on NPC afflicted cats. Dr. Vite identified that intrathecal administration of HPBCD (120 mg in 0.6 ml saline every two weeks) delayed the clinical manifestations of neurological disease

¹² Charles C. Pontikis, David J. Begley, Kings College London Blood-Brain Barrier Group, *2-Hydroxypropyl- β -cyclodextrin does not cross the blood-brain barrier (BBB) in +/- or Npc1-/- mice*, Poster Abstract, February 2010

for at least up to 24 weeks of age (an age when untreated NPC cats die) but had no effect on hepatic disease.

In addition, Dr. Vite showed that while subcutaneous therapy with HPBCD at all doses tested ameliorated liver disease, only the 8000mg/kg dose substantially affected neurological disease. In addition, 8000mg/kg dose resulted in early death due to pulmonary toxicity. Dr. Vite also identified a dose related negative effect of HPBCD on hearing function,¹³ a finding that has recently been observed in mice as well (Dr. Steven Walkley, Albert Einstein College of Medicine, personal communication). While both normal and NPC cats treated with HPBCD do experience a significant increase in hearing threshold, the overall neurological improvements seen with intrathecal delivery of HPBCD in cats is quite remarkable and far exceeds the therapeutic benefit achievable by systemic administration at any dose.

Recent preliminary pharmacokinetic data conducted on our behalf by Johnson & Johnson Pharmaceutical in NPC cats following both SQ and IT administration has been obtained (see Figure #4). The results indicate that following intrathecal administration of 125 mg, the CSF concentration of HPBCD achieved is nearly *600 times higher* than that seen with subcutaneous administration at doses as high as 8000 mg/kg one hour post injection. Although a more complete dose and time course comparison will be necessary, this data further supports the limited extent to which HPBCD crosses the blood brain barrier after systemic administration.

¹³ Charles Vite, Sarah Ward, et al. *2-hydroxypropyl-[beta]-cyclodextrin raises hearing threshold in normal cats and in cats with Niemann-Pick Type C disease*, Pediatric Research, March 2010

FIGURE #4**Research data provided by Johnson & Johnson****NPC CAT DATA**

Cat No.:	Matrix	Time point (min)	Concentration (µg/mL)	Route	Dose
7955	CSF	10	9490	Intrathecal	125 mg/cat
7956	CSF	15	13800	Intrathecal	125 mg/cat
7957	CSF	60	10700	Intrathecal	125 mg/cat
7958	CSF	57	8470	Intrathecal	125 mg/cat
8057	CSF	60	< LLQ	s.c.	1000 mg/kg
8058	CSF	60	< LLQ	s.c.	1000 mg/kg
8059	CSF	60	17.7	s.c.	4000 mg/kg
8075	CSF	60	21.2	s.c.	4000 mg/kg
8076	CSF	60	18.7	s.c.	8000 mg/kg
8078	CSF	60	23.7	s.c.	8000 mg/kg
7955	Plasma	12	190	Intrathecal	125 mg/cat
7956	Plasma	27	60.8	Intrathecal	125 mg/cat
7957	Plasma	63	70.7	Intrathecal	125 mg/cat
7958	Plasma	60	36.0	Intrathecal	125 mg/cat
8057	Plasma	60	606	s.c.	1000 mg/kg
8058	Plasma	60	487	s.c.	1000 mg/kg
8059	Plasma	60	2310	s.c.	4000 mg/kg
8075	Plasma	60	2830	s.c.	4000 mg/kg
8076	Plasma	60	3340	s.c.	8000 mg/kg
8078	Plasma	60	3630	s.c.	8000 mg/kg

Additional recent research in both mouse (Dr. Steven Walkley, Albert Einstein College of Medicine, publication pending and Dr. John Dietschy, UT Southwestern, publication pending) and feline (Dr. Charles Vite, University of Pennsylvania, publication pending) models of NPC have shown that direct administration of HPBCD into the intrathecal or intracerebroventricular (ICV) space at low concentrations has an equal or superior effect on delaying the onset of neurological symptoms as that observed following high systemic doses.

In fact, continuous ICV administration via osmotic mini pumps in NPC +/- mice appears capable of prolonging life expectancy beyond that achievable with peripheral administration (Dr. John Dietschy, University of Texas Southwestern, personal communication).

Further, in vitro experiments in neuronal cultures derived from NPC patients has shown that continuous exposure of the cells for 24 hours at concentrations of HPBCD as low as 0.1 mM are capable of reversing neuronal accumulation of cholesterol and expanding cell viability (Dr. Jean Vance, University of Alberta, publication pending).

Summary of Benefits:

HPBCD is the only substance identified to date that has been shown in animal models of NPC disease to consistently prolong life expectancy by at least 100% (mouse) and/or delay the onset of neurological symptoms (cat and mouse). While the mechanism by which this remarkable neurological effect has yet to be elucidated, it is clear that the greatest effect can be achieved by direct intrathecal or ICV administration.

Summary of Risks:

Of potential concern, experiments in the cat model of NPC involving high doses subcutaneous delivery of HPBCD have shown no prolongation of life expectancy. This finding may be confounded by the observation that repeated subcutaneous injections of such high doses (4000 – 8000 mg/kg) appear to result in pulmonary complications and death. Also, either the systemic or intrathecal administration of HPBCD appears to elevate hearing threshold in both NPC and normal healthy cats (see Reference #13) and mice (Walkley, personal communication). This hearing loss in cats and mice may be species specific, the consequence of very high dose, or may in fact be a toxic side effect of HPBCD. Further dose response studies are underway in animals to clarify this finding.

Because of the pulmonary complications and elevated hearing threshold findings in animals, we tested the Hempel twins' pulmonary function and hearing. In December 2009, bronchoscopies were performed on the Hempel twins. Very small scattered nodules were visible in both twins' lungs and could be possible xanthomas. Xanthomas have been reported in one potential case of NPC. It is unclear if these nodules are NPC mutation specific or could be as a result from HPBCD treatment. On April 13, 2010, CT scans of the twins' lungs were conducted at Children's Hospital Michigan and there was no bronchiectasis or evidence of interstitial lung disease. Detailed pulmonary reports were submitted to the FDA in our 2010 Annual Report filing.

On December 9, 2009, and April 15, 2010, the Hempel twins underwent audiological evaluations to check their hearing response.¹⁴ Hearing in the twins appears unaffected despite receiving steady state intravenous doses of 2500 mg/kg bi-weekly for over one year.

¹⁴ Two hearing reports: Children's Hospital of Oakland, December 8, 2009 and Children's Hospital Michigan, April 15, 2010, while under sedation

High subcutaneous doses in the range of 8000 mg/kg in the NPC cats are required to achieve a beneficial neurological effect. As mentioned previously, both NPC afflicted cats and normal cats without the disease experience hearing loss following treatment with HPBCD.

SAFETY OF INTRATHECAL CYCLODEXTRIN ADMINISTRATION

Very limited toxicity data exists concerning the administration of cyclodextrins into the central nervous system. Published findings, however, suggest that like systemic administration, intrathecal and ICV administration appears to be very safe.

In rats, the administration of HPBCD at concentrations as high as 20% had no effect upon the volume evoked micturition reflex, blood pressure, heart rate, or spinal reflexes.¹⁵ Studies to evaluate dose response and toxicity are ongoing in the cats (personal communication, Dr. Charles Vite) to determine if lower doses may be safely administered without compromising hearing.

The utility of cyclodextrins as vehicles for administering drugs to the CNS has also been described in detail.¹⁶ Interestingly, the complexation of opioid analgesics such as sufentanil with HPBCD has been shown to increase their epidural and intrathecal effectiveness while reducing systemic side-effects.¹⁷ The optimal concentrations of HPBCD used were 10%. The potentiation of epidural sufentanil analgesia by HPBCD has been shown using an in vitro diffusion cell model to be related to increased flux through the meninges rather than acting as a slow-release reservoir.¹⁸

CHEMICAL PROPERTIES OF HPBCD IN THE CSF

Drug Supply Reference Statement. Trappsol® brand of endotoxin controlled HPBCD will be obtained from Cyclodextrin Technologies Development, Inc. 27317 NW 78th Ave, High Springs, FL 32643 (386)-454-0887. The product is referenced in Drug Masterfile 10772. A letter authorizing reference to the DMF has been filed with the FDA in the initial IND submission. HPBCD for intrathecal administration will be compounded by the hospital pharmacy where the drug is administered. The product will consist of HPBCD in sterile saline filled under aseptic conditions and subjected to sterility and pyrogenicity testing prior to use.

¹⁵ Jang, Yaksh and Hill, Journal of Pharmacology and Experimental Therapies, *Use of 2-Hydroxypropyl-B-Cyclodextrin as an Intrathecal Drug Vehicle with Opioids*, 1992;261(2):592-600

¹⁶ Yaksh T L; Jang J D; Nishiuchi Y; Braun K P; Ro S G; Goodman M, Life Sciences, *The utility of 2-hydroxypropyl-beta-cyclodextrin as a vehicle for the intracerebral and intrathecal administration of drugs*, 1991;Volume 48, pgs. 623-633

¹⁷ Meert, TF et al Eur J. Anaesthesiol, 1992, vol 9, pgs. 399-409; Meert, TF and Melis, W., Acta Anaesthesiol Belg. 1992, 43: 79-89

¹⁸ Bernards C M, Journal of Pharmaceutical Sciences, *Effect of (hydroxypropyl)-beta-cyclodextrin on flux of morphine, fentanyl, sufentanil, and alfentanil through the spinal meninges of monkey*, 1994;83(5):620-2

The solubility of HPBCD is quite high, exceeding 600 mg/ml. Viscosity is not an issue in concentrations below 55%.

Table 1 shows the pH and osmolarity of HPBCD in saline at the concentrations proposed for intrathecal administration. By contrast, normal human CSF has a pH of 7.33 and osmolarity of 295 mOsm/Kg H₂O (Reference: Fishman, R.A. Cerebrospinal Fluid in Disease of the Nervous System. Philadelphia: Saunders, 1980)

TABLE 1. Chemical properties of HPBCD in saline (independent, duplicate measurements):

Proposed Dose	pH	Osmolarity
175 mg/6 mL	6.58	317
	6.50	310
350 mg/6 mL	6.65	343
	6.53	338
525 mg/6 mL	6.61	372
	6.77	370
700 mg/6 mL	6.68	404
	6.63	399
875 mg/6 mL	6.73	439
	6.90	432

Dose Selection

The targeted HPBCD dose is predicated upon the finding that a 0.1 - 1 mM concentration of HPBCD is capable of normalizing cholesterol in NPC neuronal cultures ***exposed continuously for 24 hrs***. Further, 20 minute incubation of hippocampal neurons with 5 mM concentration of the more toxic methyl-beta-cyclodextrin did not alter cell viability.

Following IT administration in the patients, concentrations of HPBCD are expected to decline with normal CSF clearance. In mice, a 40 mg/kg dose administered IT would be equal to 22 mM (40 mg/kg x 0.025 kg = 1 mg; 1 mg/.04 ml CSF = 25 mg/ml) while a 10 mg/kg ICV dose in the mouse would be approximately 5 mM.

In the cat, the dose tested to date (120 mg administered in 0.6 ml of saline) would be equal to 26 mM (120 mg/ 4 ml CSF= 30 mg/ml). However, as seen from Figure #4, CSF levels achieved after IT administration were around 12 mM (13.8 mg/ml) 60 min after administration, indicating rapid clearance from the CSF. In fact, recent experiments in the cat have confirmed undetectable levels of HPBCD in the CSF 24 hrs after administration. Pharmacokinetic calculations based on the cat intrathecal administration data (Table 2) show an expected half life in the CSF of approximately 2-3 hrs.

Table 2. Preliminary CSF pharmacokinetics of HPBCD in cats following 125 mg intrathecal injection.

Time point				ug/mL				
(hr)	#7955	#7956	#7957	#7958	#8095	#8096	#8098	Average
0.25	9490	13800						11645
1			10700	8470				9585
4					639	331	245	405
24					15.1	6.45	14	11.85
Cmax	11645	ug/mL						
Tmax	0.25	hr						
AUC (0-inf)	28614	ug.h/mL						
Clearance	4193	ml/hr						
t1/2	2.56	hr						

These initial HPBCD dose estimates are calculated as: MW=1400, 1.0 mM=1.14 mg/ml x 125 ml human CSF volume = 175 mg to achieve 1 mM concentration upon intrathecal injection.

INTRATHECAL HPBCD TREATMENT PLAN

Baseline Clinical Assessment

Prior to the initiation of HPBCD intrathecal injections, the following assessments have/or will be performed on the Hempel twins. These baseline tests will serve as the basis for determining the effects of intrathecal HPBCD treatment:

Brain imaging studies:

- MRI with DTI 3 T imaging (completed)
- PET Scans F-18 FDA PET, C-11-PK-11195 PET (completed)
- 24 hour VMR (completed)
- Monthly EEG recordings will be collected for safety monitoring as well as to document possible changes in seizure activity during the course of the treatment

Organs:

- Lung CT scan, Bronchoscopies (completed)
- Volumetric CT of liver and spleen (completed)

Hearing tests:

- Audiological assessments (completed)

Neurological Assessments:

- Neurological exams (visual and video recording) (in process)
- Eye exam by neuro-ophthalmologist (in process)

Additional Assessments:

- Complete physical examination (in process)
- Completion of the NIH NPC severity scale (Appendix 3)
- Renal function test and urinalysis (in process)

Baseline CSF and Plasma samples:

- Immediately prior to all intrathecal HPBCD administration, 6-8 ml of CSF will be collected for biomarker analysis by Dr. Kaj Blennow and for routine studies (cell count, glucose, protein). See Appendix 2. This volume is slightly more than the injection volume.
- Complete blood count and serum chemistry will be collected in the institution specific tube and analyzed by the local laboratory. The serum chemistries will evaluate electrolytes, bicarbonate, glucose, BUN, creatinine, magnesium, phosphate, hepatic enzymes (AST and ALT), total protein, albumin, bilirubin, and alkaline phosphatase. In addition, a complete lipid panel will be obtained.

- Additional blood samples will be collected as clinically indicated for specific biomarker analysis. See Appendix 2.

Location:

The Hempel twins will receive initial intrathecal infusions at CHRCO in Oakland, California. Following the initial intrathecal injections without adverse event, the patients may undergo subsequent treatment at Renown Regional Medical Center in Reno, Nevada, which is more convenient to their residence. Both facilities are equipped with pediatric ICUs. Dr. Caroline Hastings will oversee intrathecal infusions in both hospital locations.

Schedule and Dosage:

A complete overview of the treatment protocol and dosage of HPBCD intrathecal therapy for the Hempel twins is depicted in Appendix 1.

We propose an initial two month HPBCD intrathecal injection protocol period. During this period, current intravenous HPBCD therapy on the Hempel twins will be discontinued in order to minimize confounding the results of efficacy and safety determinations. Beginning on treatment Day 1, and for the duration of the treatment period, all concomitant medications and supplements will be recorded and held constant.

Continued safety monitoring and clinical assessment will continue as described below.

- The patients will undergo lumbar puncture in the hospital setting under general anesthesia administered by a pediatric anesthesiologist. Typically, propofol or ketamine is given intravenously with continuous airway, cardiorespiratory monitoring with blood pressure and pulse oximetry monitoring.
- The patients will be positioned in the lateral decubitus position and knees brought up to shoulders. The area over the lumbar sacral spine will be prepped and draped in a sterile manner. A 22-gauge spinal needle will be inserted at L3-L4 and CSF obtained by natural flow.
- The study medication will be transferred in a sterile manner into a sterile syringe prior to the procedure. This will be instilled intrathecally via the spinal needle.
- It is anticipated that approximately 6-8 ml of CSF will be obtained at the time of intrathecal therapy and the study medication will be given in a volume of 6 ml.

- Following the procedure, the patients will be kept in a supine position for one hour to allow distribution throughout the CSF (as is standard of practice in instilling intrathecal chemotherapy in children).
- On study Day 1, intrathecal injections will consist of 175 mg of HPBCD dissolved in 6 ml sterile Saline. The solution will be prepared by a pharmacy certified by the California or Nevada Board of Pharmacy to prepare Compounded Sterile Products in accordance with current USP chapter 797 guidelines for aseptic processing. Specifically, for a high-risk non-sterile material received in bulk the following procedure will be employed:

In a ISO class 5 or cleaner room the HPBCD will be weighed and dissolved in of sterile saline using sterile containers

Terminal sterilization of high-risk level CSPs by filtration shall be performed with a sterile 0.22- μ m porosity filter entirely within an ISO Class 5 or superior air quality environment

The sterilized solution will be placed into commercial sterile 10 ml syringes, under ISO class 5 or cleaner conditions, and labeled according to the pharmacy SOP with before use dating of 24 hrs in accordance with USP 797: *“For a sterilized high-risk preparation, in the absence of passing a sterility test, the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 24 hours at controlled room temperature.”*

The solutions of HPBCD are extremely stable at room temperature, but should be prepared for delivery to the clinic no more than two hours prior to use.

Procedure

HPBCD will be administered via a lumbar puncture (IT) on Day 1 after baseline assessments are completed. See dose information below. Patients will be continuously monitored in the hospital for at least four hours following the procedure, and are expected to remain hospitalized overnight for hourly observations for potential delayed reactions.

The patients will have vital signs (e.g., heart rate, blood pressure, temperature) monitored before, during and after the injections, and will undergo a physical examination, including neurological assessment, and clinical laboratory measurements. Immediately prior to each injection, 6-8 ml of CSF will be collected for biomarker studies as indicated above. Additional safely monitoring is outlined below.

DOSE ESCALATION SCHEDULE

Following the initial HPBCD intrathecal injection without adverse events, the patients will receive additional bi-monthly injections of HPBCD for up to two months and a total of five injections. The concentrations of HPBCD that we propose are well below the 20% concentration injected safely into animals by the intrathecal route. In the absence of significant adverse events, 6 ml intrathecal injections of HPBCD will be repeated with dose escalations on Days 14, 28, 42, 60. The solution will be administered as prepared in sterile isotonic Saline for injection.

- We will add 17.5 mg of HPBCD every two weeks as follows:
- Day 1 = 175 mg HPBCD (1mM)
- Day 14 = 350 mg HPBCD (2mM)
- Day 28 = 525 mg HPBCD (3mM)
- Day 42 = 700 mg HPBCD (4mM)
- Day 60 = 875 mg HPBCD (5mM)

Thus, by the last injection, approximate CNS concentrations of 5 mM will be achieved.

Dosage Adjustments

In the event that adverse events that are either definitely or possibly related to the investigational drug are observed at any dose level, the next two administrations will be given at the previous dose shown to be tolerated without adverse reaction. Subsequent re-challenge with higher doses will be at the treating physician's discretion. If the adverse event meets CTCAE criteria for Grade 4 toxicity, no further doses will be administered to the child (see Study Termination below).

At the completion of Day 60 without adverse events, the patients will undergo a repeat of MRI and PET imaging, NIH NPC severity scale assessment, neurological testing and CSF and plasma samples will be analyzed. Neurosurgical implantation of an Ommaya reservoir will then be considered for ongoing long term administration of HPBCD.

Safety Monitoring, Discontinuation Rules for i.t. HPBCD Administration

Vital signs including heart rate, respiratory rate, blood pressure and pulse oximetry will be monitored every 15 minutes for the first hour during initiation of IT infusion, every 30 minutes during the second hour, and then every hour. At the conclusion of the HPBCD infusion, and prior to any subsequent infusions, the patients will receive complete blood chemistry and urinalysis.

In the unlikely event that a severe allergic reaction should occur, the ***following specific plan to deal with the possibility of anaphylactic or hyper-allergic response*** will be in place:

A standard cardex for the patient in the clinic will be prepared. This card (bright orange and on top of the clinic chart at each visit) has the name, age and weight of the child. The standard drugs for resuscitation in the event of allergy or anaphylaxis are on the chart and the doses are pre-calculated. We will arrange to have these drugs available in the clinic or hospital room, at the bedside, and draw them if needed prior to administration. The protocol for initial management includes:

- Discontinue the drug
- Establish airway if necessary. Assess breathing. Supply with 100% oxygen with respiratory support as needed. Assess circulation and establish IV access. Place patient on a cardiac monitor
- Albuterol nebulized, 0.05 to 0.15 mg/kg in 3 ml NS every 15 minutes as necessary
- Diphenhydramine 1mg/kg IV or IM
- Methylprednisolone 2 mg/kg IV
- If patient is hypotensive:
 - Place in Trendelenburg position, head at 30-degree angle below feet
 - IV fluid bolus, NS or LR 20 ml/kg IV over 5 to 15 minutes. Repeat as necessary
 - Epinephrine 1:10,000, 0.01 mg/kg (0.1 cc/kg) SC or IV

In the event of any symptoms of allergy, anaphylaxis or seizures, the following patient procedures will be followed. For mild allergy, such as rash or swelling without airway or mucosal involvement, mild allergy will be managed by diphenhydramine administration and observation. Severe allergy will require intensive care and patients will be admitted to the PICU. We will then be guided by our Grade 4 stopping rules.

Study Termination

In the event clinically significant adverse events are observed, the study may be terminated at any time at the discretion of the treating physician. Throughout the study, stopping criteria will be the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), v3) Grade 4 toxicity (if the AE is possibly or definitely related to study drug).

At the end of two months treatment, the data will be reviewed with the FDA and a decision will be made as how to best move forward with HPBCD treatment.

Therapeutic Endpoints

The primary endpoint of this study will be safety, tolerability and stabilization in neurological functioning. Secondly, arrest of disease progression will be assessed based upon comparison

of pre and post treatment scores on the NIH NPC Severity Scale (Appendix 3) and independent expert review of the case report and video assessments.

At two month intervals and/or at the conclusion of the study, a complete physical and videotaped neurological exam will be performed. Changes in neurological status will be assessed using independent ratings of the videotapes examined by a pediatric neurologist. A Glucose PET will be obtained for possible changes in hypometabolism.

Changes in plasma biomarkers previously observed will be confirmed by comparison of original baseline samples at the start of this protocol. Changes in CSF biomarkers will be compared with baseline as will changes in imaging. Continued neurological examinations, with particular emphasis on seizure frequency will provide a clinical endpoint.

APPENDIX 1**INTRATHECAL HPBCD TREATMENT**

	Baseline	Day 1	Day 14	Day 28	Day 42	Day 60	Study End
HPBCD Dose (mg/6 mL) i.t.		175	350	525	700	875	
MRI (brain)	X						X
PET (brain)	X						X
EEG	X						X
NIH NPC Severity Scale	X						X
Neuro Exam by treating physician at each visit	X	X	X	X	X	X	X
Neuro Exam by neurologist	X			X			X
Physical exam	X	X	X	X	X	X	X
CBC, serum chemistries	X	X	X	X	X	X	X
CSF glucose, protein, cell counts, and Biomarkers	X	X	X	X	X	X	
Plasma Biomarkers	X	X	X	X	X	X	

APPENDIX 2

1. Blennow CSF and Plasma sampling procedures for Dr. Kaj Blennow

- Make lumbar puncture at a standardized time point, preferably 9-11 AM (or 8-12 AM)
- Take a standardized volume (e.g. 5 mL in children) of CSF in a single tube, use polypropylene tubes (e.g. Sarstedt tube #60540012)
- In the case of a marked bleeding at the puncture, the tube with the first mL of CSF should be discarded, and CSF tapped into a new tube. This should be recorded.
- Take an EDTA-tube of blood for the plasma sample (this is important for analysis of β amyloid)
- Send the CSF (placed on the ice) and blood samples immediately to the local laboratory for further processing.
- Laboratory procedures:
- Mix the CSF gently, e.g. by turning the tube upside down 2-3 times (yellow cap on)
- Centrifuge the CSF sample at 2.000xg for 10 min at +4 C, to eliminate cells and other insoluble material. Do NOT transfer CSF to another tube for centrifugation
- Centrifuge the EDTA-blood sample at 2.000xg for 10 min at +4 C, to get EDTA-plasma
- Aliquot the CSF and plasma into smaller portions (e.g. 0.5 mL or 1 mL) in polypropylene tubes with a screw cap
- Make all aliquoting with the tubes placed on crushed ice
- Label all tubes with date, patient ID and aliquot number
- Place the tubes directly in a minus 70-80 C freezer. Do NOT snap freeze
- Ship samples via FedEx on lots of dry ice and in thick frigolite box. Samples must be sent early in the week (preferably Monday/Tuesday).

Shipping Note: email for further instructions prior to shipping.

- Dr. Kaj Blennow
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2. Metabolomics Studies by Dr. Jung Suh: CHORI: Sample Preparation Protocol for thiol redox analysis in human plasma and peripheral blood mononuclear cells

- Materials Needed:
- BD Vacutainer 5 ml K3EDTA (BD product number: 367654; or Any other EDTA containing anticoagulant blood collection tubes)
- Sigma Histopaque -1077
- Thiol blocking buffer; 0.1 M Tris-Cl buffer pH 6.8 containing 20 mM Iodoacetamide
- Phosphate buffered saline (Sigma P5368)
- Buffer preparation protocol:
- M Tris-Cl buffer pH 6.8 – Make a stock 1M Tris-Cl pH 6.8 solution by dissolving 12.11 g of Tris (Sigma: T6066) in 0.1 L of HPLC grade water. To this solution, add 3.69 g of iodoacetamide (Sigma: I1149) and dissolve. Aliquot 10 ml to 15 ml conical tubes and store in -20 C. This can be prepared ahead of time and stored for upto 6 month. On the day of sample collection, take the 10 ml solution and add 90 ml of HPLC grade water.
- Blood plasma, RBC, and Peripheral blood mononuclear cell collection:
- Blood is collected by venipuncture to the antecubital vein with 23-gauge butterfly needle attached to a 5 ml BD vacutainer tube
- Mix 2 ml of whole blood and add to a tube containing 2 ml of the thiol blocking buffer and mix by inverting the tubes. Transfer 1 ml of diluted whole blood into a 1.5 ml eppendorf tube. Centrifuge at 10000 x g for 1 min. Collect the plasma and RBC pellet into two separate tubes. *This step must occur immediately following blood draw.
- 3 ml of blood left from step 2 will be used for peripheral blood mononuclear cell isolation. For this, gently overlay 1.5 ml of the diluted whole blood in step 2 on top of 3 ml of histopaque solution (prepare two 15 ml tubes). Then, centrifuge at

400 x g for 30 min. After centrifugation, carefully aspirate the upper plasma layer with a pasteur pipet. Carefully transfer the opaque cell layer and pool them in a new 15 ml tube.

- Add 5 ml of PBS to the cells and gently invert. Centrifuge for 250 x g for 10 min. Aspirate out the PBS and store the cell pellet in -80 C.
- With the remaining blood from step 1, transfer the whole blood sample to a new tube and centrifuge at 10,000 x g for 1 min and collect the plasma and RBC fractions and transfer to new tubes.
- Samples collected:
- Plasma treated with thiol blocking buffer ~0.5 ml
- RBC treated with thiol blocking buffer ~ 0.5 ml
- PBMC treated with thiol blocking buffer ~2 million cells
- Plain Plasma ~ 0.5 ml
- Plain RBC ~ 1ml

Ship to:

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APPENDIX 3

NATIONAL INSTITUTES OF HEALTH NPC SEVERITY SCALE

Eye Movement	Score	Ambulation	Score
Normal eye movement	0	Normal	0
Mild vertical supranuclear gaze palsy (detected by physician)	1	Clumsy	1
Functional vertical supranuclear gaze palsy (problems noted by parents)	2	Ataxic unassisted gait	2
Total vertical supranuclear gaze palsy	3		
Total ophthalmoplegia	5	Assisted ambulation	4
(vertical and horizontal)		Wheelchair dependent	5
Speech	Score	Swallow	Score
Normal speech	0	Normal, no dysphagia	0
Mild dysarthria		Cough while eating	1
(understood by others)	1	Intermittent dysphagia*	w/Liquids +1
Severe dysarthria			w/Solids +2
(understood by family only)	2	Dysphagia*	w/Liquids +1
			w/Solids +2
Non-verbal/functional communication skills for needs	3	Nasogastric tube or gastric tube for supplemental feeding	4
Absence of communication	5	Nasogastric tube or gastric tube feeding only	5
Fine Motor Skills	Score	Cognition	Score
Normal	0	Normal	0
Slight dysmetria/dystonia		Mild learning delay,	

(independent manipulation)	1	grade appropriate for age	1
Mild dysmetria/Dystonia			
(requires little to no assistance, able to feed self without difficulty)	2	Moderate learning delay, individualized curriculum	3
Moderate dysmetria/dystonia			
(limited fine motor skills, difficulty feeding self)	4	Severe delay/plateau, needs constant supervision, no longer acquiring new skills or no longer in school	4
Severe dysmetria/Dystonia			
(gross motor limitation, requires assistance for all activities)	5	Loss of cognitive function	5

Hearing	Score	Memory	Score
Normal hearing (all tones \leq 15 dB HL)	0	Normal	0
High frequency hearing loss (PTA** \leq 15 dB HL, > 15 dB HL in high frequencies)	1	Mild short-term memory loss (forgetful)	1
Slight-mild hearing loss (PTA 16-44 dB HL)	2	Moderate short-term memory loss (gets lost)	2
Moderate hearing loss (PTA 45-70 dB HL)	3	Difficulty following commands	3
Severe hearing loss (PTA 71-90 dB HL)	4	Unable to follow commands	4
Profound hearing loss (PTA > 90 dB HL)	5	No memory	5

Seizures	Score	Modifiers	Score
No history of seizures	0	Gelastic cataplexy	
Single seizure	1	No history	0
Rare seizures	2	Occasional	+1
Seizures, well controlled with meds	3	Frequent (every month)	+2
Seizures, difficult to control with meds	5	Narcolepsy	
		No history	0
		Occasional	+1
		Frequent (every month)	+2
		Behavior	
		No problems	0
		ADHD, aggressive	+1
		Harmful to self/others	+2
		Psychiatric	
		No problems	0
		Mild depression	+1
		Major depression,	
		Hallucinations,	
		Psychotic episodes	+2
		Hyperreflexia	
		No problems	0
		Mild	+1
		Severe	+2
		Incontinence	
		No problems	0
		Occasional	+1
		Frequent	+2
		Auditory Brainstem Response (ABR)	

Normal	0
Abnormal	+1
Absent	+2
Respiratory	
No problems	0
Hx pneumonia	+1
Pneumonia \geq 2x/year	
or active therapeutic intervention	+2

* Score is additive within these two subsections

** PTA = pure-tone average – this is reported on the audiogram

APPENDIX 4

DECLARATION OF HELSINKI

Recommendations guiding physicians in biomedical research involving human subjects

Adopted by the 18th World Medical Assembly Helsinki, Finland, June 1964 and amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, 35m World Medical Assembly, Venice, Italy, October 1983, 41st World Medical Assembly, Hong Kong, September 1989 and the 48th General Assembly, Somerset West, Republic of South Africa, October 1996

INTRODUCTION

It is the mission of the physician to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfillment of this mission. The Declaration of Geneva of the World Medical Association binds the physician with the words, "The Health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

The purpose of biomedical research involving human subjects must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the etiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research. Medical progress is based on research, which ultimately must rest in part on experimentation involving human subjects.

In the field of biomedical research a fundamental distinction must be recognized between medical research in which the aim is essentially diagnostic or therapeutic for a patient, and medical research, the essential object of which is purely scientific and without implying direct diagnostic or therapeutic value to the person subjected to the research.

Special caution must be exercised in the conduct of research, which may affect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further scientific knowledge and to help suffering humanity, the World Medical Association has prepared the following recommendations as a guide to every physician in biomedical research involving human subjects. They should be kept under review in the future. It must be stressed that the standards as drafted are only a guide to physicians all over the world. Physicians are

not relieved from criminal, civil and ethical responsibilities under the laws of their own countries.

BASIC PRINCIPLES

- Biomedical research involving human subjects must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.
- The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol which should be transmitted for consideration, comment and guidance, to a specially appointed committee independent of the Investigator and the Sponsor provided that this independent committee is in conformity with the laws and regulations of the country in which the research experiment is performed.
- Biomedical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given his or her consent.
- Biomedical research involving human subjects cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.
- Every biomedical research project involving human subjects should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the subject or to others. Concern for the interests of the subject must always prevail over the interests of science and society.
- The right of the research subject to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the subject and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject.
- Physicians should abstain from engaging in research projects involving human subjects unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits. In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports of experimentation not in

accordance with the principles laid down in this Declaration should not be accepted for publication.

- In any research on human beings, each potential subject must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at liberty to abstain from participation in the study and that he or she is free to withdraw his or her consent to participation at any time. The physician should then obtain the subject's freely given informed consent, preferably in writing.
- When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship to him or her or may consent under duress. In that case a physician who is not engaged in the investigation and who is completely independent of this official relationship should obtain the informed consent.
- In case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the subject is a minor, permission from the responsible relative replaces that of the subject in accordance with national legislation.
- Whenever the minor child is in fact able to give consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian.

MEDICAL RESEARCH COMBINED WITH PROFESSIONAL CARE (CLINICAL RESEARCH)

- In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure if, in his or her judgment, it offers hope of saving life and re-establishing health or alleviating suffering.
- The potential benefits, hazards and discomfort of a new method should be weighed against the advantages of the best current diagnostic and therapeutic methods.
- In any medical study, every patient - including those of a control group, if any should be assured of the best-proven diagnostic and therapeutic method. This does not exclude the use of inert placebo in studies where no proven diagnostic or therapeutic method exists.
- The refusal of the patient to participate in a study must never interfere with the physician-patient relationship.

- If the physician considers it essential not to obtain informed consent, the specific reasons for this proposal should be stated in the experimental protocol for transmission to the independent committee.
- The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge only to the extent that its potential diagnostic or therapeutic value of the patient justifies medical research.

NON THERAPEUTIC BIOMEDICAL RESEARCH INVOLVING HUMAN SUBJECTS (NON-CLINICAL BIOMEDICAL RESEARCH)

- In the purely scientific application of medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.
- The subject should be a volunteer - either a healthy person or patient for whom the experimental design is not related to the patient's illness.
- The Investigator or the investigating team should discontinue the research if, in his/her judgment, it may be harmful to the individual if continued.
- In research on man, the interest of science and society should never take precedence over considerations related to the well being of the subject.

PROTECTION OF HUMAN SUBJECTS (21 CFR PART 50)

Informed consent must be obtained from every patient before he or she enters a study. It must be given freely and not under duress. Consent must be documented by use of a consent form that has been approved by the IRB and signed by the patient or the patient's legally authorized representative. The Department of Health and Human Services suggests that when minors are involved, a parent or guardian should sign the consent form. If the minor is an adolescent, his or her signature should also be included. Non-English-speaking patients must be presented with a consent form written in a language that they understand. A copy of the signed consent form must be given to the patient signing it. Another copy must be kept in the Investigator's files and made available to Sponsor and FDA representatives upon request. If, for any reason, patient risk increases as the study progresses, the patient must sign a revised consent form that has been approved by the IRB. Before the study begins, a sample of the consent form must be provided to the Sponsor. The FDA may reject otherwise scientifically valid studies if proper informed consent has not been obtained from all patients.

Only in the case of a life-threatening incident may study drug be used without prior signed consent. In such an emergency situation, separate certifications must be written both by a

physician not participating in the study and by the Investigator. The certifications along with the protocol and informed consent must be sent to the IRB within five working days. In this situation, the Investigator may not administer any subsequent study drug to that patient until informed consent and IRB approval are obtained.

BASIC ELEMENTS OF INFORMED CONSENT

Every consent form must include the following eight elements:

1. A statement that the study involves research, an explanation of the purpose of the research and the expected duration of the patient's participation, a description of the procedures to be followed, and identification of any procedures that are experimental;
2. A description of any reasonably foreseeable risks or discomforts to the patient;
3. A description of any benefits to the patient or to others that may be reasonably expected from the research;
4. A disclosure of appropriate alternative procedures or course of treatment, if any, that might be advantageous to the patient;
5. A statement describing the extent, if any, to which confidentiality of records identifying the patient will be maintained and noting the possibility that the FDA and Sponsor representatives may inspect the records;
6. An explanation as to whether any compensation or medical treatments are available if injury occurs for research involving more than minimal risk. The explanation should involve a description of the compensation or treatment available or a statement describing where further information may be obtained;
7. An explanation of whom to contact for answers to pertinent questions about the research and the patient's rights, and whom to contact in the event of a research related injury; and:
8. A statement that participation is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the patient is otherwise entitled, and that the patient may discontinue participation at any time without penalty or loss of benefits to which the patient is otherwise entitled.

ADDITIONAL ELEMENTS OF INFORMED CONSENT

When appropriate, one or more of the following elements of information shall also be included in the consent form:

- A statement that the particular treatment or procedure may involve risks to the patient (or to the embryo or fetus, if the patient\ or may become pregnant) which are currently unforeseeable;
- Anticipated circumstances under which the Investigator without regard to the patient's consent may terminate the patient's participation;
- Any additional costs the patient might incur from participation in the research; The consequences of a patient's decision to withdraw from the research and procedures for orderly termination of participation by the patient;
- A statement that significant new findings developed during the course of the research that might affect the patient's willingness to continue participation will be provided to the patient; and,
- The approximate number of patients involved in the study.

Nothing in these regulations is intended to limit the authority of a physician to provide emergency medical care to the extent the physician is permitted to do so under applicable federal, state, or local laws.

Informed consent allows the patient to fully understand his or her participation and serves to protect the Investigator and Sponsor from potential negligence claims, A fully informed patient is the best protection against such claims.

The informed consent requirements in these regulations are not intended to preempt any applicable federal, state, or local laws that require additional information to be disclosed in order that informed consent is legally effective. Some states, such as California and Oregon, require further action on the Investigator's part concerning subject consent.



July 7, 2010

Ruyi He, M.D.
Acting Deputy Director
U.S. Food & Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266

RE: Addison and Cassidy Hempel(CURRENT INDs: 104,114 and 104,116)

Dear Dr. He:

As the proud and loving parents of Addison and Cassidy Hempel, we are writing to the United States Food and Drug Administration to acknowledge that we understand the risks and dangers associated with the current proposal by our doctors to deliver hydroxy-propyl-beta-cyclodextrin (HPBCD) into the central nervous system, and ultimately the brains, of our identical twin girls.

As you know, Addison and Cassidy suffer from Niemann Pick Type C disease (NPC), a devastating and progressive neurological condition which is fatal and has no approved medical treatments.

For the past year, as our doctors have treated Addison and Cassidy with infusions of HPBCD into the bloodstream, we have been funding important new research studies on cyclodextrin to determine if the compound crosses the blood brain barrier. Research by leading blood brain barrier experts shows that little to none crosses the blood brain barrier and we have unfortunately watched our children continue to decline neurologically despite intravenous administration of cyclodextrin.

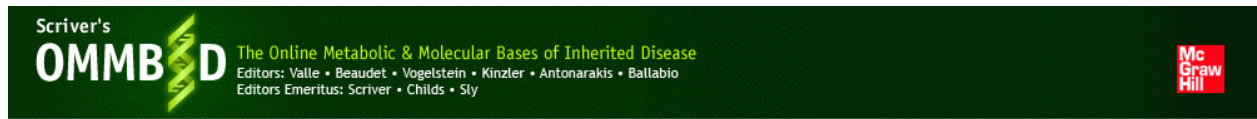
In addition, over the past year, intrathecal cyclodextrin treatments have been ongoing in NPC animal models and they are having a profound neurological effect on these NPC afflicted animals. In felines, the treatment appears to be completely arresting neurological progression of this deadly genetic cholesterol metabolism disorder.

We believe that cyclodextrin is a potentially lifesaving treatment for our twins. We fully accept the risks of this proposed treatment and give our full consent as their guardians to receive this highly experimental treatment.

We sincerely hope that after reviewing all of the data available, the FDA will agree to allow our doctors to begin this potentially life-saving treatment right away under compassionate use.

With best regards,

Hugh and Christine Hempel



PART 16: LYSOSOMAL DISORDERS

Chapter 145: Niemann-Pick Disease Type C: A Lipid Trafficking Disorder

Marc C. Patterson, Marie T. Vanier, Kinuko Suzuki, Jill A. Morris, Eugene Carstea, Edward B. Neufeld, Joan E. Blanchette-Mackie, Peter G. Pentchev

Abstract

1. Niemann-Pick disease type C (NP-C) is an autosomal recessive lipidosis with protean clinical manifestations, distinguished biochemically by a unique error in cellular trafficking of exogenous cholesterol that is associated with lysosomal accumulation of unesterified cholesterol. A majority of patients with this phenotype are linked genetically to chromosome 18, the locus of Niemann-Pick disease type 1 gene (*NPC1*). *NPC1* is a novel gene whose predicted protein product contains between 13 and 16 transmembrane domains, and a sterol-sensing domain with homologies to Patched, HMG-CoA reductase, and sterol regulatory element binding protein [SREBP] cleavage-activating protein (SCAP). A region designated the NPC domain, conserved in yeast, nematode, and mouse, contains a leucine zipper. NP-C is distinct at clinical, biochemical, and molecular levels from the primary sphingomyelin lipidoses (Niemann-Pick disease types A [NP-A] and Niemann-Pick disease types B [NP-B], respectively), with which it has traditionally been grouped. Niemann-Pick disease type D (NP-D) is allelic with NP-C, and should be regarded as a variant phenotype associated with a genetic isolate, rather than a distinct entity. A small group of patients belong to a second genetic complementation group that does not link to chromosome 18. These individuals are believed to have mutations in a gene provisionally designated Niemann-Pick disease type 2 gene (*NPC2*).
2. The clinical manifestations of NP-C are heterogeneous. Most patients with NP-C have progressive neurologic disease, although hepatic damage is prominent in certain cases, and may be lethal in some. Variable hepatosplenomegaly, vertical supranuclear ophthalmoplegia, progressive ataxia, dystonia, and dementia characterize the "classic" phenotype. These children present in childhood, and die in the second or third decade. Other phenotypes include presentations with fetal ascites, fatal neonatal liver disease, early infantile onset with hypotonia and delayed motor development, and adult variants in which psychiatric illness and dementia predominate.
3. NP-C is panethnic. Genetic isolates have been described in Nova Scotia (formerly Niemann-Pick disease type D) and southern Colorado. Complementation studies have demonstrated two distinct groups. About 95 percent of patients link to chromosome 18q11, and thus to *NPC1*; the remainder are believed to have mutations in a second gene, provisionally designated *NPC2*.
4. NP-C has an estimated prevalence of approximately 1:150,000, making it a more common phenotype than NP-A and NP-B combined. It is likely that the true prevalence of the disease has been underestimated because of confusing terminology, the lack of a definitive diagnostic test prior to the discovery of the abnormalities of cellular cholesterol processing, and failure to recognize the clinical phenotypes.
5. Foam cells or sea-blue histiocytes are found in many tissues. Such cells are not specific for NP-C and may be absent, particularly in cases lacking visceromegaly. Characteristic inclusions (polymorphous cytoplasmic bodies) may be identified in skin and conjunctival biopsies. Neuronal storage with cytoplasmic ballooning and a variety of inclusions is present throughout the nervous system. Neurofibrillary tangles, meganeurites, and axonal spheroids are also seen.

6. In most cases of **NP-C**, the primary molecular defect lies in **NPC1**. Unesterified cholesterol, sphingomyelin, phospholipids, and glycolipids are stored in excess in the liver and spleen. Glycolipids are elevated in the brain, the principal target of this disease. There is no overt increase in cholesterol in the brain in human NP-C or its animal models. Partial sphingomyelinase deficiency, observed only in cultured cells (and never in leukocytes or solid tissues), represents a variable, secondary consequence of lysosomal cholesterol sequestration. Cultured fibroblasts show a unique disorder of cellular cholesterol processing, in which delayed homeostatic responses to exogenous cholesterol loading are impaired in association with cholesterol accumulation in lysosomes.
7. The diagnosis of **NP-C** requires both documentation of a characteristic pattern of filipin-cholesterol staining and measurement of cellular cholesterol esterification in cultured fibroblasts during LDL uptake. Candidates for such testing are identified chiefly by clinical presentation with or without supportive findings from neurophysiological tests and tissue biopsies. There is considerable variability in the degree of impairment of cholesterol trafficking in NP-C. Consequently, antenatal diagnosis has been restricted to families in which the biochemical abnormalities are pronounced. Molecular diagnosis will offer a desirable alternative in families where mutations in **NPC1** have been identified in the index case.
8. Symptomatic treatment of seizures, dystonia, and cataplexy is effective in many patients with **NP-C**. Combination drug regimens have been shown to lower hepatic and plasma cholesterol levels in human NP-C. There is no evidence that such therapy alters the progression of the disease in humans or murine models.
9. Animal models with clinical, pathologic, and biochemical features resembling **NP-C** have been described in two species of mice, as well as in the cat and boxer dog. The murine ortholog of **NPC1** is mutated in the C57BLKS/J and BALB/c models.

Major advances in our understanding of Niemann-Pick disease type C (**NP-C**) have occurred since the last edition of this text. Complementation studies have demonstrated that the NP-C phenotype results from mutations in two distinct genes. The gene associated with the smaller complementation group, accounting for perhaps 5 percent of NP-C patients, has not yet been identified. This gene has been provisionally designated **NPC2**.

The **NPC1** gene was cloned in 1997, conclusively demonstrating that **NP-C** and the sphingomyelin lipidoses (Niemann-Pick disease types A and B [**NP-A** and **NP-B**, respectively]) are distinct entities at a molecular level, confirming their earlier separation on clinical and biochemical grounds. Furthermore, it was found that NP-C is allelic with Niemann-Pick disease type D (**NP-D**), justifying the elimination of NP-D as an entity separate from NP-C.

As well as providing a new tool for the investigation of cellular homeostasis, the identification of **NPC1** will also provide a new means of investigating the poorly understood pathogenesis of neurodegeneration in **NP-C**. The latter goal is a necessary condition for the development of effective therapy for this devastating neurologic disorder.

This chapter (a) traces the historic evolution of **NP-C**; (b) characterizes its distinct and diverse clinical features; (c) defines its unique cellular lesions; (d) describes and evaluates various diagnostic procedures; (e) discusses potential therapeutic strategies; and (f) speculates on the role of **NPC1** in cellular lipid trafficking.

HISTORY

The first recognizable description of NP-C appeared in Crocker and Farber's review of Niemann-Pick disease in 1958.¹ Until then, Niemann-Pick disease was restricted to infants conforming to Niemann's original description² of infantile neurodegenerative disease with hepatosplenomegaly. Crocker and Farber based their diagnosis of Niemann-Pick disease on the presence of foam cells and increased tissue sphingomyelin, thus including children with indolent or absent neurologic disease in this category. All of the classic neurologic features of NP-C were present in their patient 15, namely: vertical supranuclear gaze palsy; ataxia; dystonia; dementia; cataplexy; dysarthria; spasticity; and seizures. They also described several other presentations of NP-C, including prolonged neonatal jaundice with death in infancy (patient 2); hypotonia and delayed motor milestones (patient 9); isolated organomegaly (patient 10); seizures (patient 13); and learning and behavioral problems at school (patient 14).

Crocker later classified Niemann-Pick disease into four groups based on biochemical and clinical criteria.³ Group A (NP-A) included the classic patients with neurodegenerative disease leading to death in infancy; group B (NP-B) patients had organomegaly without nervous system disease; group C (NP-C) patients had slowly progressive neurologic illness; and group D (NP-D) closely resembled group C, except for its restriction to a genetic isolate in Nova Scotia. Nonneural tissues in the latter two groups had relatively less sphingomyelin, and more cholesterol storage than tissue from group A and B patients.

Numerous reports in the 1960s and 1970s described a disorder now clearly recognizable as NP-C. The diagnoses included atypical cerebral lipidosis;⁴ juvenile Niemann-Pick disease;⁵ dystonic juvenile idiocy without amaurosis;⁶ juvenile dystonic lipidosis;⁷⁻⁹ giant cell hepatitis;¹⁰ lactosylceramidosis;¹¹ neurovisceral storage disease with vertical supranuclear ophthalmoplegia;¹² maladie de Neville;¹³ DAF (down gaze paresis, ataxia, foam cell) syndrome;¹⁴ adult dystonic lipidosis;¹⁵ and adult neurovisceral lipidosis.¹⁶ The confused terminology notwithstanding, the salient clinical features of vertical supranuclear gaze palsy (VSGP),^{17, 18} ataxia, dystonia, and dementia were well described and clearly established NP-C as a distinct disorder.

In 1966 Brady and coworkers¹⁹ demonstrated severe generalized sphingomyelinase deficiency in NP-A, a finding that was soon extended to type B, but not to types C and D,²⁰ indicating that the two latter types constituted separate entities. Hypotheses invoking the deficiency of a specific sphingomyelinase isoenzyme²¹ or of a sphingomyelinase activator protein²² were disproved²³⁻²⁵ and the concept of secondary sphingomyelin lipidosis was strengthened by observations of multiple lipid storage in NP-C.^{23, 26} Somatic cell hybridization studies²⁷ supported the concept of NP-C as a separate entity. In 1982, a consensus was reached in Prague to separate the sphingomyelinase-deficient forms (NP-A and NP-B) from the other forms of Niemann-Pick disease (NP-C and NP-D).²⁸

In 1984, the seminal observation by Pentchev and coworkers²⁹ of defective cellular esterification of exogenously derived cholesterol in the BALB/c murine model of the disease^{30, 31} led to the discovery of an identical lesion in NP-C and further demonstration of unique abnormalities of intracellular transport of LDL-derived cholesterol with sequestration of unesterified cholesterol in lysosomes in the disease³²⁻³⁵ (see "Pathophysiology" below). From that time on, the concept of NP-C evolved from a primary sphingomyelin storage disorder to a primary cholesterol lipidosis. Strategies based on cholesterol trafficking abnormalities facilitated the early diagnosis of patients.^{36, 37} Mapping of the murine *spm* gene to chromosome 18³⁸ and subsequent correction of the metabolic lesion in mutant murine fibroblasts by human chromosome 18³⁹ facilitated linkage studies in the human disease. Genetic heterogeneity, with two complementation groups, was demonstrated in NP-C using combined cell hybridization and linkage studies.^{40, 41} The gene involved in more than 95 percent of the patients (*NPC1*) has been mapped to

18q11.^{42, 43} Definitive progress has been achieved with the isolation of the *NPC1* gene⁴³ and its murine ortholog.⁴⁴

CLINICAL MANIFESTATIONS

NP-C is a disorder with protean manifestations that can present at any time from intrauterine life to adulthood (see Table 145-1). Manifestations may be primarily hepatic, neurologic, or psychiatric. Patients with NP-C may thus present to perinatologists, pediatricians, family practitioners, hematologists, gastroenterologists, neurologists, internists, or psychiatrists, all of whom should be familiar with this disease.

Table 145-1: Clinical Presentations of NP-C

Age of onset	Presentation
Perinatal period	Fetal ascites Neonatal jaundice Benign, self-limiting Rapidly fatal Hepatosplenomegaly VSGP usually absent
Early infantile	Hypotonia Delay of motor milestones Hepatosplenomegaly VSGP usually absent
Late infantile	"Clumsy" frequent falls (ataxia) Isolated organomegaly
VSGP may be present	
Juvenile	School failure (intellectual impairment and impaired fine movements) Behavioral problems Ataxia, dysarthria, dystonia Seizures Cataplexy VSGP present
Adolescent and adult	Progressive neurologic deterioration Dementia Psychosis VSGP may be present

Classic Niemann-Pick Disease Type C

The “classic” **NP-C** patient is the product of a normal pregnancy; about half of these children have transient neonatal jaundice. Although development in early childhood is usually unremarkable, the child may be labeled as ill-behaved on entering kindergarten or school. Many years may pass before it becomes apparent that the child is slowly dementing. Meanwhile, the child is labeled as clumsy and suffers frequent falls before overt ataxia is recognized. Eye blinking or head thrusting on attempted vertical gaze may be noted. Gelastic cataplexy may appear at this time, with manifestations as subtle as head nodding or as dramatic as atonic collapse with injury. Dysarthria, dysphagia, and drooling contribute to educational problems by impairing communication and exposing the child to ridicule. Dystonia first manifests as posturing of a hand or foot when walking or running, and gradually becomes generalized. Partial, generalized, or mixed seizures may begin in childhood or later. Enlargement of the liver or spleen is often first detected in early childhood and usually regresses over time. In at least 10 percent of patients, hepatosplenomegaly is never detected.

The child suffers increasing physical and intellectual disability through late childhood and adolescence, eventually becoming chairbound and incapable of continuing at school. Psychiatric disturbances including psychosis may coincide with the onset of puberty. Severe dysphagia now imperils nutrition, and the upper airway is poorly protected. In many cases, spasticity or rigidity (or both) add to the burden of nursing care. Death from pulmonary complications occurs in the teenage years or early adulthood.

Variant Phenotypes

The “classic” clinical profile accounts for 50 to 60 percent of cases of **NP-C** in large series. Many alternative presentations have been described.

Four children with fetal ascites^{45, 46} have been described; only one survived the first year. Two died from hepatic failure and the other from respiratory failure.⁴⁷ Foam cells were found in the pulmonary interstitium in this patient,⁴⁷ and in another patient with progressive neonatal liver failure.⁴⁸ One child presented at 4 months with respiratory symptoms.⁴⁹ Further cases presenting with pulmonary involvement have been described.⁵⁰ A link between this aggressive pulmonary phenotype and complementation group two has been suggested.⁵¹

As many as half the patients with **NP-C** have neonatal cholestatic jaundice that is usually self-limiting;⁵² in as many as 10 percent of **NP-C** cases, terminal hepatic failure without neurologic symptoms occurs.^{10, 48, 49, 52–55} **NP-C** has been reported as the second most common genetic cause of liver disease in infancy in the United Kingdom, after α_1 -antitrypsin deficiency.⁵⁶ Neonatal jaundice without overt liver disease may herald a more aggressive clinical course, with neurologic abnormalities appearing in the first 4 years of life.⁵⁷ In exceptional cases, neurologic dysfunction is not apparent until adolescence.

Some children present with hypotonia and delayed motor development before 2 years of age.^{52, 58, 59} These children invariably have hepatosplenomegaly, usually do not learn to walk, develop intention tremor and generalized spasticity, and die between 3 and 5 years of age. **VSGP** is not seen. This phenotype is more frequently recognized in patients from southern Europe, the Middle East, and North Africa than in those from the U.S.⁶⁰ One child was described with symptomatic peripheral neuropathy.⁶¹ Other childhood presentations include visceromegaly discovered in the course of intercurrent illness, cataplexy with⁶² or without narcolepsy,⁶³ or a rigid-akinetic syndrome.⁶⁴ There is considerable overlap among these early onset groups.

In contrast to these aggressive presentations, insidious onset and slow progression characterize late onset cases. Cognitive and psychiatric disturbances are prominent, and may overshadow other findings. Shulman and colleagues⁶⁵ reviewed 16 cases of NP-C with adult onset, including a new case of their own. Age of onset ranged from 18 to 59 years (mean 32 years). Signs at presentation included dysarthria (44 percent), dementia (31 percent), psychosis (25 percent), limb ataxia (25 percent), and gait ataxia (25 percent). During the course of the illness, the signs observed included dementia (81 percent), gait ataxia (75 percent), limb ataxia (69 percent), dysarthria (56 percent), VSGP (56 percent), splenomegaly (50 percent), psychosis (38 percent), extrapyramidal signs (38 percent), pyramidal signs (31 percent), dysphagia (25 percent), hepatomegaly (13 percent), and seizures (6 percent).^{15, 16, 65–70} One patient was described with clinical and imaging findings mimicking multiple sclerosis.⁷¹

Two recent, biochemically well-documented observations suggest the existence of a nonneuronopathic (or, alternatively, very delayed neurologic onset) variant of NP-C. The first patient was diagnosed at the age of 46 because of an enlarged spleen that was ruptured in a traffic accident.⁷² Another patient with splenomegaly and thrombocytopenia was diagnosed at age 52, following splenectomy (Massenkeil, Harzer, and Vanier, unpublished). Other similar, albeit less well documented, patients are known.^{73, 74} This raises the question of a possible relationship with the ill-defined patients reported as Niemann-Pick type E.⁷⁵

Specific Symptoms and Signs

Certain manifestations of NP-C deserve separate comment. VSGP is the neurologic hallmark of this disease, and it has been present in all juvenile and adult cases examined by one author (M.C.P.). Early and late onset patients in whom this sign was not found have been reported.^{65, 74} Increase in saccadic latency, followed by subtle slowing of vertical saccades (upward or downward) begins in childhood, and may be accompanied by blinking or head thrusting. Older patients complain of difficulty negotiating stairs, or that their eyes become stuck at extremes of vertical gaze. Voluntary vertical gaze is completely paralyzed in the late stages of the illness, and horizontal eye movements may also be affected. Oculocephalic reflexes are preserved. The sign is easily missed if not specifically sought. Saccades should always be examined in addition to pursuit movements. For cooperative patients, an optokinetic stimulus may be helpful. Recent studies suggest that VSGP in NP-C most likely reflects selective dysfunction of vertical burst neurons in the brain stem^{76, 77} (see “Pathology” below).

Cataplexy usually occurs late in the neurologic illness,^{78, 79} but may be the presenting feature.^{62, 63} Typically, the loss of postural tone is evoked by a humorous stimulus (gelastic cataplexy), and the resulting falls may lead to repeated injury.⁷⁸ This symptom also correlates with brain stem disease.^{63, 78–83}

EPIDEMIOLOGY AND CLINICAL GENETICS

NP-C shows autosomal recessive inheritance. Clinical heterogeneity within families is limited,⁸⁴ except when one sib has a rapidly fatal cholestatic form and another sib survives to show neurologic signs.^{84, 85} Few data are available on the epidemiology of NP-C, but the disease is clearly panethnic. Two genetic isolates have been described, French Acadians in Nova Scotia (formerly NP-D)³ and Spanish-Americans in southern Colorado.⁸⁶ Their phenotypes, although variable, are indistinguishable from other patients with NP-C. A study in Yarmouth County, Nova Scotia⁸⁷ found a 1 percent incidence of NP-C and an estimated a carrier frequency between 10 and 26 percent (95 percent confidence limits). The disease is apparently much less common in the general population. A prevalence of about 1/150,000 live births has been calculated for France, West Germany, and the U.K. from the number of cases diagnosed over a 15-year period (M.T.V.). In those countries, NP-C thus appears more frequent than NP-B and NP-A

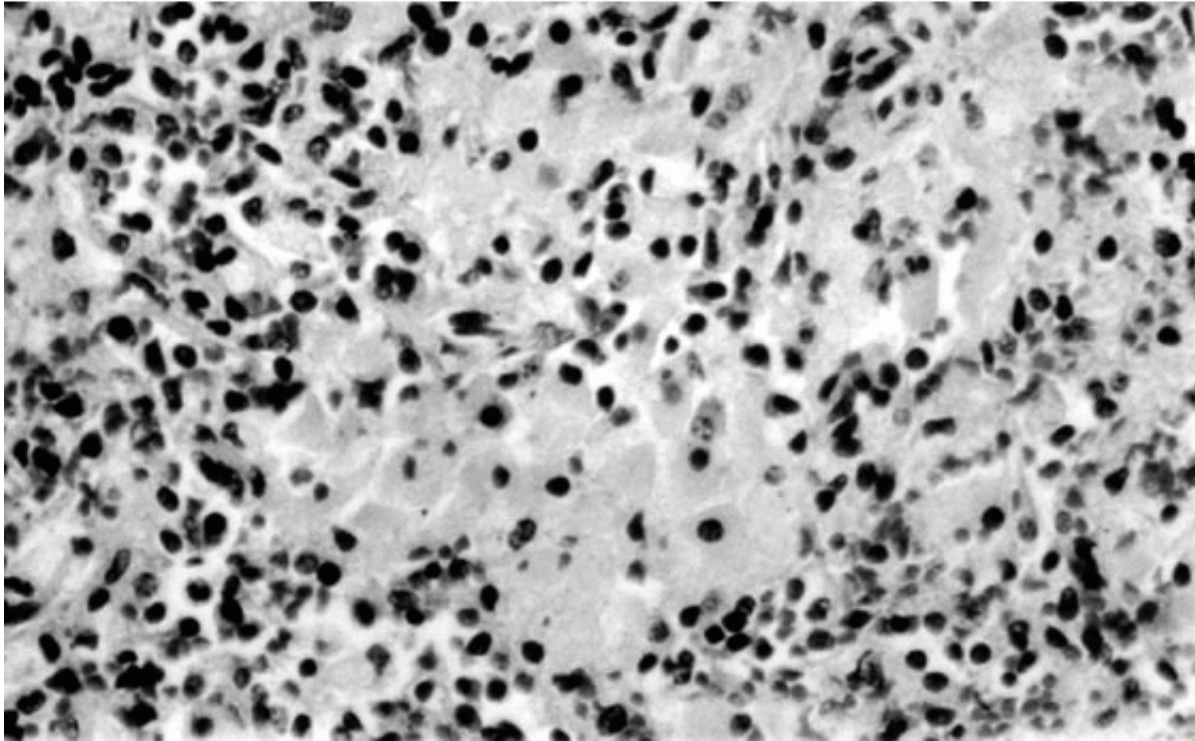
combined. The prevalence of NP-C has undoubtedly been underestimated in the past, due to confusing terminology, the previous lack of specific biochemical tests, and the many variant forms of the disease.

PATHOLOGY

Human Disease

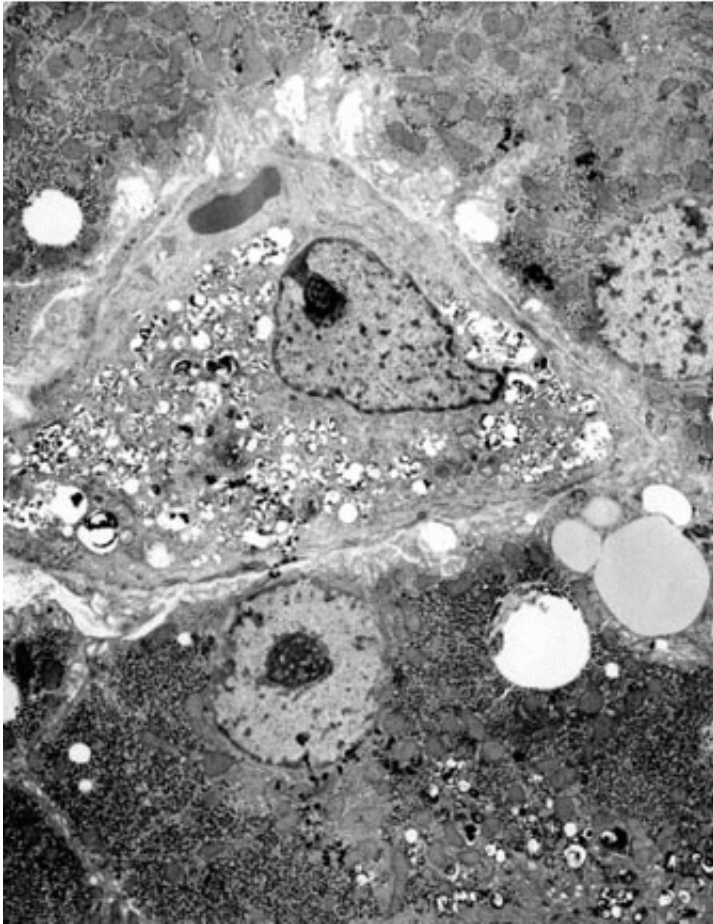
In all clinical forms of **NP-C**, the cardinal pathologic features are those of neurovisceral storage disease with foamy storage cells in the visceral organs and an accumulation of storage materials in neurons and glial cells in the nervous system. The severity of the pathologic manifestations varies considerably, reflecting the clinical heterogeneity of the disease.⁸⁸

The most notable gross pathologic feature is splenomegaly with or without associated hepatomegaly. Hepatic involvement may be prominent in early life. In most juvenile or adult patients, hepatomegaly or lymphadenopathy is not seen.⁶⁰ Histopathologically, two distinct types of cells, foamy cells (macrophages) and sea-blue histiocytes are seen in bone marrow preparations.^{12, 15, 86} Intermediate forms also occur. These cells stain variably with Luxol fast blue (LFB), periodic-acid Schiff (PAS), and Sudan black stains, and are strongly positive with the Schultz reaction for cholesterol and for acid phosphatase.^{9, 12, 89–92} Foamy cells and sea-blue histiocytes are conspicuous in spleen (Fig. 145-1), tonsils, lymph nodes, liver, and lung. These cells tend to be clustered in the red pulp in spleen and within the sinusoids in the liver. Microvacuolation may be found in the hepatocytes.^{90, 91} Hepatic pathology is usually more conspicuous in the early onset cases, in which giant cell transformation of hepatocytes (“giant cell hepatitis”), cholestasis, or both have been reported.^{10, 48, 53, 55, 93} In some infantile cases, severe pulmonary involvement causing death by respiratory failure has been reported.^{47, 50, 51} Three of these early lethal pulmonary involvement cases were found to belong to the rare genetic complementation group 2 (see “Molecular Genetics” below). In adult cases, only a few foamy cells may be found in the hepatic sinusoid without significant storage in hepatocytes.¹⁶

Fig. 145-1:

Clusters of foamy macrophages in the spleen (H&E, x520).

Dumontel and coworkers reported the pathology of a 20-week fetus including detailed ultrastructural analysis of storage cells in the liver and spleen. They found many pleomorphic lysosomes and variously shaped crystalline structures resembling cholesterol crystals.⁹⁴ Similar crystalline structures have not been reported in postnatal NP-C cases in humans, but are seen in macrophages in the brain and liver of the murine model of NP-C⁹⁵ (see “Animal Models” below). Pathologic involvement of skin, skeletal muscle, and eye may be subtle on routine histopathologic preparation, but abnormal inclusions can be found in histiocytes at the ultrastructural level.^{96–99} The cytoplasmic inclusions are polymorphic structures of various electron densities, consisting of stacked, closely packed osmiophilic membranes of varying size and thickness admixed with multiple electron-lucent vacuoles^{100, 101} (Figs. 145-2 and 145-3). Similar pleomorphic membranous inclusions can be also found in conjunctival epithelial cells; endothelial cells and pericytes; keratinocytes; lens epithelium; retinal ganglion cells; retinal pigment epithelium; pericytes; Schwann cells; smooth muscle cells; and fibroblasts.^{96–98} Sea-blue histiocytes contain concentrically arranged, tightly packed electron-dense inclusions and lipofuscin granules.^{9, 89}

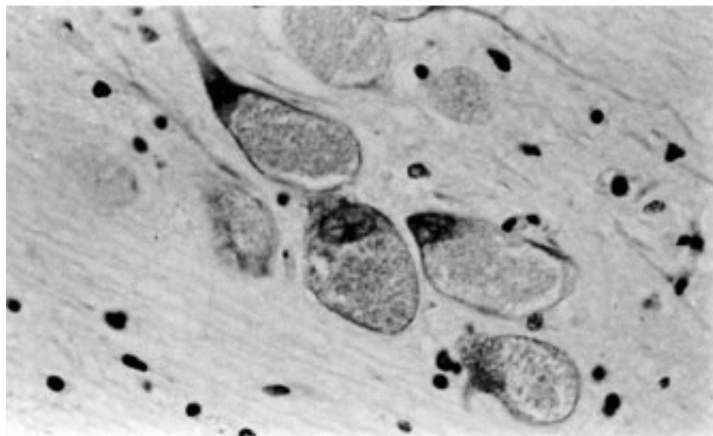
Fig. 145-2:

Electron micrograph of liver showing numerous inclusions in a macrophage (Kupffer cell) in the center and surrounding hepatocytes (×8600).

The brain is often atrophic, severely so in patients with a slowly progressive clinical course. Neuronal storage is the most conspicuous cerebral pathology (Fig. 145-4). Cortical neurons, in particular large pyramidal neurons in the deep cortical layers, show distended cytoplasm. In hematoxylin and eosin (H&E) preparations, pale gray-blue granular inclusions or fine vacuolation are noted in the perikarya, and axonal hillocks appear swollen (meganeurites). These granular inclusions are variably stained with PAS and/or **LFB** but are negative¹⁰² or only weakly positive⁸⁹ for the histochemical stain for cholesterol. In the basal ganglia and thalamus, larger neurons tend to be more affected. The degree of neuronal cytoplasmic ballooning and distribution of such storage neurons may vary considerably in individual cases.^{5, 100} Golgi preparations of the cerebral cortex reveal meganeurites, ectopic neurites, and irregular focal swellings of dendrites of affected pyramidal neurons in the deep cortical layers.^{103, 104} These features are very similar to those of storage neurons in the gangliosidoses (see Chaps. 151 and 153). Small pyramidal neurons in the layers II, III, and IV are often devoid of storage materials.^{103, 104} The cerebral white matter is usually normal, although demyelination with perivascular collections of macrophages containing sudanophilic granules has been reported.¹⁰² Neuroaxonal dystrophy in the form of axonal spheroids is found throughout the neuraxis, in particular in the thalamus (Fig. 145-5), dentate nucleus, and midbrain,

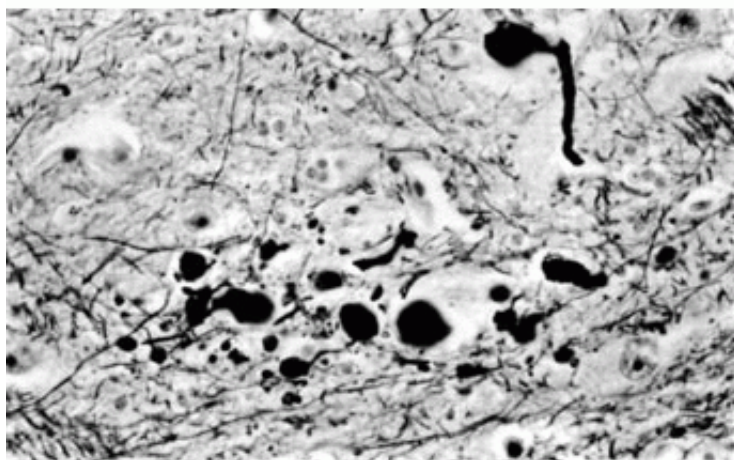
including substantia nigra.^{59, 102} The cerebellum is variably affected by this process. In severe cases, both Purkinje and granular cells are lost and replaced with dense fibrillary gliosis.^{5, 59, 100} Most of the surviving Purkinje cells and Golgi cells show the perikarya distended with storage materials. Antler-like swelling of Purkinje cell dendrites may be seen.⁵ The perikarya of both anterior and posterior horn neurons in the spinal cord are distended with storage materials. Neuronal storage is also prominent in the myenteric plexus.⁵⁹

Fig. 145-4:



Storage neurons with ballooned perikarya in the substantia nigra (Nissl stain, x520).

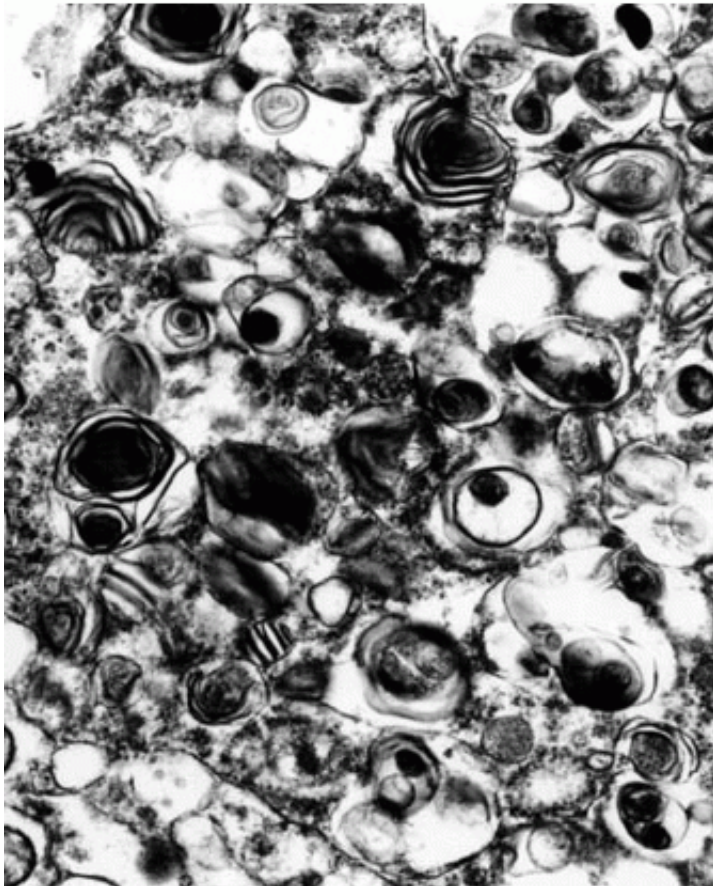
Fig. 145-5:



Clustered axonal spheroids in the thalamus (Bielschowsky stain, x520).

Ultrastructurally, neuronal inclusions consist of heterogeneous lamellar structures, called polymorphous cytoplasmic bodies (PCBs) (Fig. 145-6). Some neurons contain cytosomes resembling the membranous cytoplasmic bodies (MCBs) of gangliosidoses, “zebra bodies,” or both. Lipofuscin-like bodies or compound PCBs surrounded by a single membrane may be found in some neuronal perikarya. These complex PCBs are often associated with dilated smooth membrane profiles of Golgi apparatus. Microglia may be enlarged and often contain PCBs.^{4, 6, 9, 89, 100, 101} Similar inclusions are also found in hepatocytes (Fig. 145-4).

Fig. 145-6:



Electron micrograph of polymorphous inclusions (PCBs) in neuronal perikarya (×66,600).

Recent studies have shown that neurofibrillary tangles (NFTs) associated with neuropil threads are consistent findings in the brains of NP-C patients with a prolonged clinical course. These NFTs consist of paired helical filaments (PHF).^{67, 68, 105–108} Paired helical filament tau (PHFtau) in NP-C is similar to that of Alzheimer disease (AD).¹⁰⁹ Unlike AD, NFTs in NP-C predominantly involve the deeper layers of the cerebral cortex, thalamus, basal ganglia, hypothalamus, brain stem, and spinal cord. Entorhinal cortex, orbital gyrus, and cingulate gyrus are commonly affected. The distribution of NFTs generally parallels that of the swollen storage neurons. Paired helical filaments (PHFs) are found in the swollen perikarya as well as in the meganeurites. However, neurons without swollen perikarya may contain NFTs. NFTs can be

found in a number of non-Alzheimer cases,¹⁰⁸ and thus the presence of NFTs in NP-C cases may simply represent a nonspecific neuronal response to metabolic perturbation. However, the frequent association of a prolonged clinical course and the presence of NFTs, even in patients as young as 10 years,¹⁰⁸ may suggest that the association is more than coincidental and reflects unique neuropathology in NP-C.

Morphologic studies of the peripheral nerves are scarce. Axonal degeneration and membrane-bound lysosomal inclusions have been reported in Schwann cells, endoneurial fibroblasts, macrophages, pericytes, and endothelial cells.^{61, 110}

Animal Models of NP-C

A feline model,¹¹¹ canine model,¹¹² and two murine models (BALB/c npc^{nih} and C57BLKS/J *spm*),^{95, 113–115} are known. The C57BLKS/J mouse, also known as the “sphingomyelinosis” mouse, was reclassified as a model of NP-C rather than of NP-A.¹¹⁶ Light and electron microscopic features of these models closely resemble human NP-C. In both canine and feline models, infiltration of foamy macrophages is extensive in the lung, liver, spleen, and lymph nodes. Hepatosplenomegaly is absent in the canine model.

Neuronal storage is prominent throughout the cerebrum. Cortical pyramidal neurons in laminae II, III, and V in the feline model exhibit meganeurite formation with or without ectopic dendritogenesis.¹⁰⁴ Axonal swelling or axonal spheroids is very frequent in the feline model¹¹¹ but is relatively mild in the canine model.¹¹² These axonal spheroids demonstrate immunoreactivity for glutamic acid decarboxylase (GAD).¹⁰⁴ A marked loss of Purkinje and granular cells is documented in the cerebellum of the canine model while Purkinje cells are relatively well preserved in the feline model. Segmental myelin loss is noted in the spinal roots in feline NP-C.

Two murine models have almost identical histopathology. Hepatomegaly is not apparent. Foamy macrophages can be detected in young asymptomatic mice and their numbers increase significantly with age. These foamy cells stain positively with various lectins, indicating the heterogeneous nature of the storage material.¹¹⁷ Alveolar macrophages in younger mice contain osmiophilic dense granules and annulolamellar structures, but larger multilamellar concentric structures are found more frequently in the older mice.¹¹⁸ The brain of the affected mice is smaller than controls, with atrophy of the cerebellum and midbrain region including colliculi.^{95, 114} In both murine models, neuronal storage and axonal spheroids are very conspicuous. In BALB/c npc^{nih} mice, hypomyelination and myelin degeneration have been reported,^{95, 115} whereas myelination is well preserved in the C57BLKS/J mice.¹¹⁴ In the older mice, loss of Purkinje cells and abnormal dendritic arborization of the surviving Purkinje cells are observed.^{114, 119, 120} Ultrastructural features of these storage neurons are closely similar to those described in feline and human NP-C and numerous concentric lamellar inclusions are found in the perikarya. Inclusions are also seen in astrocytes, oligodendrocytes, microglia, and vascular endothelial cells. Myelination of the spinal roots and peripheral nerves appears normal. However, teased fiber preparations of the sciatic nerve show accumulation of myelin ovoids in practically all paranodal regions of the Schwann cells in BALB/c npc^{nih} mice,¹²¹ but myelin sheaths are reported to be preserved in C57BLKS/J mice.

Pathology in Heterozygotes

In lysosomal storage diseases, heterozygotes are usually normal. However, foamy bone marrow cells and inclusions in skin fibroblasts have been reported in the humans heterozygous for NP-C.^{122, 123} Furthermore, in feline obligate heterozygotes, occasional polymorphic membranous cytoplasmic inclusions have been reported in hepatocytes, biliary epithelial cells, and Kupffer cells.¹²⁴

PATHOPHYSIOLOGY

Tissue Lipids

Liver and Spleen.

A complex lipid storage pattern, with no compound predominating, is observed in liver and spleen in NP-C.^{23, 26} Apart from a moderate (twofold to fivefold) increase in sphingomyelin and unesterified cholesterol,^{1, 16, 26, 54, 55, 59, 75, 85, 125–127} bis(monoacylglycero)phosphate,^{8, 23, 26, 58, 126, 127} glucosylceramide,^{26, 58, 59, 89, 128, 129} and, to a lesser extent, other phospholipids and glycolipids accumulate. More lipid accumulates in spleen than in liver,²⁶ where alterations may be subtle.^{9, 130} Abnormal lipid storage is already present in the fetus and in fetal liver glucosylceramide accumulates to a greater extent in NP-C than in Gaucher disease.^{37, 59, 94, 130, 131} Very similar lipid abnormalities have been reported for the murine and feline models.^{30, 111, 132, 133}

Brain.

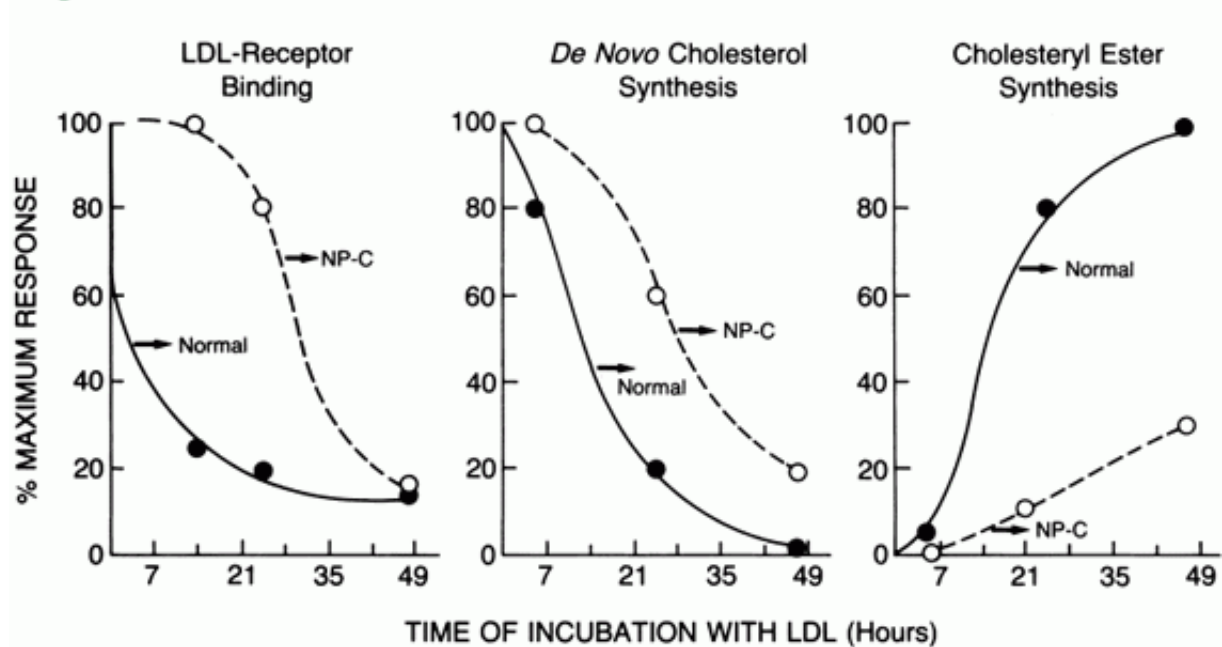
Brain lipids have been analyzed in a number of patients with NP-C, without evidence of a pathologic increase in cholesterol or sphingomyelin.^{3, 5, 53, 54, 75, 90, 91, 126, 127, 134–139} In cerebral cortex, the sole reported abnormalities pertain to glycolipids.^{9, 59, 90, 91, 102, 127, 129, 130, 137, 139–142} The most striking alterations are a many-fold increase of glucosylceramide (up to levels observed in type II Gaucher disease) and of lactosylceramide to the point that one case was initially described as lactosylceramidosis.^{11, 142} G_{M3} and G_{M2} gangliosides, as well as asialo-G_{M2} ganglioside, are significantly elevated. These alterations are not present in fetal brain and are discrete in the first months of postnatal life.^{130, 139} In white matter, these abnormalities are less marked but a varying decrease in myelin lipids has been observed. In cases with infantile onset and rapid progression of the neurologic disease, severely decreased galactosylceramide values indicate profound demyelination.^{59, 130, 136, 139} Milder changes in galactosylceramide are observed in the juvenile forms.^{9, 127, 130} Phospholipid and cholesterol concentrations are decreased in proportion to the degree of demyelination. The biochemical findings in human NP-C brains parallel those reported for the mutant BALB/c mouse.¹¹⁵

Cellular Cholesterol Lipidosis

Elucidation of the cellular basis of NP-C occurred serendipitously in the course of the study of mutant BALB/c mice that, in retrospect, had clear biochemical and pathologic similarities to human NP-C (see “Tissue Lipids” and “Animal Models” above).^{30, 133} A lesion in the intracellular processing of exogenous cholesterol was demonstrated in this murine model.²⁹ This led to the seminal discovery that cultured NP-C fibroblasts were also deficient in their ability to synthesize cholesteryl esters during endocytic uptake of LDL^{31, 32} and stored abnormal amounts of unesterified cholesterol in an intravesicular compartment when cultured in a cholesterol-enriched medium.^{33, 143, 144} Later studies from several laboratories led to more detailed understanding of the disruption in intracellular cholesterol metabolism. The subject has been reviewed by several authors.^{145–150}

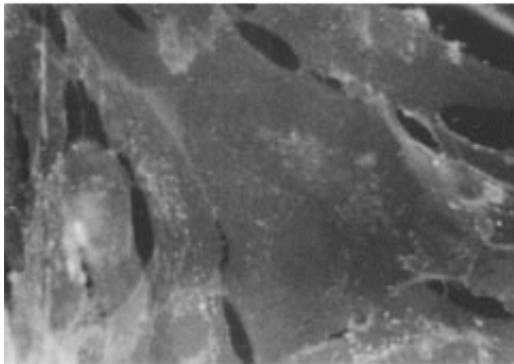
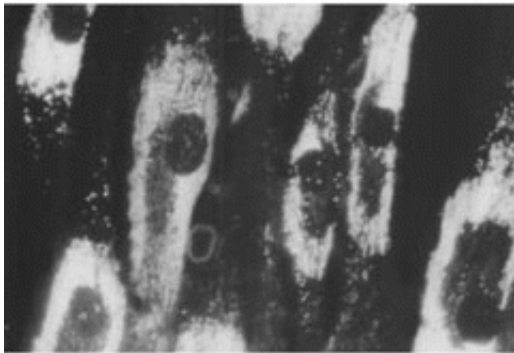
Internalization of LDL, its subsequent transport to lysosomes, and lysosomal hydrolysis of cholesteryl ester are not impaired in NP-C.^{33, 151} However, endocytosed cholesterol is sequestered in lysosomes and transport to the plasma membrane and the endoplasmic reticulum (ER) is retarded.^{33–35, 143, 145, 147, 152–154} There are three principal homeostatic responses following cellular uptake of LDL cholesterol¹⁵⁵ (see Chap. 120): (a) attenuation of *de novo* cholesterol synthesis; (b) depression of receptor-mediated LDL uptake; and (c) activation of cellular cholesterol esterification. All of these are compromised in NP-C fibroblasts³³ (Fig. 145-7), but are normal when the same cells are treated with 25-hydroxycholesterol.¹⁵¹ This indicates that the defect specifically affects the processing of endocytosed cholesterol and that the ability of the cell to respond is intact. Newly synthesized cholesterol does not appear to pass directly through the lysosome and is not affected by the sterol trafficking error.³⁵

Fig. 145-7:



Induction of cholesterol homeostatic responses in fibroblasts cultured with LDL. Normal and NP-C fibroblasts were cultured in lipoprotein-deficient serum to activate the LDL-receptor pathway. Cells were subsequently challenged with LDL to measure their ability to respond to the endocytic uptake and accumulation of the lipoprotein-derived cholesterol. (Adapted from Pentchev et al. 33 Used by permission.)

The intracellular distribution of cholesterol has been monitored with filipin, a fluorescent probe that forms specific complexes with unesterified cholesterol.¹⁵⁶ In cholesterol-depleted medium, little filipin-fluorescent staining is seen in either normal or NP-C fibroblasts. In normal cells, adding LDL to the medium results in enhanced filipin-cholesterol staining of the plasma membrane and some fluorescent staining of intracellular structures (Fig. 145-8A). In contrast, LDL uptake by NP-C cells is marked by striking filipin-cholesterol staining of perinuclear vacuoles (Fig. 145-8B), identified immunocytochemically as lysosomes.³⁴ This lysosomal sequestration of exogenous cholesterol is associated with impaired relocation to the plasma membrane.^{34, 35}

Fig. 145-8:**A****B**

Filipin-stained localization of unesterified cholesterol in fibroblasts cultured with LDL. Cholesterol-depleted normal (A) and NP-C (B) fibroblasts were cultured with LDL (50 mg/ml) for 24 h. Cells were subsequently fixed and stained with filipin to fluorescently label the intracellular distribution of LDL-derived cholesterol. A, In normal cells, cholesterol accumulation was associated with some enhanced filipin staining of the perinu...

In control fibroblasts, cholesterol egress from lysosomes may be compromised by hydrophobic amines (U-18666A, imipramine, natural sphingoid bases),^{156–158} by progesterone,¹⁵⁹ or by inhibitors of H^+ -ATPase such as bafilomycin.¹⁶⁰ The two first pharmacologic models have been widely used to study cellular trafficking of LDL-cholesterol.

The cellular consequences of the NP-C lesion may be traced in this order: the primary mutation leads to lysosomal sequestration of endocytosed cholesterol, to delayed induction of homeostatic regulation, and to cellular cholesterol lipidosis. The cellular distribution of cholesterol follows the descending concentration gradient: plasma membrane to endosomes to *trans*-Golgi to *cis*-Golgi to smooth endoplasmic reticulum to rough endoplasmic reticulum. The existence of this gradient implies the presence of specific trafficking mechanisms for its creation and maintenance. Exogenous cholesterol in the form of LDL enters the cell by receptor-mediated uptake,¹⁵⁵ and the endocytosed lipoproteins are targeted to lysosomes for hydrolysis of proteins and cholesteryl esters.¹⁵⁵ The mechanism of cholesterol efflux from lysosomes and transport to other sites in the cell is not well defined. Transfer of cholesterol to the plasma membrane from lysosomes appears rapid and constitutive,^{161, 162} but the route of trafficking is still under investigation. There is evidence for a plasma membrane-independent pathway from lysosomes to endoplasmic

reticulum.^{163, 164} Bidirectional circulation between lysosome and plasma membrane has been suggested.¹⁶⁵ The NP-C mutation has provided compelling evidence that the Golgi complex plays an active role in the transport of lysosomal cholesterol to other cellular membranes.^{148, 150, 163, 166} Filipin staining of NP-C cells following LDL uptake has revealed unusually early staining of cholesterol in the Golgi apparatus in addition to lysosomal cholesterol accumulation.¹⁵³ High-resolution electron microscopy of such cells subjected to freeze-fracture cytochemistry has shown accumulation of cholesterol in the *trans*-Golgi cisternae and deficient cholesterol relocation to *cis*-medial cisternae and adjacent condensing vacuoles.¹⁶⁶

It is not known if cholesterol first accumulates in the lysosome or the Golgi complex. Although the bulk of endocytosed unesterified cholesterol accumulates in lysosomes, the primary trafficking block in **NP-C** could affect cholesterol transport simultaneously at several organelles. Alternatively, block at an obligatory transport step at the Golgi complex could account for an upstream accumulation of cholesterol in the lysosomes. Indeed, disruption of this organelle by brefeldin-A redirects the flow of lysosomal cholesterol from the plasma membrane to the endoplasmic reticulum.¹⁶³ The abnormal distribution of cholesterol in the Golgi complex may therefore be linked to the finding that lysosomal cholesterol movement to the plasma membrane is retarded in NP-C cells.^{35, 163}

Notwithstanding these documented errors in cellular LDL cholesterol metabolism in cultured cells, the plasma lipoprotein profile of **NP-C** patients is normal. A recent study has, however, reported that intracellular trafficking of LDL-derived cholesterol is also defective in vivo in such patients.¹⁶⁷ Males with NP-C and same-sex controls were given [¹⁴C]mevalonate intravenously, as a tracer of *de novo* synthesized cholesterol, in addition to [³H]cholesteryl linoleate in LDL to monitor lipoprotein-derived sterol. The release of unesterified [¹⁴C]cholesterol into the plasma and bile was normal in controls. In marked contrast, the appearance of LDL-derived [³H]cholesterol in the plasma and bile was retarded in the affected individuals. The relative extent to which cholesterol transfer was delayed in the three NP-C patients correlated with the degree to which LDL cholesterol metabolism was affected in cultured fibroblasts derived from these same individuals. The kinetic data obtained in this fashion can be analyzed by compartmentalized modeling to provide a measure of both the size and turnover of the NP-C-induced cholesterol pools in vivo. This may provide a measurable endpoint for therapeutic interventions, if indeed extraneural cholesterol storage is relevant to disease progression (see “Treatment” below).

Sphingolipid Metabolism

Early studies attempted to link the **NP-C** mutation to a deficiency of sphingomyelin catabolism (see “History” above). Levels of sphingomyelinase activity are normal or elevated in tissues and leukocytes of NP-C patients.^{20, 75, 126, 127, 131, 168} In cultured fibroblasts, partial deficiency of sphingomyelinase activity^{86, 168, 169} with decreased degradation of exogenous sphingomyelin^{170–172} is found, albeit inconsistently.^{52, 171} It has been shown convincingly that depression of sphingomyelinase activity in NP-C-cultured fibroblasts is a secondary and reversible consequence of excessive cholesterol sequestration.^{36, 173} Glucosylceramidase activity is not decreased in solid tissues but often shows partial deficiency in cultured cells.¹⁷⁴ In contrast to human patients, solid tissues from the murine models show partial deficiencies of both sphingomyelinase and glucosylceramidase.^{30, 132}

G_{M2} ganglioside accumulates in lysosomes of cultured cells of patients, in spite of normal in vitro activities of hexosaminidase A.¹⁷⁵ There was evidence of an increased rate of biosynthesis, and accumulation occurred independently of the LDL-cholesterol uptake. Impaired cellular transport of G_{M2} ganglioside in **NP-C** has been suggested as a mechanism for this observation.^{175–177} Nevertheless, the explanation for the storage of several glycolipids, all with a short oligosaccharide chain, in human tissues (especially

brain) remains elusive, and is an area of continuing investigation.

Another intriguing observation is the many-fold elevation of free sphingoid long-chain bases, shown both in the BALB/c mouse model¹⁷⁸ and in human patients' tissues and cultured cells, that is apparently not modulated by LDL.¹⁷⁹

Other Biochemical Abnormalities

An as yet totally unexplained observation is the deficient ferritin immunoreactivity demonstrated in liver and spleen of NP-C patients.^{180, 181} Some abnormal features described in the BALB/c mouse model, such as increased dolichol phosphate¹⁸² or partial decrease of some peroxisomal β -oxidation enzymes and catalase activity in brain and liver,¹⁸³ have not been investigated in humans. One NP-C patient from a highly consanguineous family was described with additional defective peroxisomal β -oxidation of branched-chain substrates,¹⁸⁴ but the most likely explanation was that the patient was expressing mutations in two separate genes. Very long chain fatty acids in plasma studied in 6 NP-C patients showed normal results (Vanier, unpublished data).

Some other reported abnormalities most likely pertain to the alterations in trafficking of membrane lipids. Cystine has been shown to accumulate in lysosomes of tissues and cultured cells from both the murine BALB/c model and human NP-C, possibly as a consequence of cholesterol storage.¹⁸⁵ Other observations made by individual groups include high levels of caveolin-1 expression observed in liver of heterozygote mutant BALB/c mice¹⁸⁶ or fibroblasts of NP-C heterozygotes,¹⁸⁷ defective processing of apolipoprotein D found in astrocytes of the BALB/c mutant mouse,¹⁸⁸ and the decrease in membrane fluidity observed in cultured cells from the C57BLKS/J mouse model and from human patients.¹⁸⁹

Two observations suggest some impairment of cell signaling in NP-C: the attenuated elevation of cytoplasmic calcium concentration following LDL uptake¹⁹⁰ and the correlation between free sphingosine levels and inhibition of phorbol dibutyrate binding in NP-C cells.¹⁹¹ Alterations in neurotransmitters (serotonin, glycine, glutamate, and GABA) have also been described in the cerebellum and cortex of the BALB/c mouse model.¹⁹²

MOLECULAR GENETICS

Genetic Heterogeneity in NP-C

The 11 patients included in the initial cell hybridization study were found to belong to the same complementation group,⁶⁹ but subsequent work⁴⁰ disclosed the existence of a minor second complementation group. A larger investigation including genetic linkage analysis definitively established that there were two separate genetic loci responsible for NP-C disease.⁴¹ The gene defective in the large majority of NP-C families (approximately 95 percent) has been localized to chromosome 18, cloned, and designated *NPC1* (see "Identification of the *NPC1* Gene" below). The *NPC2* gene, defective in, at most, 5 percent of NP-C families (only six separate families known to date), has not yet been mapped or identified. Crossbreeding¹⁹³ and complementation¹⁹⁴ experiments in the C57BLKS/J and the BALB/c murine models have shown that both have allelic mutations and belong to the same complementation group as human *NPC1*.

The phenotypes of patients belonging to the two complementation groups have been thoroughly studied. Half of the patients in the *NPC2* group had suffered from the rare, rapidly fatal form with prominent lung involvement.⁴¹ Apart from this fact, variability in the clinical and cellular phenotypes occurred in both

groups, and no biochemical marker could be found that was specific to one of the groups.^{41, 175} This strongly suggests that the two respective gene products may function in tandem or sequentially.^{41, 43}

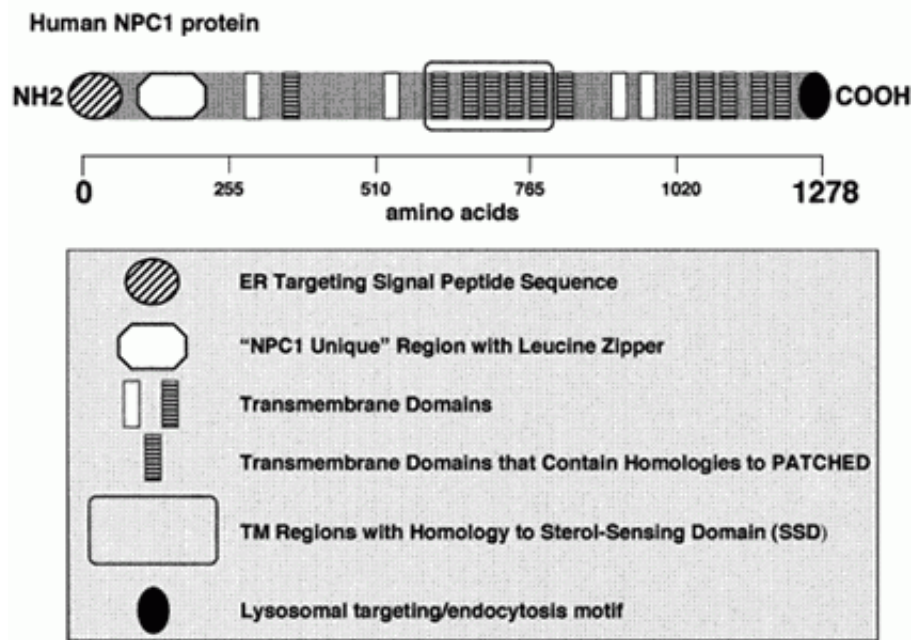
Identification of the *NPC1* Gene

The *NPC1* gene was identified through human positional cloning with the aid of the NP-C murine models. The studies of human NP-C have consistently paralleled characterization of the murine disease. In 1991, investigators linked the murine NP-C mutation in the *spm* mouse model to chromosome 18.³⁸ Human genomic regions that were syntenic to mouse chromosome 18 included portions of human chromosomes 5 and 18. Thirty-one stringently diagnosed NP-C families participated in a linkage study that mapped the NP-C gene to the pericentromeric region of chromosome 18.^{42, 43} Microcell transfer of human chromosome 18 into NP-C mouse derived 3T3 cells resulted in the restoration of cholesterol transport.³⁹ Linkage studies in Nova Scotia Niemann-Pick disease type D localized to 18q11, confirming that type C and type D were allelic.¹⁹⁵

The NP-C critical interval on chromosome 18 was refined through genetic examination of an extended pedigree of Bedouin Arab descent, which exhibited extensive consanguinity, suggesting a homoallelic mutation. Within this family a genetic recombination narrowed the *NPC1* interval to a 1-cM region defined by markers D18S44 to D18S1388.⁴³ The NP-C interval was further characterized with a yeast artificial chromosome (YAC) physical map consisting of three overlapping genomic clones. These genomic clones were introduced into NP-C-like mutant CHO cells via spheroplast fusion.¹⁹⁶ One of the three YACs complemented the NP-C phenotype. Transcripts were identified within the YAC-defined interval through sequence, exon trapping, and database analyses. The *NPC1* gene was identified based on mutation analysis of affected individuals as well as the ability to rescue the normal phenotype with the introduction of the NP-C cDNA into cultured human NP-C cells.⁴³ Identification of a major deletion in the murine ortholog confirmed the character of the *npc^{nih}* murine model.⁴⁴ The cloned human NP-C transcript was 4673 bp in length.⁴³

Translation of the open-reading frame of the *NPC1* gene predicts a protein of 1278 amino acids with a molecular weight of 142 kDa. Based on amino acid sequence analysis, the NPC1 protein is predicted to be an integral membrane protein with 13 to 16 putative transmembrane domains (Fig. 145-9). The N-terminus contains a peptide sequence associated with targeting to the endoplasmic reticulum. At the C-terminus, there is a lysosomal/endocytic-targeting signal (LLNF). Amino acids 73 to 94 comprise a leucine zipper motif. This lies within a region from amino acids 55 to 165 that has high homology with other *NPC1* orthologs. A potential tyrosine phosphorylation site is located at amino acid 506. The human and mouse NPC1 proteins have 14 potential glycosylation sites. The NPC1 human protein has high homology (percent identity/percent similarity) to other NP-C orthologs: mouse (85/93), yeast *Saccharomyces cerevisiae* (34/57), and the nematode *Caenorhabditis elegans* (30/55).⁴³

Fig. 145-9:



Structure of the human NPC1 protein.

NPC1 has extensive homology to Patched. This homology lies within 12 of the 16 predicted transmembrane domains of NPC1.¹⁹⁷ Patched is the defective protein in basal cell nevus syndrome; it is also known to be involved in the Sonic hedgehog signaling pathway. The significance of this homology has yet to be defined.

The **NPC1** protein also has homology to other proteins that are involved in cholesterol homeostasis. Within a five-transmembrane region of amino acids 615 to 797, there is significant homology to the sterol-sensing domains of HMG-Co A reductase, involved in cholesterol synthesis, and to the sterol regulatory element-binding protein (SREBP) cleavage-activating protein, **SCAP**, an activator of a transcription factor in cholesterol biosynthesis.^{198, 199} The sterol-sensing domains of these proteins are thought to be involved in their regulation based on the levels of sterols within the cell. It is not known whether cholesterol binds to this domain.

NP-C is a panethnic disorder with only two described founder populations, French Acadians in Nova Scotia^{1, 3, 200} and Spanish Americans in Colorado.⁸⁶ Outside of these populations, ongoing studies in several laboratories found that a majority of patients have private mutations and are compound heterozygotes, which will complicate genotype/phenotype correlations. Eight mutations in nine unrelated individuals were defined for the identification of the **NPC1** gene, including two deletions, one insertion, and five missense mutations.⁴³ Mutations in the Japanese populations from 11 unrelated NP-C families have been identified, of which 2 are splicing errors.²⁰¹ The mutation in the French Acadian population of Nova Scotia has been recognized as a missense mutation.²⁰⁰

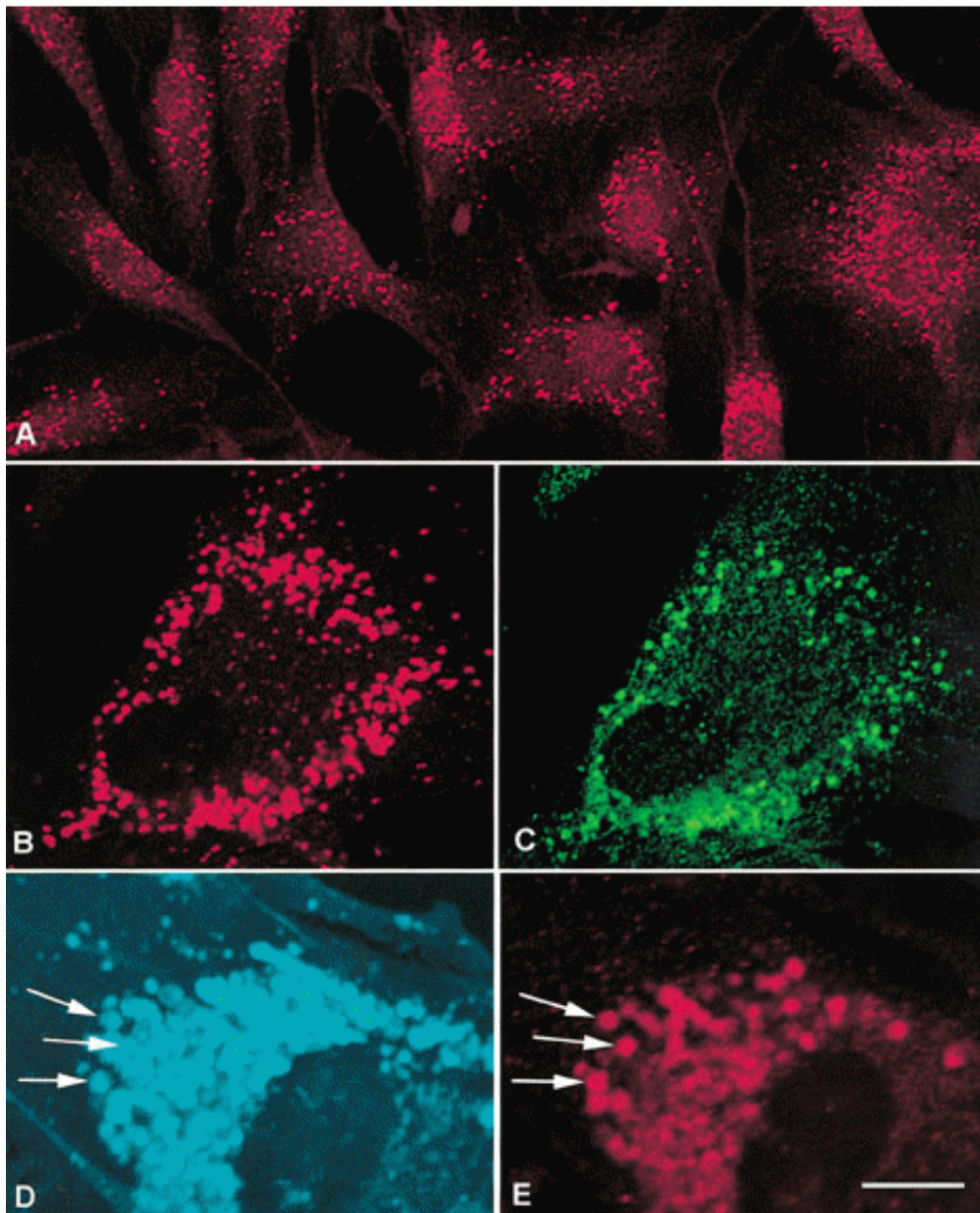
Murine Mutations

The npc^{nih} murine mutation is an insertion of an 824-bp retrotransposon-like sequence along with a 703-bp deletion of the genomic sequence consisting of 44 bp of exon sequence and 659 bp of intron sequence.⁴⁴ The resulting *NPC1* transcript has a frameshift mutation resulting in a truncated protein that is one-third the size of the NP-C protein, and that excludes 11 of the 13 transmembrane domains and the putative sterol-sensing domain. The availability of genotyping enhances the value of the npc^{nih} mouse for the study of NP-C.

CURRENT RESEARCH

The identification of the *NPC1* gene (see “Molecular Genetics” above) and its protein product promises to provide further insights into the mechanisms governing lysosomal cholesterol mobilization, and other aspects of intracellular lipid trafficking. In one study, polyclonal antiserum to a C-terminal region (amino acid residues 1256 to 1274) of the NPC1 protein has been used in immunocytochemical studies to determine its intracellular localization in cultured human fibroblasts.²⁰² In control cells, as well as NP-C cells whose mutations do not affect the expression of this particular epitopic region, NPC1 colocalizes with lysosome-associated membrane protein (lamp)-positive vesicles in the perinuclear region of the cell (Fig. 145-10A, B, and C). These vesicles are distinct and separate from the lamp-positive vesicles in which cholesterol accumulates after LDL uptake (Fig. 145-10B, and C). This segregated distribution of NPC1 and endocytosed cholesterol into separate lamp-positive vesicles is abolished when drugs are added that block lysosomal cholesterol egress.²⁰² The addition of the hydrophobic amine U-18666A¹⁵⁶ to cultured fibroblasts during the endocytic uptake of LDL causes both cholesterol and NPC1 to accumulate in the same lamp-positive vesicles (Fig. 145-10D and E). This altered relocation of NPC1 into cholesterol-engorged lysosomes suggests that NPC1 vesicles normally interact transiently with the cholesterol-enriched lysosomes to affect a relocation of this sterol pool. Further characterization of these NPC1 vesicles remains a focal point of current research.

Fig. 145-10:



A, Control human fibroblasts incubated with LDL for 24 h, immunostained with anti-peptide antibodies to the C-terminus of NPC1 protein, and viewed with confocal fluorescence microscopy. NPC1 immunofluorescence is present in small granules that are distributed throughout the cytoplasm of cells. B and C, Control fibroblast incubated with LDL for 24 h and immunostained with both (B) antibodies to a lysosomal membrane glycoprotein...

Recent studies in NP-C cells show that the cellular defect encompasses a more global transport error than targeted disruption of cholesterol trafficking alone. Because a majority of lysosomal cholesterol relocates to the plasma membrane,²⁰³ intracellular routing of endocytosed cholesterol can be viewed, at least in part, as tracing a retroendocytic vesicular pathway in which lysosomal components are transported to the plasma membrane.²⁰⁴ This consideration encouraged a reevaluation of the trafficking defect in NP-C. Measurement of the cellular egress of an endocytosed fluid phase marker ([¹⁴C]sucrose) was carried out in cultured control and NP-C fibroblasts to study trafficking through this retroendocytic vesicular pathway.²⁰² A discernible delay in the loss of [¹⁴C]sucrose from the monolayers of NP-C fibroblasts was noted. Compartmentalized kinetic modeling of the data suggests that the difference in [¹⁴C]sucrose clearance from normal and NP-C cells can be accounted for by delayed movement from a vacuolar compartment separate from, but in direct communication with, the mature lysosomes. The extent of delayed movement of sucrose through this NP-C targeted compartment (50 percent of normal)²⁰² closely approximates the measured delays in cholesterol relocation from lysosomes to the plasma membrane in NP-C cells.^{35, 163}

A model for the intracellular distribution of lysosomal cholesterol can be proposed based on these and other data. Mobilization of a majority of the lysosomal cholesterol to other membranes is likely to occur through a vesicular-mediated pathway because both specific-drug perturbations¹⁶⁴ and critical temperature transitions²⁰² known to affect vesicular trafficking markedly suppress sterol movement. Several inferences can be drawn from the [¹⁴C]sucrose egress studies. The data strongly reinforce the notion of vesicular-mediated sterol trafficking because the vesicular-dependent clearance of sucrose is defective to the same extent as cholesterol in NP-C cells. That NPC1 mutations affect the intracellular trafficking of two substances as disparate as sucrose and cholesterol suggests that the primary functional defect may target a vesicular trafficking pathway shared by these two substances. This, in turn, suggests that metabolites other than cholesterol might assume the role of offending metabolites in cells where their trafficking between the lysosomes and plasma membranes is of major physiologic significance. Solid tissues and cultured cells in NP-C contain a heterogeneous profile of accumulating material that includes several lipids in addition to cholesterol (see “Tissue Lipids” above). The possibility of alternate metabolites may explain the absence of cholesterol accumulation in the brain in humans or animals with NP-C (see “Tissue Lipids” above). Cholesterol for synthesis of myelin appears to be made locally, not imported into brain.²⁰⁵ The brain in vivo has little capacity or opportunity to metabolize lipoproteins rich in cholesterol through receptor-mediated endocytosis targeted to lysosomal processing.²⁰⁶ On the other hand, gray matter from NP-C brains shows extensive accumulation of glycolipids (see “Tissue Lipids” above) that are normal components of the plasma membrane^{207, 208} and are enriched in this organ. It might be hypothesized that the NP-C lesion leads to impaired vesicular trafficking that in turn results in cholesterol accumulation in peripheral tissues and glycolipid accumulation in the brain, where G_{M2} in particular may play an important role in pathogenesis.^{209, 210}

DIAGNOSIS

Accurate diagnosis of NP-C requires recognition of the many clinical phenotypes, narrowing of the differential diagnosis by the use of ancillary testing, and final confirmation by biochemical testing. The NPC1 gene has been cloned, but to date, the majority of affected individuals are compound heterozygotes with private mutations. Thus, molecular diagnosis is not a suitable tool for primary diagnosis, although it holds great promise for improvements in antenatal diagnosis and identification of heterozygotes in probands' families.

The known clinical presentations of **NP-C** are protean (see “Clinical Manifestations” above). The differential diagnosis is broad (see Table 145-2). A frequent clinical conundrum is posed by the classification of individuals with hepatosplenomegaly and foam cells in the bone marrow. Such individuals could have **NP-B**, but also NP-C prior to the onset of neurologic symptoms. Sphingomyelinase always shows a normal activity in leukocytes of NP-C patients, so this assay can be used to exclude sphingomyelinase-deficient types of NPD. Depression of fibroblast sphingomyelinase in NP-C is variable, but not as pronounced as in NP-B. The blood count, biochemical profile, plasma lipids, urinalysis, and cerebrospinal fluid (cells, glucose, and protein) are normal in NP-C, except in patients with hypersplenism or cholestatic jaundice.

Table 145-2: Differential Diagnosis of NP-C [Separate Window](#)

Clinical manifestations	Alternative diagnosis
Severe neonatal jaundice	Biliary atresia, congenital infections, α_1 -antitrypsin deficiency, tyrosinemia
Isolated splenomegaly	Leukemia, lymphoma, histiocytosis, storage diseases (e.g., Gaucher, NP-A, NP-B), infections (e.g., malaria)
VSGP	G _{M2} gangliosidosis, mitochondrial diseases, glycine encephalopathy, maple syrup urine disease, dorsal midbrain syndrome
School difficulty	Attention-deficit disorder, learning disabilities, absence seizures, other dementing illnesses
Dystonia	Idiopathic torsion dystonia, doparesponsive dystonia, Wilson disease, amino and organic acidopathies (e.g., glutaric aciduria type 1), G _{M2} gangliosidosis
Dementia	Pseudodementia (depressive disorder), neuronal ceroid lipofuscinosis, subacute sclerosing panencephalitis, HIV encephalopathy
Cataplexy	Other sleep disorders, seizures, syncope, periodic paralysis

Modified with permission from Schiffmann.²⁴⁷

Imaging and neurophysiologic studies are nonspecific. MRI and CT scans may be normal or show atrophy, in particular cerebellar atrophy. Changes in the periventricular white matter have been seen in some cases.⁹⁹ Proton magnetic resonance spectroscopic imaging (MRSI) in 10 patients has shown evidence of diffuse brain involvement in **NP-C**, beyond that apparent on routine imaging studies.²¹¹ Patients with NP-C had significantly decreased N-acetylaspartate (NA)/creatine plus phosphocreatine (Cre) ratios in the frontal and parietal cortices, centra semiovale, and caudate, and significantly increased choline/Cre in the frontal cortex and centra semiovale, when compared to controls. The authors also noted some correlation between proton MRSI findings and clinical stage of disease as previously defined.⁵⁷ One earlier study had included a single patient who showed loss of an abnormal lipid peak on proton MRS

at 13 and 19 months of cholesterol-lowering therapy.²¹² Owing to differences in technique, this case cannot be readily compared to the more recent series.

The electroencephalogram may show diffuse slowing or a variety of epileptiform discharges. The latter do not always correlate with the occurrence of seizures. Central conduction times may be slowed in somatosensory and visual evoked potentials.²¹³ The audiologic profile, in particular the acoustic reflex, may provide evidence of brain stem dysfunction early in the course of the illness.²¹⁴ Changes in the brain stem auditory-evoked response begin with inversion of the wave V:I amplitude ratio, followed by increase in the wave I to V interpeak latency, progressing, finally, to loss of late wave forms.

Several characteristic pathologic findings have been described (see “Pathology” above). Foam cells and sea-blue histiocytes have been detected in many tissues in NP-C, but they are nonspecific.²¹⁵ Failure to demonstrate these cells in biopsies does not rule out the diagnosis of NP-C. Cases have been reported in which bone marrow biopsy was initially unrevealing,²¹⁶ only to show storage cells when the test was repeated years later.²¹⁷ Ultrastructural studies of tissue biopsies are more specific than light microscopy, and may strongly support the diagnosis of NP-C.^{99, 218} In early onset cases in which a liver biopsy is performed, the diagnosis of NP-C can rapidly be confirmed using as little as 5 mg of frozen tissue analyzed by thin-layer chromatography of the lipids.

Biochemical Diagnosis

The diagnosis of NP-C should no longer rely on histopathologic or tissue lipid findings alone. The discovery of abnormal LDL-cholesterol processing has established the rationale for specific biochemical testing.^{32, 144} The tests are usually performed on cultured fibroblasts,^{32, 36, 88, 110, 219, 220} although lymphocytes have been used.²²¹ To date, the diagnosis is best achieved by the concomitant demonstration of (a) intralysosomal accumulation of unesterified cholesterol as shown by a characteristic pattern of intense perinuclear fluorescence after challenge with LDL-enriched medium and staining with filipin (Fig. 145-8B), and (b) abnormal intracellular cholesterol homeostasis as defined by impaired LDL-induced cholesterol esterification.^{36, 60, 220} A majority of NP-C cell lines (approximately 80 percent) express profound alterations of cholesterol homeostasis (classical biochemical phenotype),^{32, 36, 52, 88, 110, 223} but some have milder changes, especially in their esterification ability (intermediate and variant phenotypes).^{36, 52, 130, 154, 219, 220, 224, 225} The filipin test is more sensitive than cholesterol esterification assays in detecting patients with the variant phenotype.^{36, 60, 154, 220, 226} Filipin staining also increases specificity because impairment of cholesterol esterification may be found in other disorders such as acid lipase deficiency, familial hypercholesterolemia, and I-cell disease. The filipin pattern in I-cell disease is abnormal, but can be differentiated from NP-C in expert hands.^{36, 227} In the experience of one author (M.T.V.), slight lysosomal cholesterol storage may be seen in cells from patients with sphingomyelinase-deficient types of Niemann-Pick disease.

In comprehensive investigations, no strict correlation has been observed between the severity of alteration in intracellular cholesterol homeostasis and the clinical phenotype, especially in the late infantile and juvenile onset forms.^{69, 74, 220} A trend for adult onset patients to show a “variant” biochemical phenotype has been reported.⁷⁴ On the other hand, a consistent biochemical phenotype has been the rule within a sibship.^{36, 84}

More than half the obligate heterozygotes tested show a level of LDL-induced lysosomal cholesterol accumulation and possibly of cholesterol esterification intermediate between normal individuals and affected homozygotes.^{36, 52, 74, 143, 220} Abnormalities in a few cases may be as pronounced as in some “variant” patients.²²⁶ The remaining heterozygotes overlap with controls. Thus, generalized NP-C carrier

screening using this strategy is not possible. Typical storage cells in bone marrow or inclusions in skin biopsies have been reported in a few parents of children with NP-C.^{122, 123}

Provided marked abnormalities (classical phenotype) have been demonstrated in the index case, similar tests can be used for prenatal detection of affected fetuses using cultured chorionic villus cells or amniotic fluid cells.^{37, 228} Because some heterozygotes may show significant alterations, prior study of both parents is advisable. By the summer of 1998, at least 150 at-risk pregnancies had been monitored in our laboratories and in those of Drs. D. Wenger, M. Ziegler, A. Fensom, and O. Van Diggelen.

Analysis of the lipids in a frozen liver biopsy (see “Tissue Lipids” above) is generally diagnostic, but occasionally may be inconclusive. Partial deficiency of sphingomyelinase activity in cultured skin fibroblasts is nonspecific and inconstant. Chitotriosidase has been found to be modestly elevated in plasma in several lysosomal diseases, although marked elevations of diagnostic value are seen only in Gaucher disease.²²⁹ The usefulness of this enzyme as a disease marker is diminished by its relatively frequent deficiency in the general population.²³⁰

Molecular Diagnosis

In NP-C patients belonging to complementation group 1, at least four laboratories have initiated mutational analysis of the *NPC1* gene. In the *NPC1* cloning paper,⁴³ eight different mutations were reported. Most of the patients were compound heterozygotes. Subsequently, the mutation in the Nova Scotia isolate of NP-C was identified.²⁰⁰ Preliminary results from the authors' laboratories and the laboratory of Dr. K. Ohno in Japan have shown that a transcript is made in most patients and that mutations occur at various locations of the gene. Most of the mutations appear to be private. A number of polymorphisms have already been observed. Consequently, with current technology, direct molecular diagnosis is not practical and may be misleading, except in specific populations such as the French Acadians in Nova Scotia and the Hispanics from southern Colorado. Nevertheless, identification of the mutation(s) in a demonstrated NP-C case is important for subsequent genetic counseling in the proband's family. Finding the mutations will make prenatal diagnosis possible for the so-called “variant” families, in which biochemical tests were not reliable. In other families, this would permit prenatal diagnosis using uncultured chorionic villi, and thus provide a result a month earlier than traditional biochemical methods. Mutational analysis would also allow accurate heterozygote testing in the proband's family.

TREATMENT

There is no specific treatment for NP-C. The identification of *NPC1* and its predicted protein product will eventually lead to new therapeutic strategies. The *NPC1* gene product appears to be an integral membrane protein, which is not likely to be readily replaced from exogenous sources including tissue transplantation. Indeed, attempts at nonspecific replacement therapy by tissue transplantation have been disappointing. In the C57BLKS/J murine model of NP-C, bone marrow transplantation^{231–233} and combined liver and bone marrow transplantation²³³ partially reversed tissue storage of cholesterol and sphingomyelin, but did not influence the continued neurologic deterioration.

Liver transplantation in a 7-year-old girl with NP-C and cirrhosis was similarly successful in restoring hepatic function, but failed to slow neurologic progression.²³⁴ There has been one report of transplantation of fetal liver cells into BALB/c mutant mice, with apparent correction of the phenotype in many cells.^{235, 236} This study, which has not been replicated, must be interpreted cautiously, as it was performed prior to description of the murine ortholog of *NPC1*, so that genotyping of the mice prior to transplantation was not possible. Transplantation of fetal liver cells has been used in two humans.²³⁷ The

one patient for whom follow-up was available to us was engrafted twice at the ages of 8.5 and 9 years, without significant improvement of the natural history of the disease. To date, there have been no reports of bone marrow transplantation in human NP-C.

Treatment strategies to reduce intracellular cholesterol accumulation were formulated based on the hypothesis that cholesterol is an offending metabolite in NP-C. Several lines of evidence supported this assumption. Cholesterol is stored in excess in the nonneural tissues in NP-C, and the defect in cholesterol trafficking is closely associated with the primary mutation. Combinations of cholesterol-lowering agents were found to reduce hepatic stores of unesterified cholesterol in NP-C patients,²³⁸ but there is no evidence that their outcome was improved, and several patients who elected to continue the diet long-term have subsequently succumbed to NP-C. There is a case report of a child treated with a cholesterol-lowering regimen in whom serial magnetic resonance spectroscopy studies were interpreted as showing diminution of stored cerebral lipids.²¹² Follow-up data have not been published. Cholesterol-lowering drugs may be ineffective in influencing neurologic disease because cholesterol is not an offending metabolite in the brain, or, alternatively, because the agents employed do not penetrate the blood-brain barrier. Dimethyl sulfoxide, which corrects partial sphingomyelinase deficiency²³⁹ and substantially reverses the cholesterol-trafficking abnormalities²⁴⁰ in NP-C fibroblasts, has been used as a single agent in two patients. Stabilization of neurologic disease was reported in an 8-year-old girl,²⁴¹ but a 3-year-old boy showed no apparent benefit.²⁴²

Regardless of the availability of specific treatments for NP-C, a great deal can be offered to patients and their families in the form of symptomatic management. We found that dystonia and tremor respond to anticholinergic agents in some patients, and seizures can be controlled or diminished in frequency with antiepileptic drugs, at least early in the course of the illness. No controlled studies of the effectiveness of such treatments are available. Protriptyline,^{78, 79} clomipramine, and modafinil are effective in controlling cataplexy and managing accompanying sleep disturbances.

Therapy to delay or prevent puberty has been proposed as a means of ameliorating the progress of NP-C. The rationale for this approach is based on the observations (a) that progesterone can induce an NP-C like phenotype in control fibroblasts,¹⁵⁹ (b) that progesterone exacerbates the biochemical abnormalities in NP-C fibroblasts,²⁴³ and (c) that symptoms of NP-C often worsen at the time of menarche. Gonadotrophin-releasing hormone analogs have been administered to some girls in an uncontrolled fashion. No data on clinical efficacy are available.

Two siblings with late onset disease, presenting with psychiatric symptoms, were treated with prednisolone with apparent improvement in symptoms, and recurrence of symptoms after steroid withdrawal.²⁴⁴

Physical therapists, speech therapists, and occupational therapists should all be involved in the care of the patient with NP-C, in order to address problems in the areas of impaired mobility, contractures, dysarthria, dysphagia, and occupation. Medical therapy notwithstanding, support of the patient and family in the course of NP-C, following long-established principles,²⁴⁵ is of paramount importance. Counseling services should be available for patients and family members. Support for families with NP-C is available through umbrella organizations for children with inherited or metabolic diseases in many countries. Support groups devoted to Niemann-Pick disease have been established in the U.S. (<http://www.nnpdf.org>), the U.K. (<http://www.nnpdf.org/npdg-uk>) France (<http://www.aafnp.org>), and Germany (<http://www.niemann-pick.de/wirveberuns.html>).

Animal models will be critical for future studies of potential therapies. The genotyping of the BALB/c murine model of **NP-C** will facilitate its use in such experiments. Prior studies focused on reduction or removal of the accumulated offending metabolite. A new approach that has shown promise in the murine model of Tay-Sachs disease is to inhibit synthesis of a known offending metabolite, in this case G_{M2} ganglioside, prior to its accumulation.²⁴⁶ Given the evidence for the role of G_{M2} ganglioside in NP-C, a trial of this agent (*N*-butyldeoxynojirimycin) in the npc^{nih} mouse may be worthwhile.

ADDENDUM

Since the manuscript for this chapter was submitted, substantial advances have occurred in our knowledge of **NP-C**. These are summarized in the following paragraphs.

PATHOLOGY

Falk and co-workers²⁴⁸ investigated developmental expression of the **NPC1** (*Npc1*) mRNA with a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The transcript was expressed in the cerebellum in vivo and in vitro during early postnatal life, as well as in the adult cerebellum. In in vitro studies, Purkinje cells and some glial cells were immunoreactive to the antibody to anti-Npc1 antibody. They concluded that since *Npc1* is expressed at similar levels throughout the development in both neurons and glia, the vulnerability of Purkinje cells in **NP-C** is likely to involve disruption of an interaction with other developmentally regulated proteins. In mouse brain, Prasad and co-workers²⁴⁹ detected *Npc1* mRNA in neurons as early as embryonic day 15. In this study, the **NPC1** gene was widely expressed in neurons, the highest levels occurring in the cerebellum and pons. The high expression in the cerebellum may account for the vulnerability of the Purkinje cells in NP-C.

Henderson et al.²⁵⁰ demonstrated that embryonic striatal neurons in primary cultures from npc^{nih} mice can take up LDL-derived cholesterol but with significantly lower hydrolysis and re-esterification than in wild type neurons. Cholesterol is thought to be critical for membrane localization and function of many signaling proteins in membrane domains or "lipid rafts." These cultured **NP-C** neurons failed to increase neurite outgrowth in response to brain-derived neurotrophic factor (BDNF), in contrast to the response of wild type neurons. Western blotting revealed that Trk receptor expression was similar in striatal neurons from wild type and mutant mice, yet BDNF-induced tyrosine phosphorylation of Trk was essentially eliminated in neurons from npc^{nih} mice. Trk activation was also abolished in cholesterol-depleted wild type neurons. Thus, abnormal cholesterol metabolism occurs in neurons of npc^{nih} mice, even at embryonic stages, and is associated with notable lack of BDNF responsiveness. Given the importance of BDNF in neuronal survival, growth, and differentiation, the lack of BDNF responsiveness might contribute to the loss of neural function in NPC.

In contrast to the diffuse expression reported in rodents, an immunoelectron microscopic investigation of the brains of macaque monkeys found that **NPC1** was expressed predominantly in astrocytic process located in the presynaptic region, rather than in neurons.²⁵¹ These investigators also reported that the terminal fields of axons and dendrites were the earliest sites of degeneration in npc^{nih} mice, based on studies with amino-cupric-silver staining. This work remains unconfirmed to date.

Wu and co-workers²⁵² have found that increasing numbers of neurons were lost by apoptosis in **NP-C** brains with progression of the disease process in both humans and mice. Apoptosis was demonstrated in Purkinje cells in the cerebellar vermis, in cerebral cortical neurons, and in some cells in the white matter as early as 30 days of age in NP-C mice, prior to detectable neurological symptoms. The numbers of apoptotic cells increased with age.

In **NP-C** brains, gangliosides G_{M2} and G_{M3} accumulate. The possible role of these accumulated gangliosides in the dysfunction and eventual death of neurons in NP-C was tested by generating double knockout mice. *npc^{nih}* and *GalNAcT -/-* mice (that lack the activity of the β -1,4-GalNAc transferase responsible for the synthesis of complex gangliosides) were interbred, and the clinical, pathologic, and biochemical characteristics of their double knockout offspring were studied.²⁵³

Positive stains for filipin and PAS best demonstrate neuronal storage in *npc^{nih}* mice. Neither filipin nor PAS stains were positive in the neurons of the double knockout mice, indicating absence of neuronal storage. Electron microscopic study found no neuronal inclusions typical of **NP-C**, and thin layer chromatography confirmed the absence of G_{M2} and complex gangliosides in the double mutant brain. The double knockout mice showed no improvement of the clinical course or neuronal degeneration compared to *npc^{nih}* controls. Macrophages and microglia containing cholesterol were still numerous in the brain, and visceral pathology was unchanged. Apoptotic neuronal loss appeared more pronounced and loss of Purkinje cells was clearly observed in the double knockouts by comparison with *npc^{nih}* controls. The absence of G_{M2} ganglioside storage from conception does not significantly influence the clinical phenotype of the NP-C in this model system.

Animal Models of NP-C

The feline model of **NP-C** was demonstrated to belong to the **NPC1** complementation group²⁵⁴ and the generation of mutations of the homologue of *NPC1* in the nematode *C. elegans* has provided a new model system allowing studies of NP-C disease in a thoroughly characterized animal that reproduces rapidly.²⁵⁵

PATHOPHYSIOLOGY

Cell Biology

The exact function of the **NPC1** gene product is not yet elucidated and this area is under active investigation by several groups. A clear consensus has yet to emerge, but major findings may be summarized as follows.

Neufeld et al. found that the **NPC1** protein was localized to late endosomes immunocytochemically and that the clearance of endocytosed ^{14}C -sucrose as well as cholesterol was defective in **NP-C** cells studied biochemically.²⁰² Both general retroendocytic trafficking and mobilization of multiple lysosomal cargo appeared defective at a late endosomal trafficking step. This mechanism would account for the accumulation of multiple lipids in NP-C cells and tissues. Site-directed mutagenesis of the NPC1 protein targeting domains of the protein responsible for its cholesterol transport function caused loss of function.²⁵⁶ Confocal microscopy of living cells, using an NPC1-green fluorescent chimeric protein, documented an unusual mode of tubular membrane trafficking for the NPC1 endocytic compartment that appears linked to the NPC1 protein and cellular cholesterol content.²⁵⁷ Using cytochemical techniques, it was found that cellular cholesterol levels modulate the glycolipid profile of the *NPC1* compartment. The results indicate that the *NPC1* compartment serves as a sorting station in the endocytic trafficking of both cholesterol and glycolipids. Enriching the cholesterol content of lysosomes recruits the NPC1 protein into endocytic vesicles containing glycolipids. In the presence of elevated cholesterol levels, certain glycolipids are restricted from entering the lysosomal compartment for degradation and are efficiently recycled in NPC1 sorting vesicles to the plasma membrane. Glycolipids that accumulate in NP-C cells and tissues, such as G_{M2} , are those sorted through the NPC1 compartment, while non-accumulating glycolipids such as CTH and G_{D3} are shown to traffic on to the lysosomes for probable degradation.²⁵⁷ Other workers have drawn similar conclusions.^{258–260}

Studies in CT60 and CT43 CHO mutants²⁶¹ disclosed that the initial movements of LDL-derived cholesterol to the plasma membrane did not require participation of the **NPC1** protein. The authors concluded that the NPC1 protein cycles cholesterol from an intracellular compartment to the plasma membrane or to the endoplasmic reticulum after (and not prior to) newly hydrolyzed LDL-derived cholesterol appears in the plasma.²⁶¹ A further report concluding that cholesterol moves freely from the lysosomes to the plasma membrane in **NP-C** cells has appeared.²⁶² The latter authors considered that cholesterol accumulation in NP-C lysosomes results from an imbalance in the brisk flow of cholesterol among membrane compartments. While most authors consider that in NP-C cells, cholesterol essentially accumulates in lysosomes,^{202, 257, 262} two reports conclude that accumulation takes place in late endosomes.^{261, 263}

Cholesterol balance and metabolism were quite extensively studied in mutant *npc^{nih}* mice. The cholesterol pool was found to expand continuously from birth in homozygous affected animals.²⁶⁴ While cholesterol entering tissues through the coated-pit pathway became sequestered in the lysosomal compartment and was metabolically inactive, cholesterol that was newly synthesized or that entered cells through the SR-BI pathway was metabolized and excreted normally.^{265, 266}

Several reviews have addressed the subject of the role of the **NPC1** protein in the broad context of intracellular cholesterol trafficking.^{267–269} In general, current opinion favors a key role for the NPC1 protein in modulating vesicular trafficking of both glycolipids and cholesterol.

MOLECULAR GENETICS

NPC1 Gene Organization, **NPC1** Protein Structure and Topology, Functional Domains, and **NPC1** Mutations

The genomic organization of **NPC1** is now known. The gene spans greater than 47 kb and contains 25 exons.²⁷⁰ Key promoter regions have been defined.^{270, 271} The NPC1 protein appears to be N-glycosylated, and shows a size of 170 and 190 kDa by western blotting, in CT-60 CHO cells transfected with wild-type NPC1²⁵⁶ or as native protein in human skin fibroblasts.²⁷² There is further evidence that the NPC1 protein resides in late endosomes and interacts transiently with lysosomes and the *trans*-Golgi network.²⁵⁸ Topological analysis of NPC1 has revealed that this glycoprotein contains 13 transmembrane domains, 3 large and 4 small luminal loops, 6 small cytoplasmic loops, and a cytoplasmic tail.²⁷³ The putative sterol-sensing domain has the same orientation as those in HMG-CoA reductase and **SCAP**.²⁷³ Information regarding the functional role of different domains of the protein has been deduced from clinical studies, and by transfection of CT-60 cells with mutant NPC1. The N-terminal domain and the lysosome-targeting motif appear essential for cholesterol mobilization.²⁷⁴ Mutations in the leucine-zipper motif and sterol-sensing domain inactivate the protein.²⁵⁶ Homozygote missense mutations in the sterol-sensing domain correlate with a severe infantile neurological onset disease and absence of detectable NPC1 protein.^{275, 276} Studies in patients revealed that a cluster of mutations was located on the third large luminal, cysteine-rich loop, which is highly conserved in NPC1 orthologues, and has a sequence showing resemblance to the RING finger motif of protein kinase C.²⁷⁷ This domain was shown to bind zinc²⁷¹ and also appears of particular functional interest.

Taking into account recent studies^{201, 272, 275, 277, 278} and data from seven groups presented in the International Workshop “The Niemann-Pick C lesion and the role of intracellular lipid sorting in human disease” (National Institutes of Health, Bethesda, MD, October 1999), the number of **NPC1** mutations known to date exceeds 100. Mutations have been found widely distributed on the gene (leucine zipper domain excepted), with, however, about 1/3 of them located in the cysteine-rich luminal loop. With few exceptions, most mutations appear to be private. In Caucasians, only one common mutant allele, I1061T,

has been identified.²⁷⁸ This mutation, which in the homozygous state correlates with a juvenile neurological onset form of the disease, constitutes about 15% of alleles in patients of Western European (especially British and French) descent. The same mutation is prevalent in the Hispanic-American isolate of Colorado and New Mexico, suggesting a founder effect.²⁷⁸ Mutational analysis of the *NPC1* gene is complicated by the large number of polymorphisms already recognized.^{201, 270}

NPC1 Gene Homologues

By EST screening, a new gene with homology to *NPC1*, named *NPC1L1*, has been isolated. It has been suggested that *NPC1* and *NPC1L1* form part of a new family of proteins that may have similar functions at different subcellular locations.²⁷⁹

TREATMENT

Bone marrow transplantation was performed on an NP-C patient aged 3 years and 5 months. Regression of hepatosplenomegaly and decreased infiltration of foamy macrophages in the bone marrow and lung were described 6 months after the transplant. Predictably, given the nature of the *NPC1* gene product, and the early onset of disease in this patient, neurological status continued to deteriorate, with progressive brain atrophy on serial magnetic resonance imaging (MRI). The authors concluded that bone marrow transplantation is unlikely to be an adequate treatment for NPC.²⁸⁰

Erikson et al.²⁸¹ reported that modifications of somatic cholesterol do not significantly alter the neurological course in *npc^{nih}* mice, confirming earlier studies.

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Review

The pathophysiology and mechanisms of NP-C disease

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Abstract

The molecular isolation of NPC1 and NPC2, the genes defective in patients with Niemann–Pick disease type C (NP-C), has heralded in an exponential increase in our understanding of this syndrome and thus of human intracellular sterol transport. Despite this, neither the mechanisms of action nor the substrates for these putative transporters have been defined. In this overview, we describe our perspectives on the current awareness of the genetic determination and cellular biology of this syndrome, with emphasis on the underlying events that lead to neurodegeneration and the manner in which they might eventually be treated.

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Unesterified sterols modulate the function of all eukaryotic cellular membranes by lending rigidity to the phospholipid bilayer. Optimal membrane function is regulated by maintaining an equilibrium between sterol biosynthesis, uptake, and metabolism. Superimposed upon this is the need to adequately balance sterols with phospholipids, specifically those of the sphingolipid group particularly in plasma membrane sub-domains (“rafts”). In addition, sterols are stored as steryl esters and act as building blocks for bile acids, steroid hormones and, notably, neurosteroids. Niemann–Pick diseases (A, B, and C) are all characterized by defects in the activity of sphingolipid degrading enzymes. The NP-A and NP-B subtypes arise due to mutations in the acid sphingomyelinase (SMase) structural gene [1]. Niemann–Pick disease type C (NP-C) is commonly, and perhaps too simplistically, considered to be a syndrome of defective cholesterol transport from the lysosome with a secondary

defect in SMase activity (reviewed in Refs. [2,3]). This is particularly pronounced in liver and spleen cells, but more deleteriously in brain, where the lipid accumulation correlates with severe neuronal dysfunction that is ultimately fatal.

Our understanding of NP-C has progressed dramatically in the past ~8 years [4] with the strong encouragement and support of family-centered foundations such as the Ara Parseghian Medical Research Foundation. Following isolation of the NPC1 and NPC2 genes [5,6] responsible for this disease and the resulting burgeoning of cell biological data, we now have a reasonable appreciation of its molecular nature. Although we know the players in this game, it is clear that we do not yet know either the rules by which the game is played or even the shape of the ball! In this brief overview, we describe our perspectives on the current state of this field and speculate on the future directions that this research may take.

2. Genetic determination

The isolation of the NPC1 gene, which is defective in the majority of individuals afflicted with this disease, provided

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an important molecular key to understanding the disease and the process of subcellular cholesterol transport [7,8]. Some 4 years later, label and colleagues identified NPC2 as the determinant of the rarer form of NP-C and subsequently created a murine model of the disorder (see review Vanier and Millat [9]). The NPC1 and NPC2 proteins share no sequence identity, but both have hallmarks of genes involved in cholesterol homeostasis and likely act in the same pathway. Moreover, both genes have counterparts in virtually all eukaryotes [10], suggesting a primordial function. Thus, the tools are in place to examine how these proteins work, yet many questions remain unanswered.

For example, do genes other than NPC1 or NPC2 cause this syndrome? Probably not; genetic linkage, cell fusion studies [11] and nucleotide sequence analyses [12–16] indicate that the vast majority of causes of NP-C are associated with mutations at the chromosome 18 and 14 loci. However, there are isolated examples of “mystery” forms of the disease that at the nucleotide level do not appear to be NPC1 or NPC2 alleles, at least within coding sequences [13,17]. Furthermore, there is evidence for the presence of modifiers or suppressors of these pathways [18] and rare instances of “mistaken identity” suggest the existence of phenocopies of the NP-C syndrome. Conversely at the cell biology level it is possible to suppress the NPC1 syndrome by mis-expression of mediators of endosomal/lysosomal trafficking ([8,19–21]).

Another major question is what accounts for the surprising soft link between disease alleles and phenotype in NP-C disease. Although some correlation of alleles with either the severe or “variant” disease phenotypes has been suggested, there is no founder effect for most kindreds with this panethnic disorder and most mutations are private. Even within a family, where the NPC1 mutant alleles are identical, there may be striking variation in age of onset, severity of symptoms and progression of the disease. The molecular isolation of the genetic and environmental factors that cause this variation would undoubtedly enlighten our understanding of the mechanism of lipid movement and provide currently untapped targets for therapeutic intervention. Approaches in model systems (yeast, fruit flies, nematodes), which have maintained remarkable conservation of this gene in terms of structure and function, may represent an appropriate and fruitful strategy in this regard [10].

3. Cellular biology

Although the predominant phenotype in NP-C mutant liver cells and fibroblasts is an accumulation of free cholesterol, several classes of sphingolipids also amass [22,23]. In addition, it is clear that gangliosides such as GM1, GM2 and GD3 are transported by the NPC1 defined pathway in cell culture models [24,25]. A striking aspect of NP-C disease is the panoply of biochemical and physiological disturbances that have been reported in the

literature. These include changes in cholesterol and sphingolipid/ganglioside accumulation, membrane microdomains (“rafts”), sphingomyelinase activity, caveolin and annexin II expression, peroxisomal function, copper metabolism, apoptosis, and neurotrophin response. What is/are the primary defect(s) that arise from loss of this gene as opposed to secondary consequences? NPC pathways transport other molecules in addition to sterols and sphingolipids. General markers of endocytosis such as sucrose and DiIC₆ are mislocalized by NPC mutant fibroblasts [26]. Thus, is there one primordial metabolite that *must* be transported by the NP-C pathways? Is there a single toxic metabolite that accumulates in this disorder, the removal of which might represent a therapeutic approach?

With respect to the basic function of this protein in the cell, we know a considerable amount about the subcellular itinerary of the culprit proteins [9,21]. Evolution provides a clue to understanding the mechanism of NPC1 function and pathophysiology. Unlike NPC2, the NPC1 molecule shows remarkable conservation, with bona-fide representatives (sometimes more than one per genome) in yeasts, worms, insects, plants and mammals [10]. The genetic conservation may be even more ancient; Ioannou and colleagues (Ref. [27] and this volume [8]) made the breakthrough observation that NPC1 is a eukaryotic member of the resistance-nodulation-division (RND) family of prokaryotic permeases. These proteins are well studied in numerous bacteria and use proton-motive force to remove hydrophobic molecules from the cell. Expression of NPC1 in *Escherichia coli* facilitated the transport of metabolites such as acriflavine and oleic acid, but not cholesterol or cholesteryl oleate, across the bacterial membrane. In yeast, despite structural and functional conservation with the mammalian protein, the primary role of the NPC1 ortholog is not to transport sterols [10,28]. These studies point to a very basic function of the protein that predates sterol transport and remains elusive. That is not to say accumulation of sterols is irrelevant to the pathophysiology of this disease or that they are not the offending metabolites (as will be discussed at a later point in this overview). However, this protein did not evolve as a low density lipoprotein derived cholesterol transporter as it significantly predates many components of mammalian sterol homeostasis. Admittedly, there are multiple camps that champion different molecules as being the most significant substrate for NPC1. To add to the complexity, the recent identification [29] of the mammalian NPC1 relative, NPC1-L1, as an intestinal transporter of cholesterol suggests that the substrate for this protein could even be tissue-specific. Clearly, any satisfying explanation of NPC1 function must account for all of the observed phenomena, but identification of the primordial cargo molecule for NPC1 could be of practical value, as this so far elusive molecule could represent a prime target for intervention. At present there is no biochemical basis (e.g.,

protein–ligand interaction) for distinguishing fatty acids from sterols or from sphingolipids as substrates. Indeed, other cargos may be relevant; it is possible that defective transmembrane movement of substrate X (where $X \neq$ cholesterol, fatty acids or sphingolipids) could be the underlying trigger to the consequences of loss of the NPC1 proteins. It would be particularly exciting if this substrate were amenable to simple treatment, but the challenge is to identify it.

A unifying hypothesis to the apparent pleiotropy of this syndrome is that NPC1 is a component of a signal transduction cascade. This is suggested by the striking sequence conservation between NPC1 and the proteins that function in the hedgehog morphogen cascade of signal generation for development. Indeed, the strongest full-length homology of NPC1 is with *Patched* (*Ptc*), a protein involved in morphogenesis in *Drosophila* and humans. *Ptc* achieves its biological function, i.e., the induction of genes involved in morphogenesis, via the Sonic Hedgehog signal transduction pathway and a direct interaction with *Smoothed* [30,31]. Despite an immense accumulation of data on this process, no direct link has yet been made between NPC1 and the manner in which *Ptc* works.

Alternatively, the pleiotropy of NPC1 mutant phenotypes may arise from the aberrant initiation of signaling cascades that derive from or respond to sterol (e.g., the sterol regulatory element binding protein SREBP and/or oxysterol LXR/RXR pathways), ceramide- or sphingosine-1-phosphate (e.g., which induces inflammatory responses and pro-apoptotic events); all of which significantly accumulate in NP-C disease. Indeed, it is clear that these responses are aberrant compared to normal cells [32–34], although it remains to be seen whether intervention at these pathways represents treatments for the disease (see below).

The steps in lipid transport through the lysosomal–endosomal system may be conferred by the interactions of NPC1 with NPC2 and other currently obscure “partners”. However, it is interesting that the NPC2 protein, a member of a large family of molecules that bind many divergent lipids in higher eukaryotes [35], is a demonstrably avid binder of cholesterol, and yet is poorly conserved in simple organisms. It may be that convergent evolution has produced multiple methods to maintain these hydrophobic molecules in a soluble and innocuous form, once they have permeated a membrane in an NPC1-dependent manner. Interestingly, the sequences of mammalian NPC1, NPC1-L1 [36], *C. elegans* NPC1 (a and b) and yeast Ncr1 retain the characteristic NH₂-terminal “NPC1-domain” that distinguishes this gene family from the Patched and SCAP-proteins. The sequence may mediate an association with other components of lipid transport, particularly as in some instances it encompasses a leucine zipper interaction motif.

An exciting new insight regarding the link between NPC1 dysfunction and cholesterol trafficking came from manipulations of small GTPases belonging to the Rab gene

family. This large family of proteins (63 members) control vesicle targeting and fusion between all endomembrane compartments and hence control flow of protein and lipid movement in the cell. Strikingly, overexpression of a subset of Rab proteins has dramatic effects on the NPC1 phenotype. Overexpression of Rab 7 and 9, GTPases, which in cell culture systems regulate trafficking between the cell surface and endosomal compartments, resulted in complete reversal of the cholesterol/sphingolipid accumulation (see [13,14] and [8]). Thus it is apparent that the NPC1 defect leading to accumulation of cholesterol/sphingolipid in unusual late endosomal compartments, can be normalized by altering the dynamics of these Rab regulated pathways. These results suggest that in NPC1 disease, lipid accumulation occurs in response to blocking a function of these critical catalysts of membrane transfer. Alternatively, the steady-state accumulation of cholesterol/sphingolipid is dependent on Rab-independent mechanisms that can be modulated by altering the dynamics of endosomal membrane trafficking pathways. Knowledge of the importance of these Rab-dependent pathways may represent a significant step towards understanding and treating this complex disease.

4. Neurodegeneration

The absence of the NPC1 protein has striking effects on sterol homeostasis in all cells. LDL-derived, and perhaps in some cells endogenously synthesized [37], sterols are mislocalized. As described above, and at multiple points throughout this volume, NP-C disease is not only a cholesterol lipidosis; multiple lipids accumulate in multiple tissues [38]. In the context of the syndrome, this initially presents as hepatosplenomegaly in early onset cases, which typically resolves as neurodegeneration becomes symptomatic and ultimately lethal. Thus, to adequately deal with this syndrome, we must understand the processes impacted by NPC1 and NPC2 in the brain. Moreover, any models of the neurodegeneration must explain the marked and selective sensitivity of brainstem and cerebellar neurons to loss of these proteins. Several reviews in this volume [38,39] elegantly define the events that happen in the brains of affected individuals. Although it is clear that Purkinje cells are most compromised in this cerebellar disorder, it remains to be defined how they die [40], although markers of both apoptosis and necrosis have been identified [41]. The events leading to apoptosis and also an ER-stress salvage pathway, known as the unfolded protein response, have all been related to sterol and sphingolipid metabolism. Indeed, cells ablated for NPC1 activity (i.e., heterozygotes or hydrophobic amine drug treated) are protected from the toxic effects of sterols [42,43]. It remains to be determined whether these pathways are directly relevant to the neuropathogenesis associated with homozygous forms of this disorder, although it seems plausible.

5. Therapeutic intervention

As described elsewhere in this volume [44], the NP-C syndrome is a challenge to the physician. Current treatments are largely symptomatic and preventive at best. The unraveling of the genetic basis of this disease created high but unrealistic expectations that effective interventions based on an understanding of fundamental mechanisms would soon follow. It was immediately apparent that protein transduction approaches would not be applicable to NPC1, a multipass-transmembrane protein. Indeed, the results of hepatic and bone marrow transplantation in humans and mice had already suggested as much. In contrast, the soluble, cholesterol binding NPC2 protein appears as an excellent candidate for replacement therapy, either as a recombinant protein or as a protein expressed by donor cells such as mobile monocytes, which could then be secreted and resorbed by neurons. Such a therapeutic trial for NPC-2 disease would be highly desirable but is unlikely given the low frequency of occurrence of this specific disorder. For the more common NPC-1 phenotype, gene therapy or stem cell replacement approaches are clearly the ideal and curative strategy. Gene therapy for this and similar neurodegenerative diseases cannot be pursued until formidable technical barriers are overcome, including bypassing the blood barrier, attaining transduction efficiency, establishing and maintaining appropriate, regulated gene expression and preventing neoplastic transformation.

The limited therapeutic trials that have been performed have been based on efforts to eliminate or limit the putative offending metabolite(s). A clinical trial of cholesterol-lowering therapy did not show evidence of neurologic benefit despite substantial reductions in hepatic unesterified cholesterol in humans. Animal studies have been similarly disappointing [45]. The failure of this approach might reflect the inability of such approaches to change brain cholesterol metabolism or simply that cholesterol is not the offending metabolite.

More recently, the significance of glycosphingolipids in the brain in NP-C disease [46–48] has been reemphasized [38]. The role of specific sphingolipids such as GM2 in promoting morbid anatomic changes has been described in NP-C and related diseases, and the inhibition of glycosphingolipid synthesis by agents such as N-butyldeoxynojirimycin (NB-DNJ), an inhibitor of glucosylceramide synthase, has been tested as treatment [49]. NPC1-mutant mice and cats showed delayed onset of neurological symptoms, increased life span, and reduced ganglioside accumulation. NB-DNJ (miglustat) has been approved in Europe and the United States for treatment of type 1 Gaucher's disease, and is currently in phase I/II trials for patients with NP-C at medical centers in New York and Manchester.

The defect in NP-C results in deficiency states as well as toxic accumulations of metabolites. Thus, the sequestration of sterols at the lysosome has the consequence of

limiting the substrates for pathways such as bile acid metabolism and steroid hormone production. In particular, the synthesis of neurosteroid hormones, dihydropregesterone and allopregnanolone is markedly compromised in cells and animals lacking NPC1 [50]. Treatment of NPC1 mutant mice with parenteral allopregnanolone resulted in delayed onset of symptoms, prolonged life span and improved neurological function, comparable to that seen with NB-DNJ [50].

In contrast to the above approaches focused on understanding and correcting directly the manifestations of deficient NPC1/2 function, the observation that modulation of Rab-dependent dynamics of the endosomal trafficking pathways can reverse cholesterol/sphingolipid intracellular accumulation, points toward a completely different corrective strategy. This therapeutic strategy would be aimed at modulating Rab-dependent steps, thereby potentially markedly reducing the severity of onset of disease by lowering lipid accumulation in endosomal compartments.

Finally, for the person (scientist or parent) not absorbed by this specific syndrome, it is worth recalling that understanding NP-C disease goes beyond a small community. Late stages in the neuropathy of NP-C and Alzheimer's disease have compelling similarities, particularly with regard to the formation of neurofibrillary tangles consisting of paired helical filaments and hyperphosphorylated tau protein [51,52]. Moreover, there is a marked appreciation of the role of cholesterol homeostasis in AD progression [53]. Similarly, concepts of sterol toxicity and its sequelae with respect to atherosclerosis will likely be impacted by understanding this complex syndrome. Thus NP-C research will impact many more of the population than first thought likely for an orphan disorder.

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Discovery of a deep intronic mutation in Intron 9 of the NPC1 gene in twin females with classic Niemann-Pick Type C.

Patrick Lundquist, Mark E. Gelsthorpe, Ph.D., Daniel S. Ory, M.D., and D. Brian Dawson, Ph.D.

Niemann-Pick Type C (NPC) is an autosomal recessive neurodegenerative disorder characterized by defective intracellular cholesterol transport. NPC classically presents in infants or early childhood with death commonly occurring in the first two decades of life. Approximately 90% of patients diagnosed with NPC have mutations within the NPC1 gene and 4% have mutations within the NPC2 gene. Fibroblasts from twin three year old females, who presented with clinical symptoms consistent with classic NPC, showed decreased cholesterol esterification and abnormal filipin staining consistent with a diagnosis of classic NPC. DNA sequencing and Multiplexed Ligation-dependent Probe Amplification (MLPA) were performed for both NPC1 and NPC2, and demonstrated only one heterozygous alteration, 1920delG in exon 12 of the NPC1 gene, in both twins. While the mother was found to carry the 1920delG, no alteration was found in the father. Quantitative RT-PCR analysis of NPC1 mRNA revealed a 0.31-0.65 fold reduction as compared to a reference cell line. Separation of the alleles from each patient into their respective daughter alleles in a mouse/human hybrid background, followed by cDNA analysis of exons 8-13 revealed an aberrant higher MW species. DNA sequence analysis demonstrated the 1920delG mutation and, for the higher MW band, an insertion of 193bp between the Exon 9/10 intron-exon boundaries. A G>A missense mutation was also noted. None of these alterations were present in the reference sample. Genomic DNA analysis indicated that the two patients and the father carry an IVS9-1009G>A missense mutation. *In silico* analysis of the IVS9-1009G>A alteration predicted that this missense change would enhance the splicing of a cryptic splice site within intron 9 leading to insertion of the 193 bp sequence. The 193bp insertion causes a frameshift leading to a premature stop codon which would be predicted to result in a truncated protein. NPC1 protein expression, normalized to actin, was approximately 15 to 21% compared to a reference cell line. Together, these data indicate that this deep intronic missense change is the second pathogenic alteration leading to NPC in these two patients.

Medical History Summary For Addison and Cassidy Hempel

Compiled by Chris Hempel, Mother

	Addison Ruth Hempel	Cassidy Helene Hempel
DOB	<ul style="list-style-type: none"> January 23, 2004 – Twin A 	<ul style="list-style-type: none"> January 23, 2004 – Twin B
Delivery	<ul style="list-style-type: none"> Via c-section at 35 weeks, two vessel umbilical cord 	<ul style="list-style-type: none"> Via c-section at 35 weeks, single umbilical cord
Birth weight	<ul style="list-style-type: none"> 1968 grams 	<ul style="list-style-type: none"> 2085 grams
Hospitalization	<ul style="list-style-type: none"> Hospitalized for 10 days for feeding issues 	<ul style="list-style-type: none"> Hospitalized for 8 days for feeding issues
Bilirubin at birth	<ul style="list-style-type: none"> Maximum total bilirubin = 9 No problems with hyperbilirubinemia, no photo therapy 	<ul style="list-style-type: none"> Maximum total bilirubin = 9.4 No problems with hyperbilirubinemia, no photo therapy
Early Development/Growth	<ul style="list-style-type: none"> Received Synagis for remainder of Feb 2004 RSV season Received all immunizations Normal growth and development noted at well child visits until two years of age 	<ul style="list-style-type: none"> Same
January 2006	<ul style="list-style-type: none"> Afflicted with flu like virus 	<ul style="list-style-type: none"> Same
February 2006	<ul style="list-style-type: none"> Splenomegaly noted at approximately 2 years of life – noted at ill-visit following viral illness, splenomegaly thought to be related to EBV infection February 16, 2006: Spleen measures 13.6cm x 11.0cm x 5.6cm Liver measures 9.6cm long axis and is homogenous in echotexture Right kidney 7.9cm, left kidney 7.5cm, normal with no mass or obstruction Complete blood count significant for mildly decreased WBC of 4.2, mild anemia and hemoglobin of 11.0, noted microcytosis, macrocytosis, ovalocytes, tear drop cells and spherocytes 	<ul style="list-style-type: none"> Splenomegaly noted at approximately 2 years of life – noted at ill-visit following viral illness, splenomegaly thought to be related to EBV infection February 16, 2006: Spleen 10.3x9.2x7.3cm Liver is unremarkable Complete blood count significant for mildly decreased WBC of 4.0, normal hemoglobin 12.2, noted microcytosis, macrocytosis, ovalocytes, tear drop cells and spherocytes Mild elevation of AST of 79 (normal 25-45)

	<ul style="list-style-type: none"> • Mild elevation of AST of 101 (normal 25-45), and also ALT of 51 (normal 2-50) • EBV panel positive for recent infection • Hereditary spherocytosis completed and negative 	<ul style="list-style-type: none"> • EBV negative, repeat testing and test positive • Hereditary spherocytosis completed and negative
March 27, 2006	<ul style="list-style-type: none"> • Spleen is about 12.4cm, prior examination spleen was 13.6, difficult to compare as spleen is larger than size of ultrasound transducer 	<ul style="list-style-type: none"> • Abdominal ultrasound conducted 3/27/06. Spleen in 13.4cm and doctor feels they underestimated the last ultrasound measurement at 10.3cm
March 31, 2006	<ul style="list-style-type: none"> • Evaluated at Stanford, Gaucher and Niemann Pick A/B ruled out, Niemann Pick Type C not tested • Abdominal ultrasound conducted, spleen measuring 13 cm in 	<ul style="list-style-type: none"> • Evaluated at Stanford, Gaucher and Niemann Pick A/B ruled out, Niemann Pick Type C not tested •
September 2006	<ul style="list-style-type: none"> • Re-evaluated at Stanford for persistent splenomegaly, immunology evaluation normal, including T and B lymphocyte panels and Immunoglobins) 	<ul style="list-style-type: none"> • Re-evaluated at Stanford for persistent splenomegaly, immunology evaluation normal, including T and B lymphocyte panels and Immunoglobins) • Spleen measured again and is 12.3cm, no other abnormalities noted on abdominal ultrasound
September 7, 2006	<ul style="list-style-type: none"> • Spleen measures 13cm in length, left kidney 6.7cm and right kidney 7.7cm and they are normal in appearance, besides enlarged spleen, unremarkable abdominal ultrasound 	<ul style="list-style-type: none"> • Spleen measured again and is 12.8cm in diameter, no other abnormalities noted on abdominal ultrasound • Left kidney 7.1cm, right kidney 7.6cm
October 30, 2006	<ul style="list-style-type: none"> • Liver/spleen scan conducted, 2.22 millicuries of Tc 99m sulfur colloid administered intravenously, spleen is enlarged, the intensity of activity in the liver is much greater than within the spleen, no evidence of colloid shift or focal defects identified 	<ul style="list-style-type: none"> • Liver/spleen scan conducted, 2.04 ,Ci of Tc 99m sulfur colloid administered intravenously, spleen enlarged, no focal defects
April 4-7,2007	<ul style="list-style-type: none"> • Hospitalized for Rotavirus 	<ul style="list-style-type: none"> • Same

March 22, 2007	<ul style="list-style-type: none"> Spleen remains enlarged measuring 13cm in length, kidneys normal in appearance (left 7.5 cm, right 7.7 cm) 	<ul style="list-style-type: none"> Spleen remains enlarged measuring 12.8cm in diameter, kidneys normal in appearance (left 7.1 cm, right 7.6 cm)
June 11-13, 2007	<ul style="list-style-type: none"> Hospitalized for Prolonged Fever, Mild leucopenia (wbc 1.9) Ophthalmologic exam normal CT scan of abdomen done to look for appendicitis Marked splenomegaly with spleen measuring 16cm in size with mesenteric lymph nodes up to a centimeter and a half in size, spleen is more enlarged from 3/22/2007 	<ul style="list-style-type: none"> Hospitalized for Prolonged Fever as well, no scans.
September 7, 2007	<ul style="list-style-type: none"> Hepatomegaly first noted radiographically on abdominal CT, 8.3cm x 16.7cm x 12.8cm Throat culture reveals rare growth <i>S. Agalactiae</i> (group B beta strep) and unusual pathogen 	<ul style="list-style-type: none"> Enlarged spleen of 12.3 cm. No focal abnormalities are noted in the liver, gallbladder, pancreas, aorta, or inferior vena cava.
September 18, 2007	<ul style="list-style-type: none"> First signs of hepatosplenomegaly CT scan of abdomen without contrast, spleen enlarged: 5.3 cm maximum transverse dimension x 10.6 cm maximum AP dimension, 15.7 cm superior to inferior dimension. Liver measures 8.3 cm in transverse dimension and 16.7 cm in AP dimension and about 12.8 cm in superior to inferior dimension Kidneys are normal with no nephrolithiasis or hydronephrosis. Skeletal Survey also conducted. No bony abnormalities 	<ul style="list-style-type: none"> First signs of hepatosplenomegaly CT scan of abdomen without contrast, spleen enlarged: 5.1 cm maximum lateral dimension x 10.4 cm maximum lateral dimension, 14.1 cm superior to inferior dimension. Liver measures 7.9 cm in transverse dimension and 16.6 cm in lateral dimension and about 12.5 cm in superior to inferior dimension Kidneys are normal with no nephrolithiasis or hydronephrosis Skeletal Survey also conducted. No bony abnormalities
September 26, 2007	<ul style="list-style-type: none"> NeuroPsychology tests performed by Dr. Carina Grandison at 3 years, 8 months old: Gross Motor 22 months, Fine Motor 27 Months, Visual Reception 33 months on Mullen Scale 	<ul style="list-style-type: none"> NeuroPsychology tests performed by Dr. Carina Grandison at 3 years, 8 months old: Gross Motor was 22 months, Fine Motor 22 Months, Visual Reception 33 months on Mullen Scale

October 2-3, 2007	<ul style="list-style-type: none"> • Video EEG, Brain MRI, neurology consultation - parents complain of ataxia and seizure activity, neurology consult says developmental delay, mild ataxia, questionable oculomotor apraxia and mild dysmetria • MRI with brain with and without contrast, study on G.E. Signa 1.5 Telsa scanner- calvaria normal, no extra axial fluid collections, ventricle system and basal cisterns are within normal limits, no areas of abnormal signal in brain substance, no acute hemorrhage, mass lesion or evidence of acute infarction 	<ul style="list-style-type: none"> • Same, essentially negative brain MRI for age
October 15, 2007	<ul style="list-style-type: none"> • Niemann Pick Type C diagnosis through electron microscopy from Stanford/Mayo Clinic • Evidence of polymorphic cytoplasmic bodies 	<ul style="list-style-type: none"> • Same
October 26, 2007	<ul style="list-style-type: none"> • Visit to Marc Patterson, Mayo Clinic. Speech still intact at this time. Abnormalities found were splenomegaly, vertical supranuclear gaze palsy, with possible increase and horizontal saccadic latency, ataxia of gait, stance and limb movement with normal power • Iturriaga scale: 6/18 • NIH severity Scale: 5/45 with no modifiers 	<ul style="list-style-type: none"> • Visit to Marc Patterson, Mayo Clinic. Speech still intact at this time. Abnormalities found were splenomegaly, vertical supranuclear gaze palsy, with possible increase and horizontal saccadic latency, ataxia of gait, stance and limb movement with normal power • Iturriaga scale: 6/18 • NIH severity Scale: 5/45 with no modifiers
November 2007	<ul style="list-style-type: none"> • Visited NIH to participate in Natural History Study • Miglustat 100mg 2x per day therapy started along with supplements such as Fish Oil and NSAIDS 	<ul style="list-style-type: none"> • Same
January 2008	<ul style="list-style-type: none"> • Elevated Serum DHEA (2.8) Normal .4-1.5ng/ml 	<ul style="list-style-type: none"> • Elevated Serum DHEA () Normal .4-1.5ng/ml
February 1, 2008	<ul style="list-style-type: none"> • Complete abdominal ultrasound conducted, spleen is 14.7cm x 12.2cm x 5.1cm, gall bladder normal, pancreas unremarkable , no other abnormalities 	<ul style="list-style-type: none"> • Complete abdominal ultrasound conducted, spleen is 12.9x10.05x4.75 cm, gall bladder normal, pancreas unremarkable , no other abnormalities

June 13, 2008	<ul style="list-style-type: none"> • Neuro-Ophthalmology Exam conducted by Dr. Gerard Hershewe 	<ul style="list-style-type: none"> • Same
October 24, 2008	<ul style="list-style-type: none"> • Liver is normal is size, no definite hepatomegaly. • Gallbladder and pancreas appear normal • Spleen is enlarged: 15.4 x 16 x 5.6 cm • Right kidney measures 9.0 and the left kidney measures 8.2 cm in length 	<ul style="list-style-type: none"> • Abdomen complete survey. Liver is homogenous without biliary dilatation. Gallbladder and portal venous flow and the IVC flow are normal. Spleen is enlarged measuring 14.7 x 13.7 x 5.2 cm. Pancreas and abdominal aorta and both kidneys appear normal. Right kidney measures 6.7 and the left kidney measures 7.9 cm in length
April 7, 2009	<ul style="list-style-type: none"> • CT Abdomen with contract • Liver is enlarged measuring 17.4 x 9.9 cm in maximal transverse dimension x 13.9 cm in cephalocaudal dimension. In comparison to prior study on 9/18/07, there is no significant change • Spleen is enlarged measuring 11.2 x 6.1 cm in greatest transverse dimension, and 16.7 cm in cephalocaudal dimension. These are also not significantly changed from 9/18/07. 	<ul style="list-style-type: none"> • CT Abdomen with contract • Liver is enlarged measuring 16.3 x 8.4 cm in greatest transverse dimension x 12.2 cm in cephalocaudal dimension. In comparison to prior study on 9/18/07, there is not significant change • Spleen is enlarged measuring 10.0 and 4.9 cm in greatest transverse dimension, and 15.5 cm in cephalocaudal dimension. These are also not significantly changed from 9/18/07.
April 2009	<ul style="list-style-type: none"> • Cyclodextrin IV therapy started with dose escalation (see submissions for additional data) 	<ul style="list-style-type: none"> • Same
August 2009	<ul style="list-style-type: none"> • XMRV active retrovirus infection confirmed by Whittemore Petersen Institute 	<ul style="list-style-type: none"> • Same
	<ul style="list-style-type: none"> • CT Scan of Abdomen and Pelvis with contrast, comparison to 4/07/2009. • Liver measures 13.3 in cephalocaudal dimension X 16.6cm in anteroposterior dimension and 6.6cm in transverse dimension for calculated volume of 681.8 cubic centimeters • Spleen measures 15.3cm in cephalocaudal dimension x 9.6 in anteroposterior dimension and 5.6cm 	<ul style="list-style-type: none"> • CT Scan of Abdomen and Pelvis with contrast, comparison to 4/07/2009. • Liver measures 12.7 in cephalocaudal dimension x 15.1cm in anteroposterior dimension and 9.6cm in transverse dimension for calculated volume of 375.6 cubic centimeters

	in transverse dimension for a volume of 276.5 cubic centimeters	<ul style="list-style-type: none"> Spleen measures 14.7cm in cephalocaudal dimension x 10.6 in anteroposterior dimension and 4.2cm in transverse dimension for a volume of 295.4 cubic centimeters
February 16, 2010	<ul style="list-style-type: none"> Video EEG shows seizure activity, multiple abnormalities, left side more than right, Keppra started 	<ul style="list-style-type: none"> Video EEG reveals seizure activity, multiple abnormalities, right side more than left, Keppra started
April 10-15, 2010	<ul style="list-style-type: none"> Children's Michigan visit; PETs reveal increased hypometabolism in Addi and Cassi's brains Taken off Keppra and put on Clonazepam 	<ul style="list-style-type: none"> Same



**Addi and Cassi Hydroxy-Propyl-Beta-Cyclodextrin Plan
Compassionate Use Clinical Study
Treatment Plan Version # 2
February 22, 2009**

Sponsor: Caroline Hastings, M.D.

Study Site: Renown Hospital, Reno NV

Patients: Addi and Cassi Hempel

Niemann Pick Type C disease (NPC) is an autosomal recessive lysosomal storage disease characterized by progressive neurodegeneration. More than 250 mutations of the NPC1 gene located on Chromosome 18 have been identified. Although NPC has a highly variable phenotype, the classic presentation occurs in middle to late childhood with insidious onset of ataxia, vertical supranuclear gaze palsy, and dementia. Other features include dystonia, dysarthria and dysphagia eventually becoming disabling. Death typically occurs in the late second or third decade from aspiration pneumonia. Definitive diagnosis of NPC is based upon impaired cholesterol esterification and positive filipin staining in cultured fibroblasts.

For expert review of NPC please see:

<http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=npv> and
<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=257220>

Addi and Cassi Hempel (DOB 1/23/2004), identical twin Caucasian females, received a definitive diagnosis of Niemann Pick Type C in October, 2007. The girls have been receiving Miglustat (Zavesca®) 100 mg BID for almost one year. In addition, the children are receiving nutritional supplements including Curcumin.

Both children have been participating in a study at the National Institutes of Health designed to track the progression of NPC disease. The children were last examined at the NIH in July of 2008. Both children continue to exhibit hepatosplenomegaly and clinical deterioration consistent with disease progression as manifested by severe neurological symptoms including cerebellar ataxia, dysarthria, dysphagia, vertical supranuclear palsy and cataplexy as well as progressive dementia.

The proposed HPBCD treatment plan is as follows:

	Baseline (-10 – 14 days)	Week 1	Weeks 2-5	Weeks 6-10	Weeks 11-15	Subsequent Weeks
Dose		80 mg/kg/d; 24 hrs x 4	160 mg/kg/d; 8 hrs x 1/wk	320 mg/kg/d; 8 hrs x 1/wk	400 mg/kg/d; 8 hrs x 1/wk	TBD pending protocol amendment
Mediport	X					
Physical exam	X	X	X	X	X	
Neruological exam	X	X	X	X	X	
Volumetric CT	X					X
Renal Function (GFR)	X					X
Vital sign	X	Q 15 min x 4 + Q 30 min x 2 + Q 240 min x 5	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	
Renal labs ¹	X	X	X	X	X	
Hematology ²	X	X	X	X	X	
Chemistry ³	X	X	X	X	X	
Biomarker sample (15 ml whole blood)	X	X	X	X	X	
Urinary cholesterol sample 10 ml	X	X	X	X	X	

¹ Serum BUN/Cr; am spot urine Pr/Cr; UA

²CBC with Platelets

³Amylases, AST, ALT, bilirubin

- **Catheterization.** A MediPort™ central venous catheter will be surgically implanted into the subclavian vein 10 days to 2 weeks prior to initial HPBCD infusion.
- **Baseline Clinical Assessment.** Prior to the initiation of HPBCD infusion both children will have the following assessments performed:
 - Complete physical and neurological exam which will be videotaped
 - Complete blood chemistry
 - Renal function test
 - Urinalysis
 - Volumetric CT of liver and spleen
 - A 15 ml blood sample and 10 ml urine sample will be retained for experimental purposes related to biomarkers and urinary cholesterol determinations.
- **Initial infusion.** Both children will receive a four day continuous infusion of HPBCD in the pediatric ward at Renown Medical center hospital in Reno, NV a facility equipped with a pediatric ICU. Dosage will be **80 mg/kg/day** at a rate of 20 ml/hr. The solution will be administered as prepared in sterile water for injection. The final concentration will be precise based upon the weight of the patients.

The solutions will be prepared by a pharmacy certified by the Nevada Board of Pharmacy to prepare Compounded Sterile Products in accordance with current USP chapter 797 guidelines for aseptic processing. Specifically, for a high-risk non-sterile material received in bulk the following procedure will be employed:

- In a ISO class 5 or cleaner room the HPBCD will be weighed and dissolved in 500 ml of sterile water using sterile containers.
 - Terminal sterilization of high-risk level CSPs by filtration shall be performed with a sterile 0.22-µm porosity filter entirely within an ISO Class 5 or superior air quality environment.
 - The sterilized solution will be placed into commercial infusion bags, under ISO class 5 or cleaner conditions, and labeled according to the pharmacy SOP with before use dating of 24 hrs in accordance with USP 797: *“For a sterilized high-risk preparation, in the absence of passing a sterility test, the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 24 hours at controlled room temperature.”*
 - The solutions will be delivered to the clinic no more than 4 hours prior to use.
- **Safety Monitoring.** Vital signs will be monitored every 15 minutes for the first hour during initiation of IV infusion, every 30 minutes during the second hour, and then every four hours. At the conclusion of the HPBCD infusion, and prior to any subsequent infusions, the patients will receive complete blood chemistry and urinalysis. In the unlikely event that a severe allergic reaction should occur, the following specific plan to deal with the possibility of anaphylactic or hyper-allergic response will be in place:

A standard cardex for each patient in the clinic is prepared. This card (bright orange and on top of the clinic chart at each visit) has the name, age and weight of the child. The standard

drugs for resuscitation in the event of allergy or anaphylaxis are on the chart and the doses are pre-calculated. We then arrange to have these drugs available in the clinic or hospital room, at the bedside, and draw them if needed prior to administration. The protocol for initial management includes:

- Discontinue the drug.
- Establish airway if necessary. Assess breathing; Supply with 100% oxygen with respiratory support as needed. Assess circulation and establish IV access. Place patient on a cardiac monitor.
- Albuterol nebulized, 0.05 to 0.15 mg/kg in 3 ml NS every 15 minutes as necessary.
- Diphenhydramine 1mg/kg IV or IM
- Methylprednisolone 2 mg/kg IV

- If patient is hypotensive:
 - Place in Trendelenburg position, head at 30-degree angle below feet.
 - IV fluid bolus, NS or LR 20 ml/kg IV over 5 to 15 minutes. Repeat as necessary.
 - Epinephrine 1:10,000, 0.01 mg/kg (0.1 cc/kg) SC or IV.

In the event any symptoms of allergy or anaphylaxis occur, the patient will be admitted to the PICU for observation and further management.

- **Subsequent infusions.** Following the initial HPBCD infusion without adverse events, the patients will receive weekly 8 hour infusions of HPBCD for three months. Additional infusions will require a protocol amendment and review by the FDA. The patients will initially receive **160 mg/kg/day**. If well tolerated the patients will receive additional weekly infusions. The patients will have vital signs (e.g., heart rate, blood pressure, temperature) monitored as above during the infusions, and will undergo a physical examination, including neurological assessment, and clinical laboratory measurement weekly.
- **Dosage adjustment.** In the absence of side effects or adverse events (defined as serum creatinine greater than or equal to 2.5 times baseline values or liver transaminases (AST and ALT) greater than 3 time baseline), and upon review by the FDA of laboratory values, and at the discretion of the investigator, a **second** dosage increases to **320 mg/kg/day** will be made. Downward adjustments in dosage may be made on a **weekly** basis at the discretion of the investigator. Similarly, a **third** monthly increase to **400 mg/kg/day** will be subject to FDA review and the discretion of the investigator. If laboratory values should be elevated above the limits, the drug will be discontinued until they return to acceptable levels. The drug may then be re-started at a dose of 10 mg/kg/day less than the most recent dose.
- **Study termination.** In the event clinically significant adverse events are observed, the study may be terminated at any time at the discretion of the investigator. Throughout the study stopping criteria will be the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), v3) Grade 4 toxicity. At the end of three months treatment the data will be reviewed with the FDA and a decision whether to continue treatment will be made.

- **Therapeutic endpoints.** The primary endpoint of this study will be improvement in neurological functioning. Secondly, arrest of disease progression will be assessed based upon independent expert review of the case report and video assessments. At three month intervals and/or at the conclusion of the study a complete physical and videotaped neurological exam will be performed. Changes in neurological status will be assessed using independent ratings of the videotapes examined by a pediatric neurologist. A volumetric CT of the liver and spleen will be obtained for possible changes in hepatosplenomegaly. Following each infusion a 15 ml blood sample and 10 ml urine sample will be retained for experimental purposes related to biomarkers and urinary cholesterol determinations. Weekly assessment by the parents will examine quality of life measures and a global impression of change will be made.

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Addi and Cassi's Hydroxy-Propyl-Beta-Cyclodextrin Compassionate Use Clinical Study

Protocol Extension 1.0

July 7, 2009

Sponsor: Caroline Hastings, M.D.

Study Site: Renown Hospital, Reno, Nevada

Patients: Addi and Cassi Hempel

IND #: 104,114 (Addison Hempel) and 104,116 (Cassidy Hempel)

Addi and Cassi Hempel (DOB 1/23/2004), identical twin Caucasian females, received a definitive diagnosis of Niemann Pick Type C in October, 2007. The girls have been receiving miglustat (Zavesca®) 100 mg BID for almost one year. In addition, the children are receiving nutritional supplements including Curcumin.

Both children have been participating in a study at the National Institutes of Health designed to track the progression of Niemann Pick Type C disease. The children were last examined at the NIH in July of 2008. Both children continue to exhibit hepatosplenomegaly and clinical deterioration consistent with disease progression as manifested by severe neurological symptoms including cerebellar ataxia, dysarthria, dysphagia, vertical supranuclear palsy and cataplexy as well as progressive dementia.

On April 13, 2009 under the above compassionate use INDs the children began receiving intravenous infusions of hydroxyl-propyl-beta-cyclodextrin (HPBCD) starting at a dose of 80 mg/kg/day. The dose of HPBCD has been escalated on a monthly basis to current levels of 400 mg/kg/day administered as a weekly eight hour infusion in a hospital setting.

Because the treatment has failed to result in significant observable clinical improvement or in adverse events, increased dosing frequency (twice/week) and rate of dose titration (100 mg/kg/infusion) will be increased starting July 16, 2009.

Attached is the proposed treatment plan for the next 12 weeks is as follows:

	Baseline* Week 13 core protocol	Week 1	Week 2	Week 3	Subsequent Weeks 4- 12	Discontinuation Week 13
Dose Escalation. Following normalization of AST, dose will be held constant for subsequent infusions.		500 mg/kg/d; 8 hrs x1 + 3-4 days 600 mg/kg/d; 8 hrs x1	700 mg/kg/d; 8 hrs x1 + 3-4 days 800 mg/kg/d; 8 hrs x1	900 mg/kg/d; 8 hrs x1 + 3-4 days 1000 mg/kg/d; 8 hrs x1	Add 100 mg/kg/d; 8hrs Every 3-4 days	
Physical exam	X	X	X	X	X	
Neurological exam	X	X	X	X	X	
Volumetric CT						X
Renal Function (GFR)						X
Vital sign	X	Q 15 min x 4 + Q 30 min x 2 + Q 240 min x 5	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	Q 15 min x 4 + Q 30 min x 2 + Q 240 min	
Renal labs ¹	X	X	X	X	X	
Hematology ²	X	X	X	X	X	
Chemistry ³	X	X	X	X	X	
Biomarker sample (15 ml whole blood)	X	X	X	X	X	
Urinary cholesterol sample 10 ml	X	X	X	X	X	

¹ Serum BUN/Cr; am spot urine Pr/Cr; UA

² CBC with Platelets, lipid panel

³ Amylases, AST, ALT, bilirubin

*Baseline values can be used from Week 13 of the core protocol.

- **Baseline Clinical Assessment.** Prior to the initiation of HPBCD infusion both children will have the following assessments performed:
 - Complete physical and neurological exam which will be videotaped
 - Complete blood chemistry
 - Renal function test
 - Urinalysis
 - Volumetric CT of liver and spleen
 - A 15 ml blood sample and 10 ml urine sample will be retained for experimental purposes related to biomarkers and urinary cholesterol determinations.
- **Initial infusion.** Both children will continue to receive infusions of HPBCD in the pediatric ward at Renown Medical center hospital in Reno, NV a facility equipped with a pediatric ICU. Dosage will be increased by 100 mg/kg/ day from the previous level and will be infused over 8 hours at a rate of 20 ml/hr. The initial infusion will be **500 mg/kg/day**. The solution will be administered as prepared in sterile water for injection. The final concentration will be precise based upon the weight of the patients.

The solutions will be prepared by a pharmacy certified by the Nevada Board of Pharmacy to prepare Compounded Sterile Products in accordance with current USP chapter 797 guidelines for aseptic processing. Specifically, for a high-risk non-sterile material received in bulk the following procedure will be employed:

- In a ISO class 5 or cleaner room the HPBCD will be weighed and dissolved in 500 ml of sterile water using sterile containers.
 - Terminal sterilization of high-risk level CSPs by filtration shall be performed with a sterile 0.22-µm porosity filter entirely within an ISO Class 5 or superior air quality environment.
 - The sterilized solution will be placed into commercial infusion bags, under ISO class 5 or cleaner conditions, and labeled according to the pharmacy SOP with before use dating of 24 hrs in accordance with USP 797: *“For a sterilized high-risk preparation, in the absence of passing a sterility test, the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 24 hours at controlled room temperature.”*
 - The solutions will be delivered to the clinic no more than 4 hours prior to use.
- **Safety Monitoring.** Vital signs will be monitored every 15 minutes for the first hour during initiation of IV infusion, every 30 minutes during the second hour, and then every four hours. At the conclusion of the HPBCD infusion, and prior to any subsequent infusions, the patients will receive complete blood chemistry and urinalysis. In the unlikely event that a severe allergic reaction should occur, the following specific plan to deal with the possibility of anaphylactic or hyper-allergic response will be in place:

A standard cardex for each patient in the clinic is prepared. This card (bright orange and on top of the clinic chart at each visit) has the name, age and weight of the child. The standard drugs for resuscitation in the event of allergy or anaphylaxis are on the chart and the doses are pre-calculated. We then arrange to have these drugs available in the clinic or hospital room, at the bedside, and draw them if needed prior to administration. The protocol for initial management includes:

- Discontinue the drug.
- Establish airway if necessary. Assess breathing; Supply with 100% oxygen with respiratory support as needed. Assess circulation and establish IV access. Place patient on a cardiac monitor.
- Albuterol nebulized, 0.05 to 0.15 mg/kg in 3 ml NS every 15 minutes as necessary.
- Diphenhydramine 1mg/kg IV or IM
- Methylprednisolone 2 mg/kg IV

- If patient is hypotensive:
 - Place in Trendelenburg position, head at 30-degree angle below feet.
 - IV fluid bolus, NS or LR 20 ml/kg IV over 5 to 15 minutes. Repeat as necessary.
 - Epinephrine 1:10,000, 0.01 mg/kg (0.1 cc/kg) SC or IV.

In the event any symptoms of allergy or anaphylaxis occur, the patient will be admitted to the PICU for observation and further management.

- **Subsequent infusions.** Assuming the previous HPBCD infusion occurs without adverse events, the patients will receive additional 8 hour infusions every three to four days (i.e., twice/week). Dosage of all subsequent infusions will increase by 100 mg/kg/day until either liver transaminase return to normal levels or signs of toxicity are observed. Following normalization of transaminase levels, dosage will be held constant for subsequent infusions. The patients will have vital signs (e.g., heart rate, blood pressure, temperature) monitored as above during the infusions, and will undergo a physical examination, including neurological assessment, and clinical laboratory measurement weekly.

- **Dosage adjustment.** In the absence of side effects or adverse events (defined as serum creatinine greater than or equal to 2.5 times baseline values or worsening of liver transaminase (AST and ALT) greater than 3 time baseline dosage will continue to be increased. If laboratory values should be elevated above the limits, the drug will be discontinued until they return to acceptable levels. The drug may then be re-started at a dose of 100 mg/kg/day less than the most recent dose.

- **Study termination.** In the event clinically significant adverse events are observed, the study may be terminated at any time at the discretion of the investigator. Throughout the study stopping criteria will be the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), v3) Grade 4 toxicity. At the end of three months treatment the data will be reviewed with the FDA and a decision whether to continue treatment will be made.

- **Therapeutic endpoints.** The primary endpoint of this study continuation will be improvement liver transaminase (AST consistently 2x normal in both patients as a concomitant of NPC disease) and in neurological functioning. Secondly, arrest of disease progression will be assessed based upon independent expert review of the case report and video assessments. At three month intervals and/or at the conclusion of the study a complete physical and videotaped neurological exam will be performed. Changes in neurological status will be assessed using independent ratings of the videotapes examined by a pediatric neurologist. A volumetric CT of the liver and spleen will be obtained for possible changes in hepatosplenomegaly. Following each infusion a 15 ml blood sample and 10 ml urine sample will be retained for experimental purposes related to biomarkers

and urinary cholesterol determinations as reflected in the sample collection protocol below. Weekly assessment by the parents will examine quality of life measures and a global impression of change will be made.

- **Biomarkers.** Collection of biomarkers and pharmacokinetic samples will be performed using baseline specimen collections and at eight week intervals. Additional samples will be collected at study conclusion.
For pharmacokinetic estimates, samples will be obtained from the MediPort at baseline, and via venipuncture at 1-2 hours after the start of the infusion, and at 10-15 minutes post infusion.

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Office of Orphan Products Development
Food and Drug Administration
Building 32, Room 5271
10903 New Hampshire Avenue
Silver Spring, MD 20993

MAY 17 2010

Children's Hospital Oakland Research Institute
Department of Pediatric Hematology Oncology
747 Fifty Second Street
Oakland, California 94609-1809

Attention: Caroline Hastings, M.D.
Director, Fellowship Program

Re: Designation Request # 10-3039

Dear Dr. Hastings:

Reference is made to your request for orphan-drug designation dated February 26, 2010, of hydroxy-propyl-beta-cyclodextrin (trade name: TrappsolTM) for "treatment of Niemann-Pick Disease, Type C." Please also refer to our letter dated March 5, 2010.

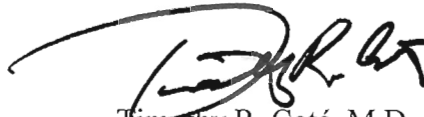
Pursuant to section 526 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 360bb), your request for orphan-drug designation of hydroxy-propyl-beta-cyclodextrin is granted for *treatment of Niemann-Pick Disease, Type C*. Please be advised that it is the active moiety of the drug and not the formulation of the drug that is designated.

Please note that if the above drug receives marketing approval for an indication broader than what is designated, it may not be entitled to exclusive marketing rights under section 527 (21 U.S.C. 360cc). Therefore, prior to final marketing approval, we request that you compare the drug's designated orphan indication with the proposed marketing indication, and submit additional information to amend the orphan-drug designation if warranted.

Please submit to the Office of Orphan Products Development a brief progress report of drug development within 14 months after this date and annually thereafter until marketing approval (*see* 21 C.F.R. 316.30). Finally, please notify this Office within 30 days of a marketing application submission for the drug's designated use.

If you need further assistance in the clinical development of your drug, please feel free to contact Diane Centeno-Deshields, R.Ph., at (301) 796-8674. Please refer to this letter as official notification. Congratulations on obtaining your orphan-drug designation.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Timothy R. Coté', with a large, sweeping flourish extending from the end of the signature.

Timothy R. Coté, M.D., M.P.H.
Director, Office of Orphan Products Development



US 20090286272A1

(19) **United States**

(12) **Patent Application Publication**
Ory et al.

(10) **Pub. No.: US 2009/0286272 A1**

(43) **Pub. Date: Nov. 19, 2009**

(54) **BIOMARKERS FOR NIEMANN-PICK C
DISEASE AND RELATED DISORDERS**

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(21) Appl. No.: **12/385,529**

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Related U.S. Application Data

(60) Provisional application No. 61/071,074, filed on Apr.
11, 2008.

Publication Classification

(51) **Int. Cl.**
C12Q 1/02 (2006.01)

(52) **U.S. Cl.** **435/29**

(57) **ABSTRACT**

Methods for screening or diagnosing subjects for disorders involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, Niemann-Pick C(NPC) disease, lysosomal storage diseases, cholesterol trafficking diseases, and neurodegenerative diseases. Also provided are methods for screening or diagnosing subjects (including infants and neonatal subjects) for NPC disease, methods for monitoring the progression, remission, and clinical status of NPC disease, and methods for evaluating the efficacy of therapeutic treatment of NPC disease.

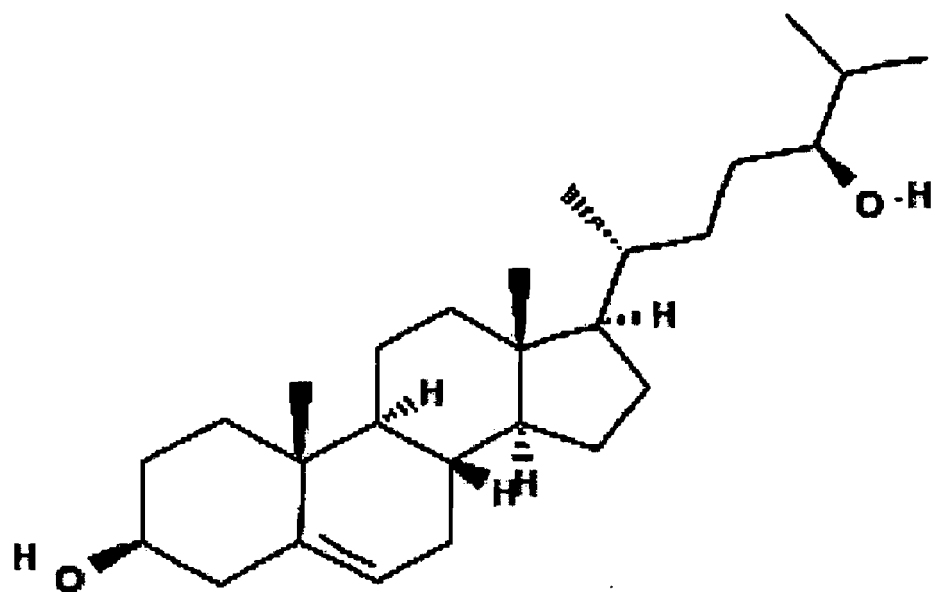


FIG. 1A

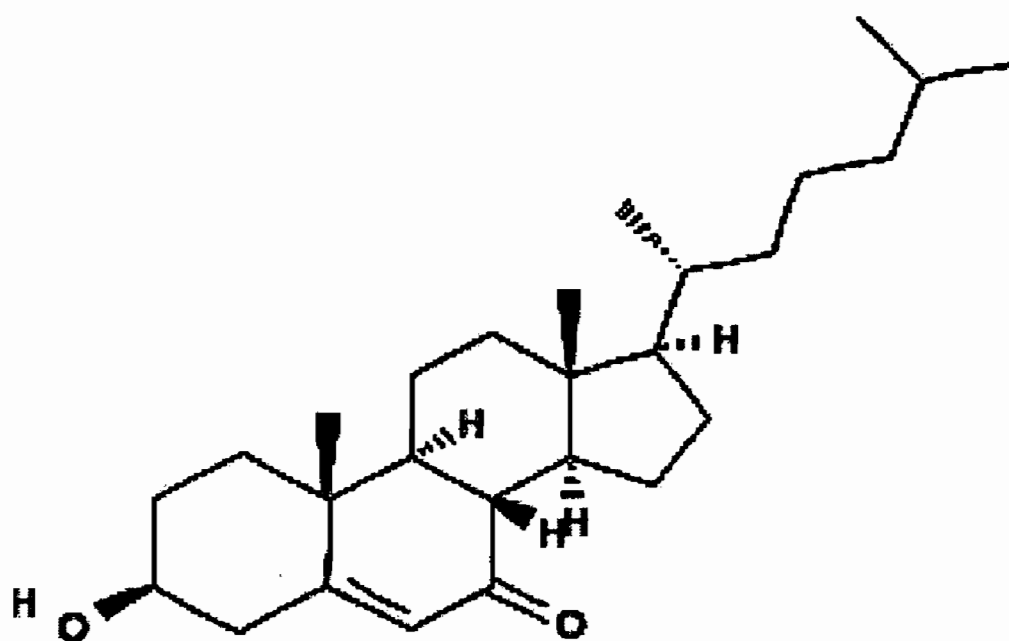


FIG. 1B

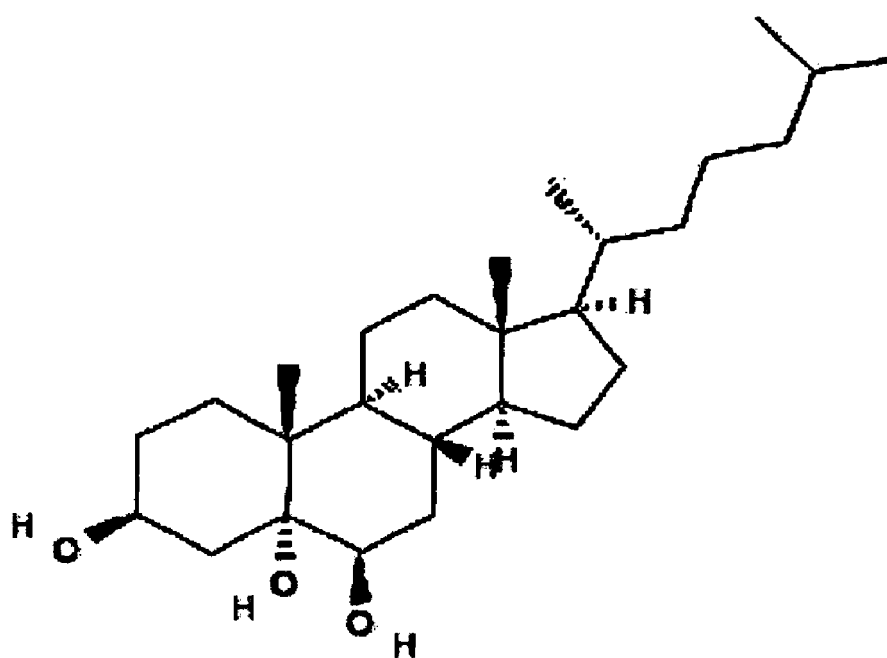
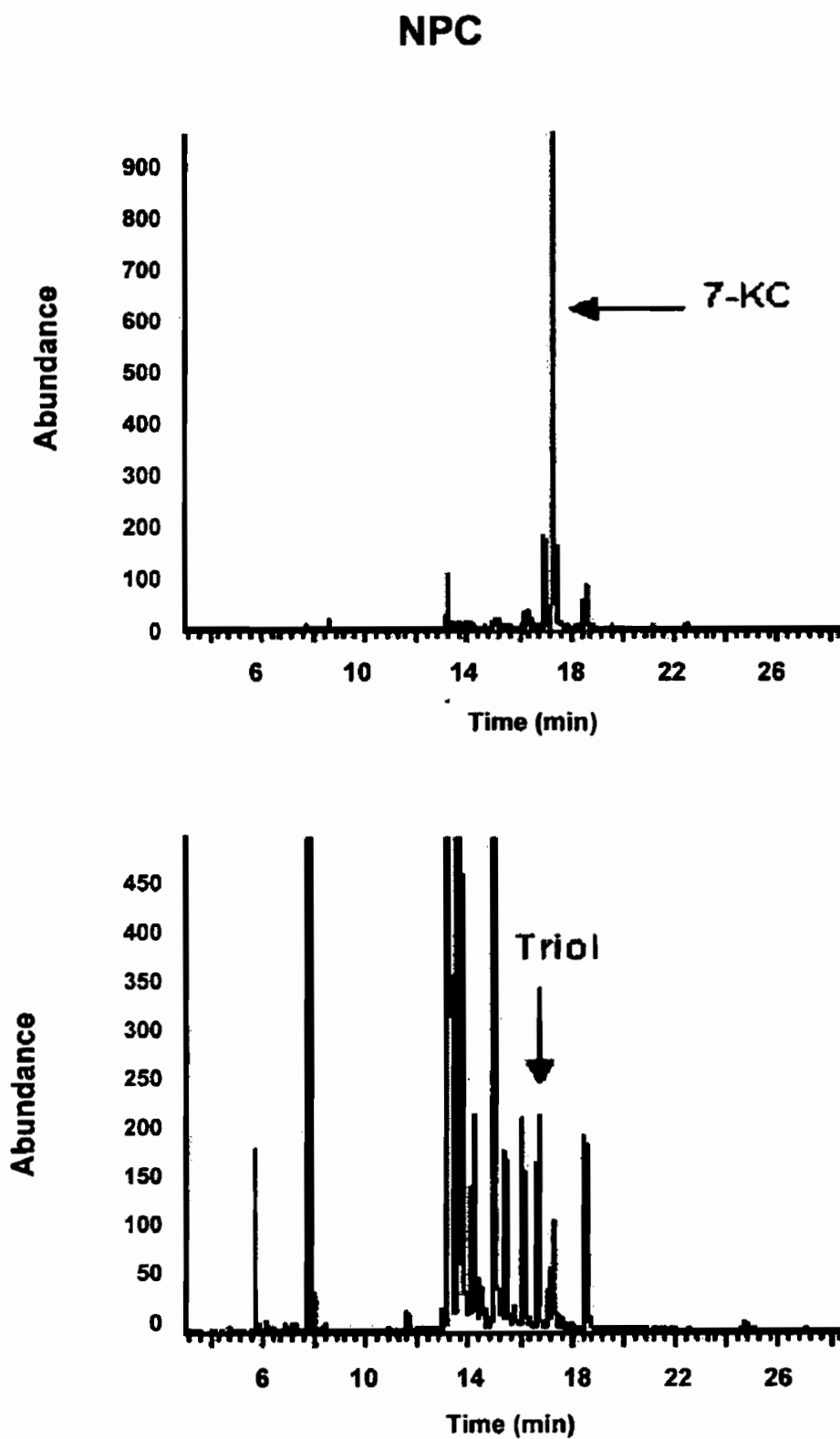
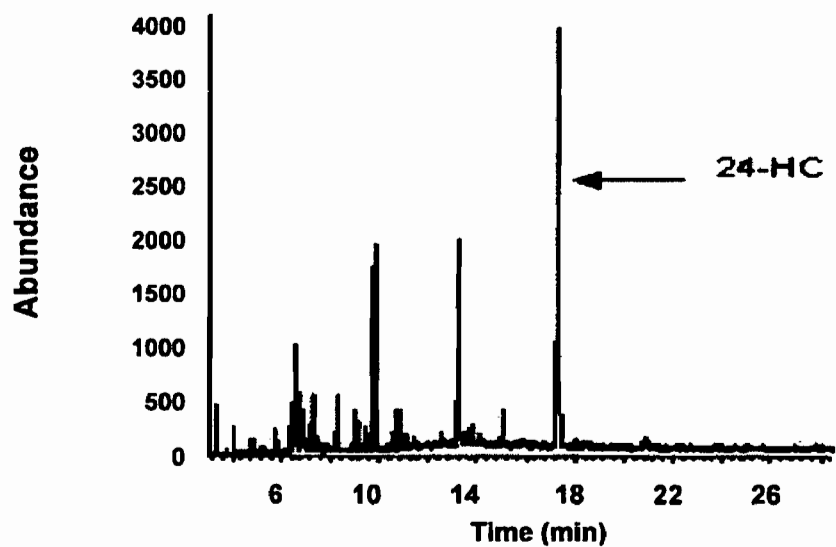
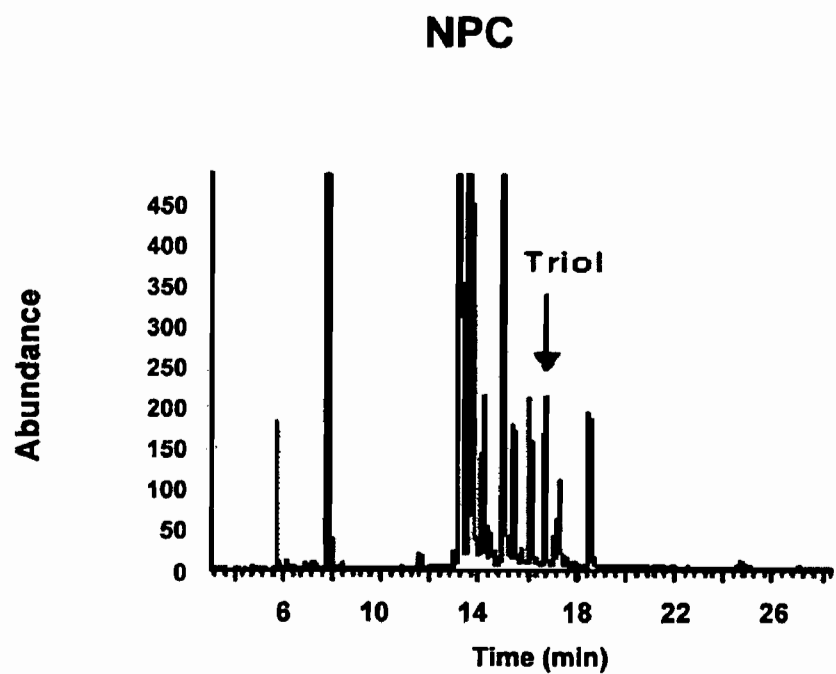


FIG. 1C

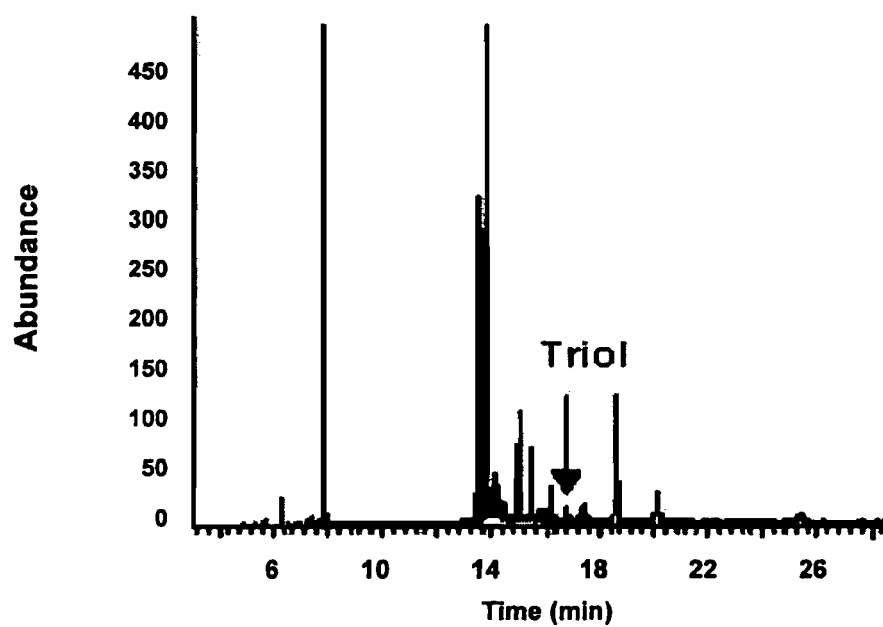
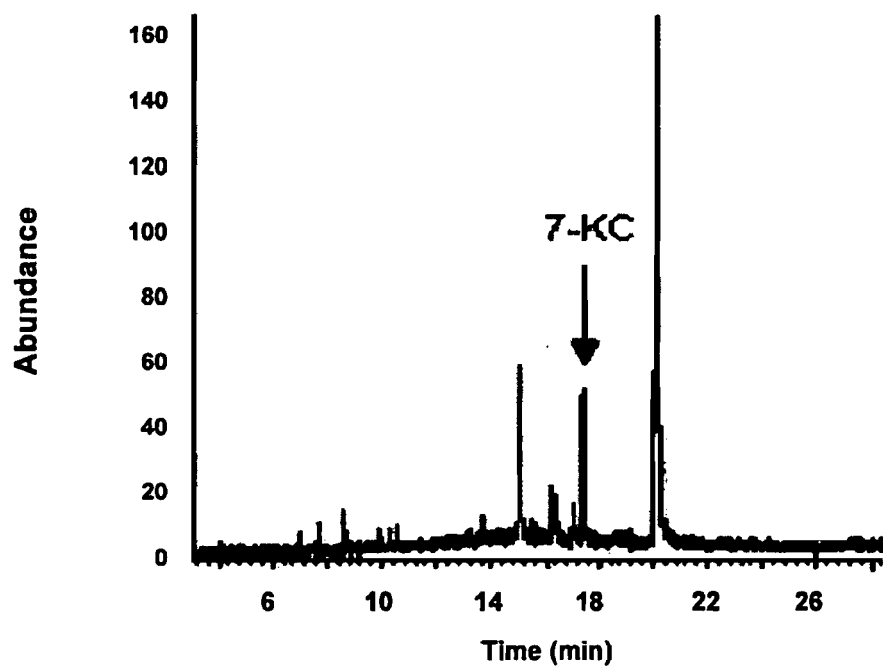
**FIG. 2A**

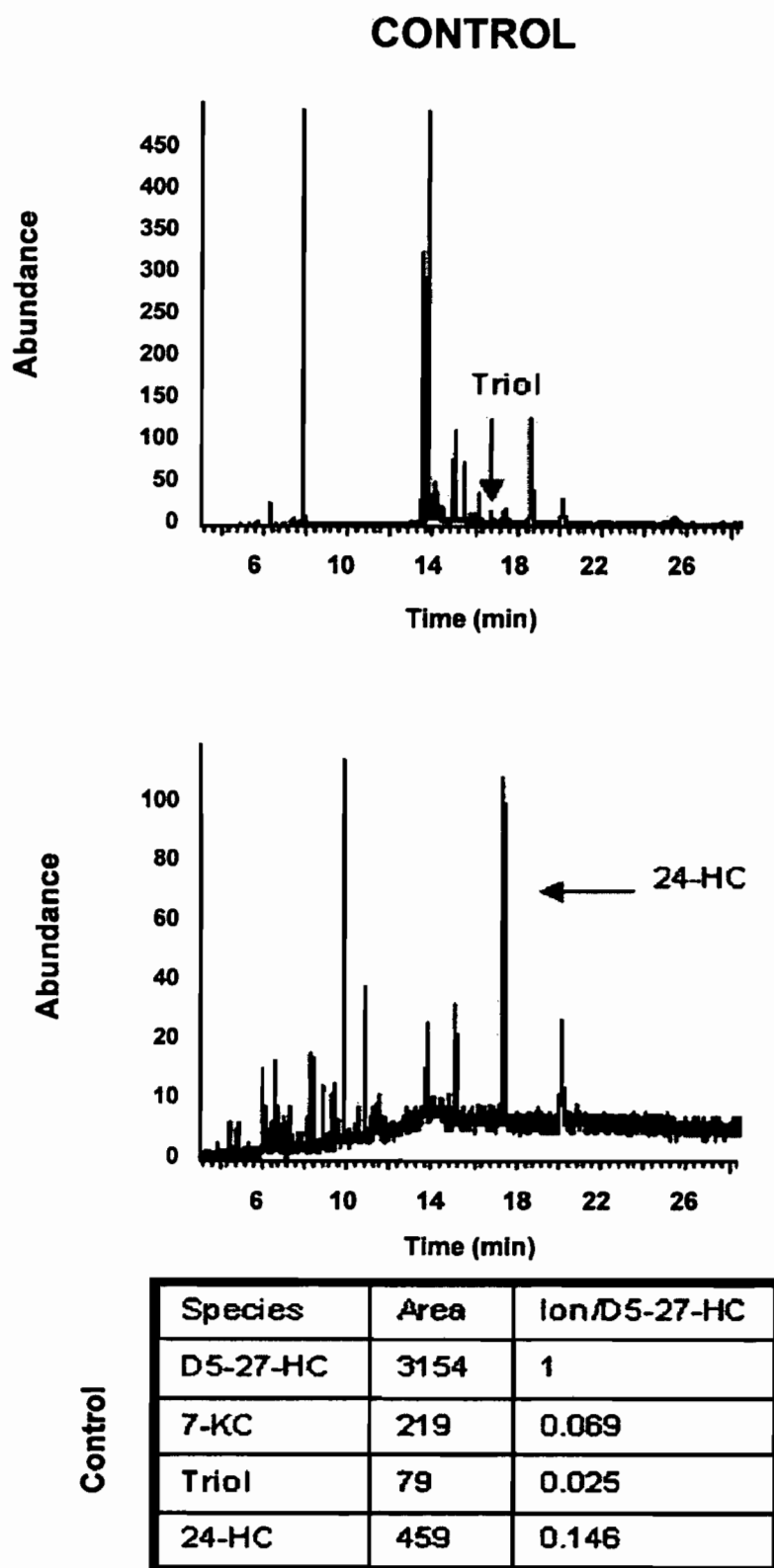


NPC

Species	Area	Ion/D5-27-HC
D5-27-HC	3522	1
7-KC	4675	1.327
Triol	972	0.276
24-HC	2026	0.575

FIG. 2B

CONTROL**FIG. 2C**

**FIG. 2D**

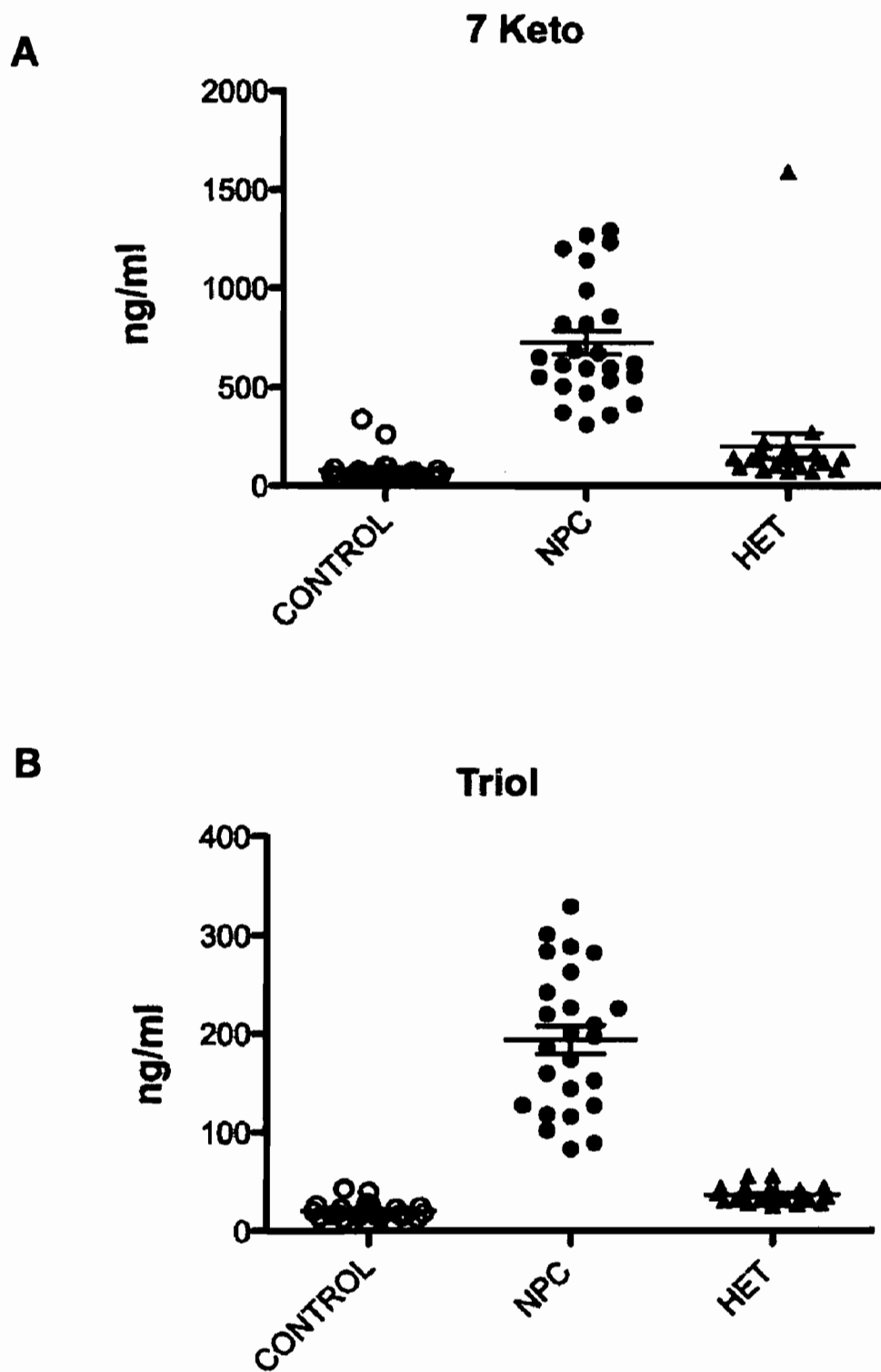


FIG. 3

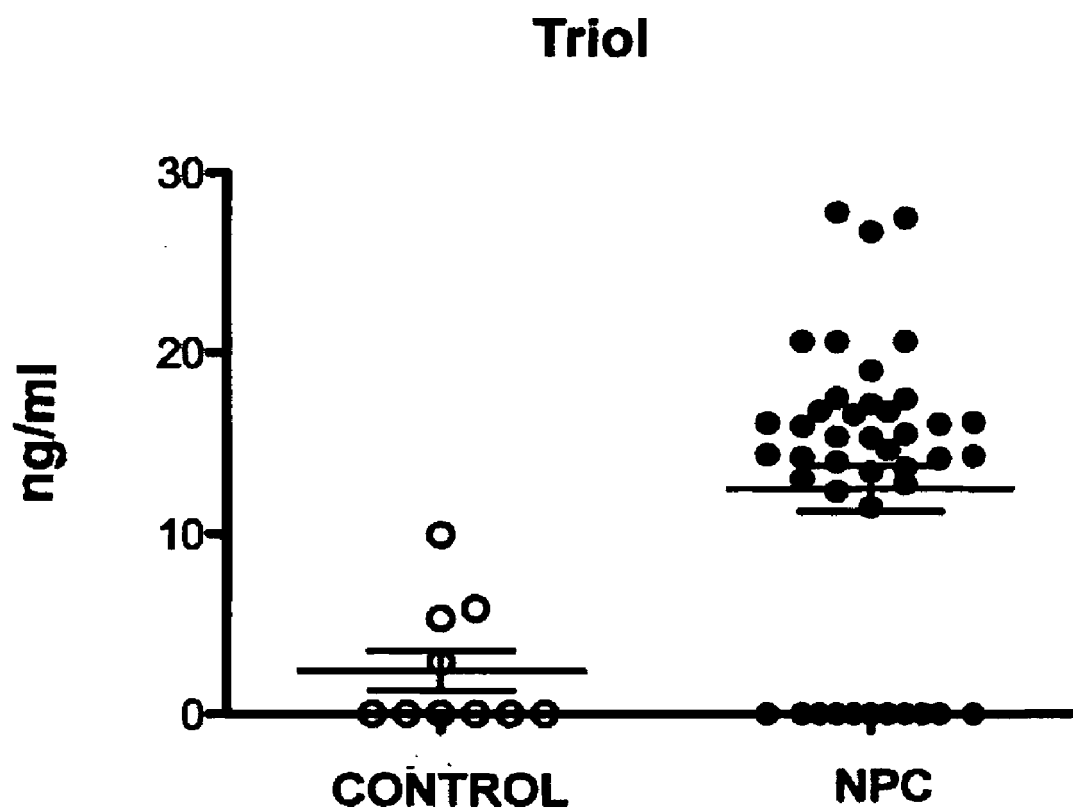
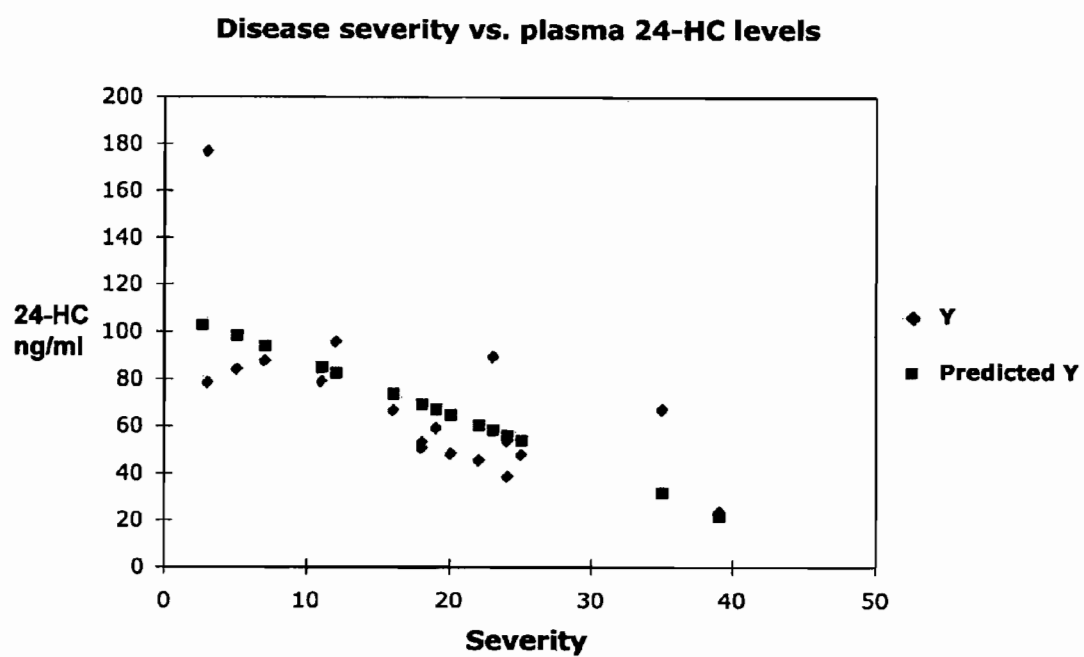
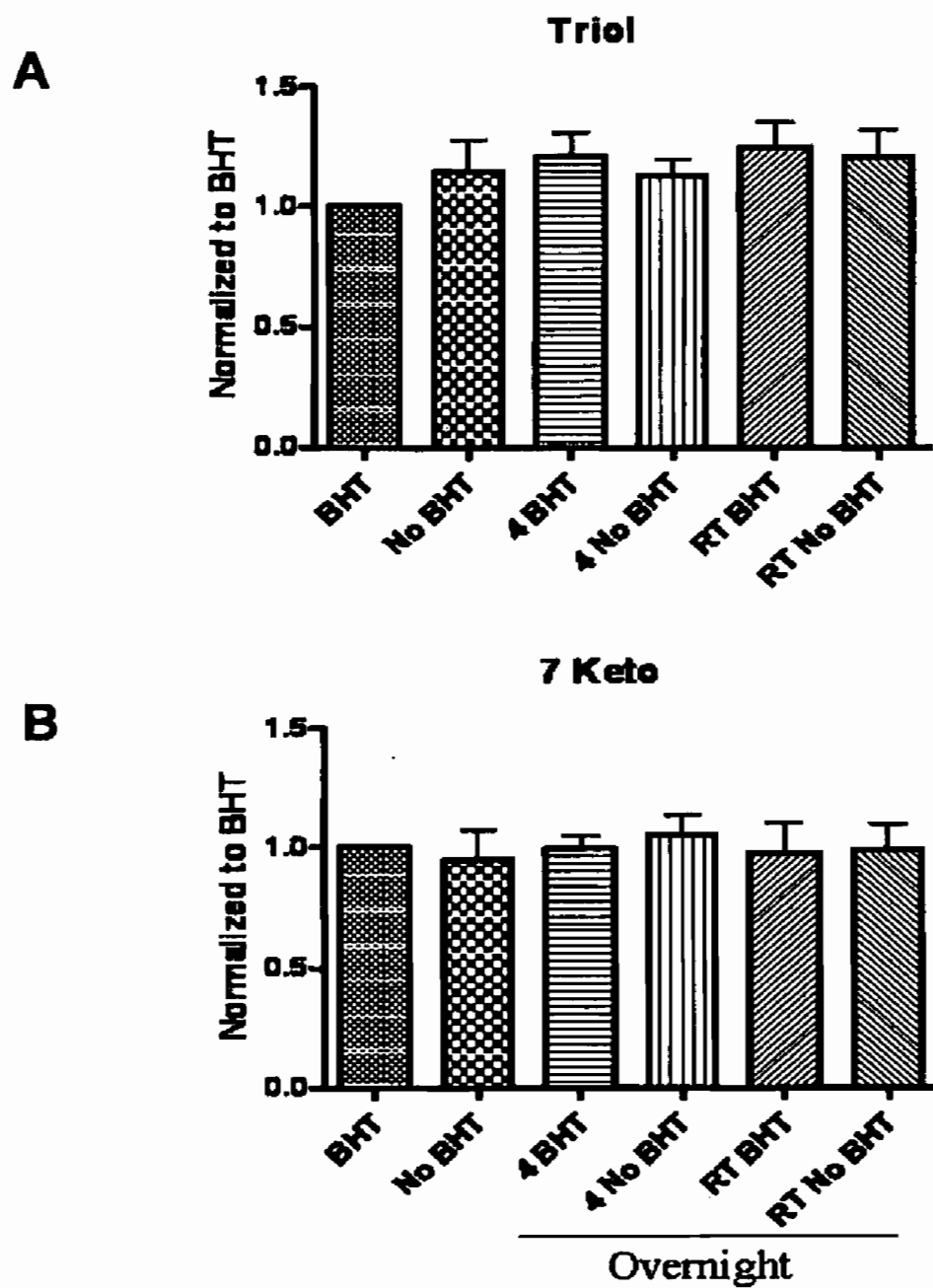
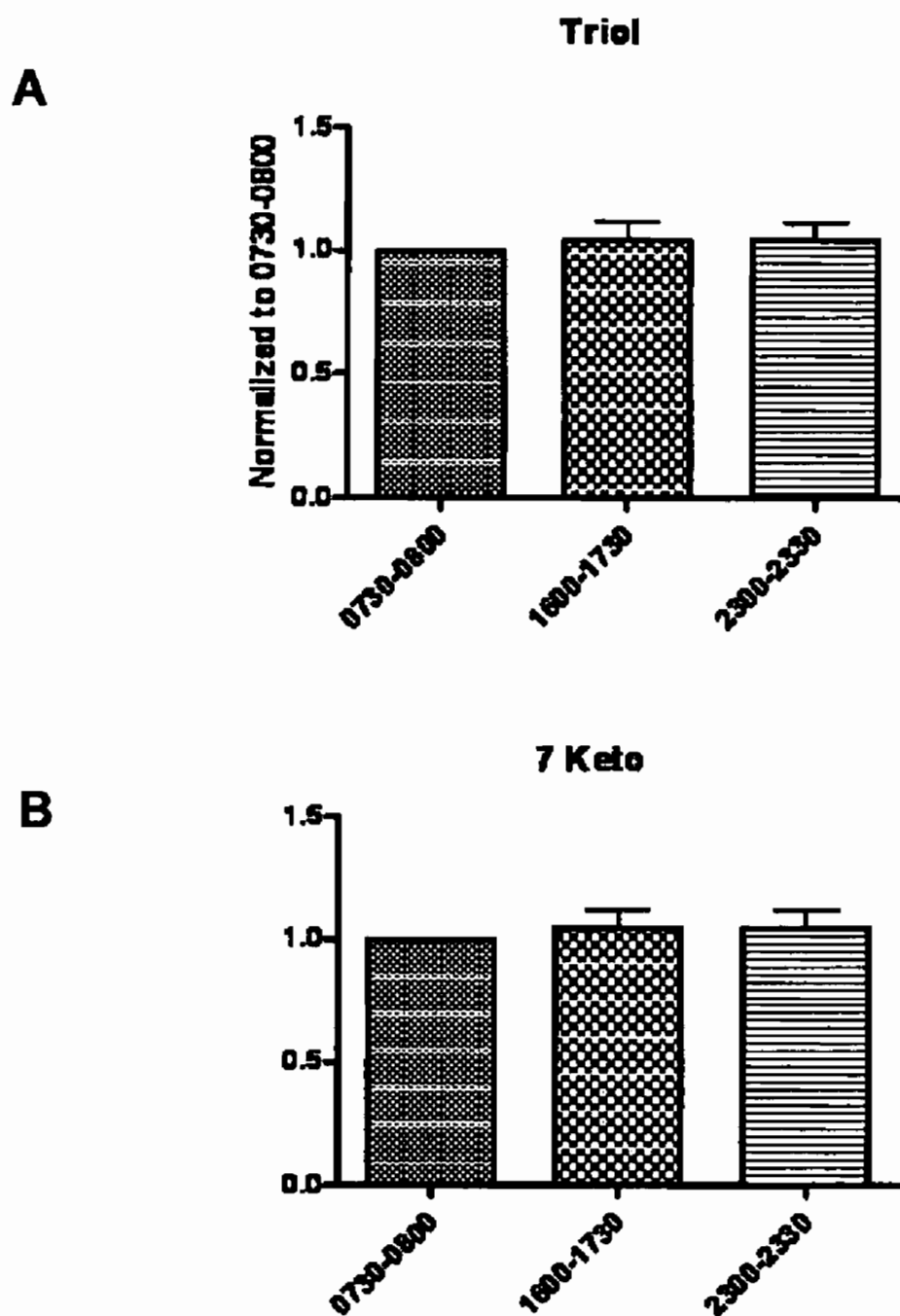


FIG. 4

**FIG. 5**

**FIG. 6**

**FIG. 7**

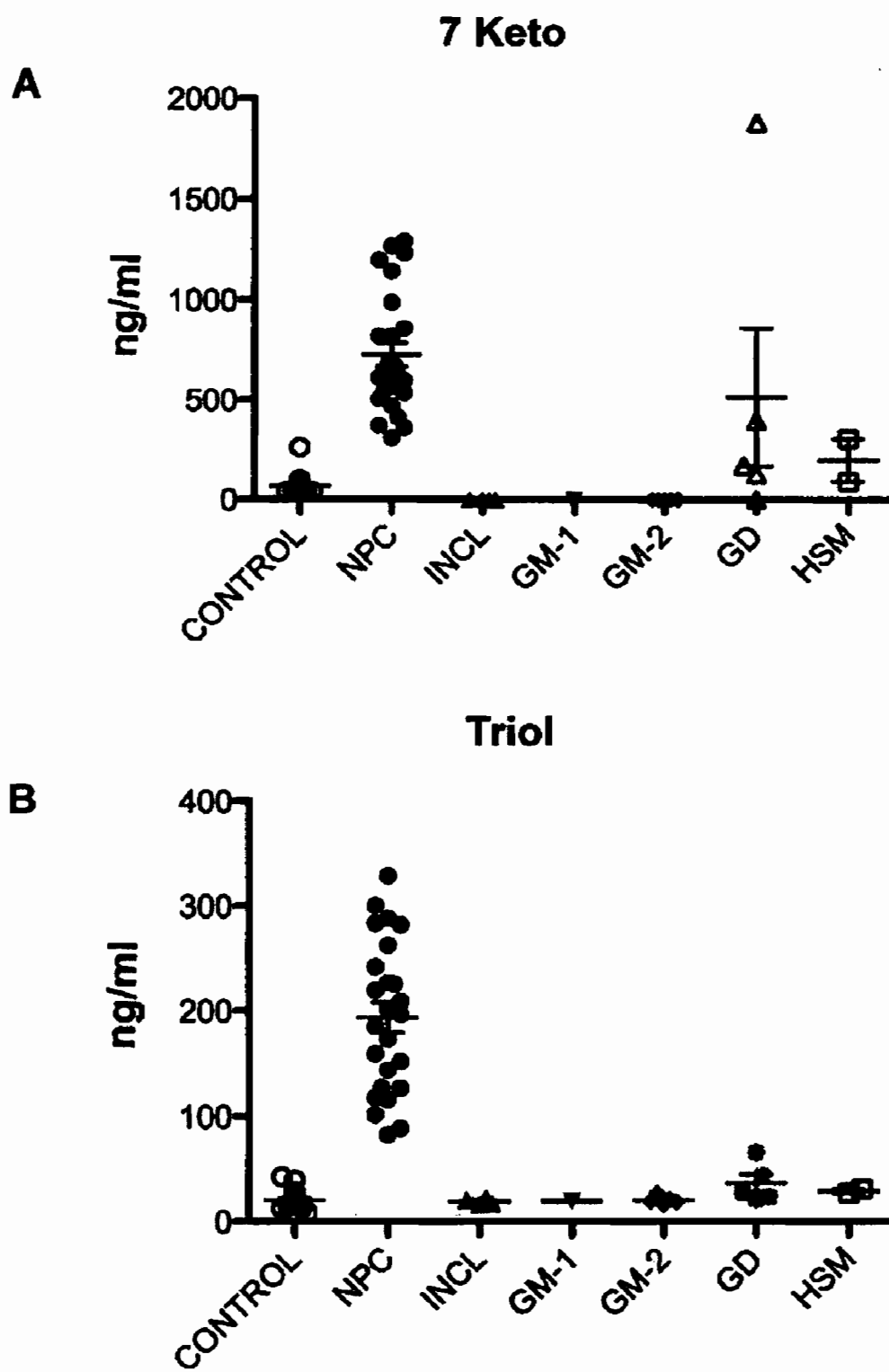


FIG. 8

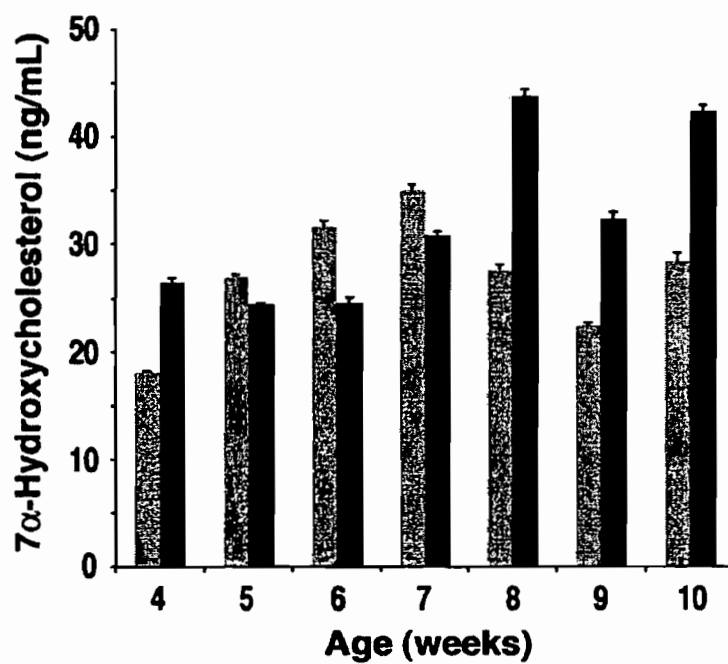


FIG. 9A

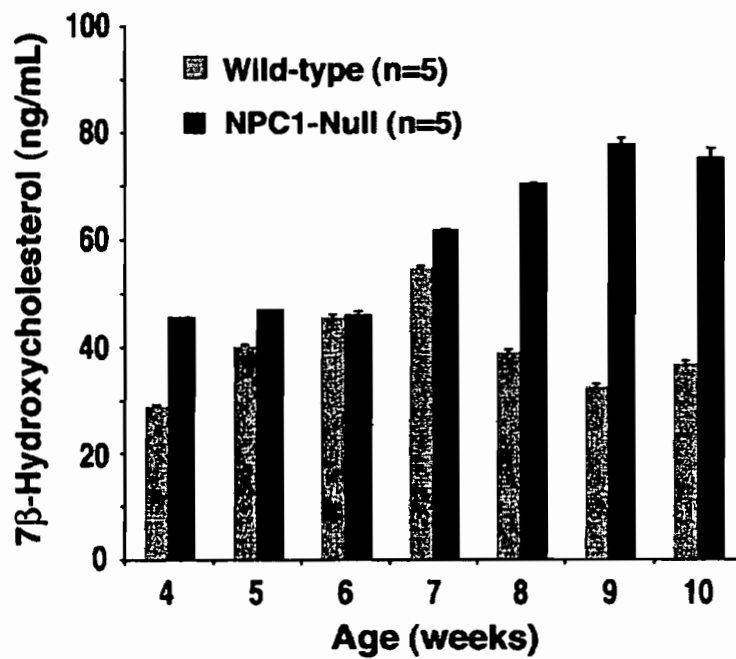


FIG. 9B

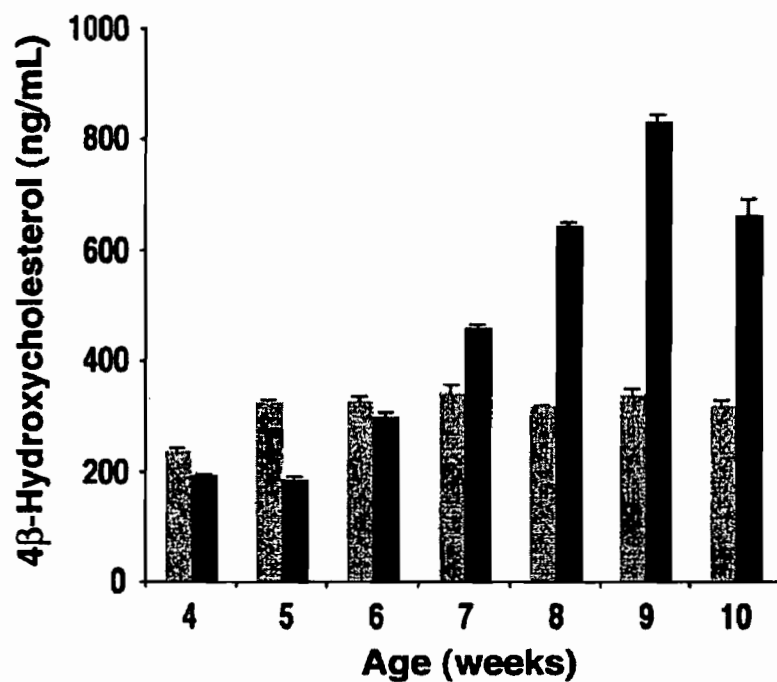


FIG. 9C

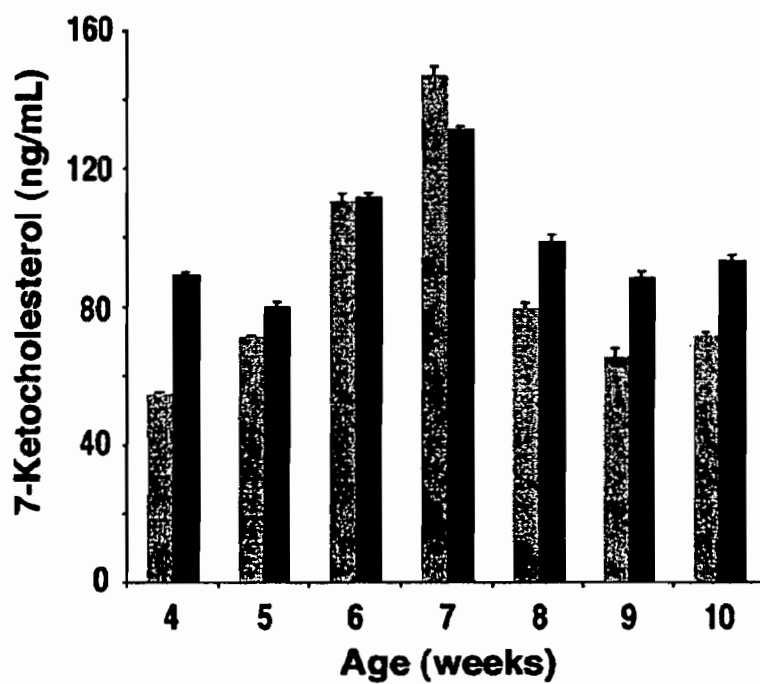


FIG. 9D

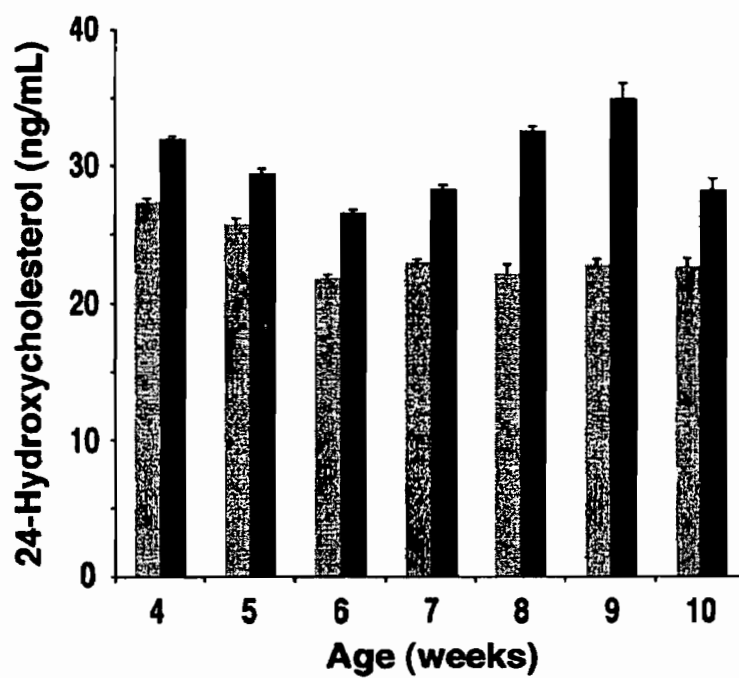


FIG. 9E

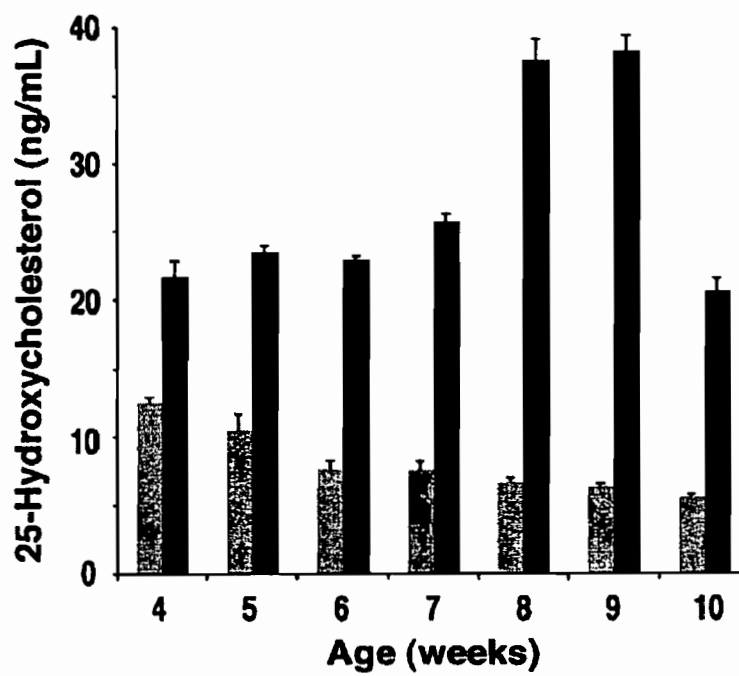


FIG. 9F

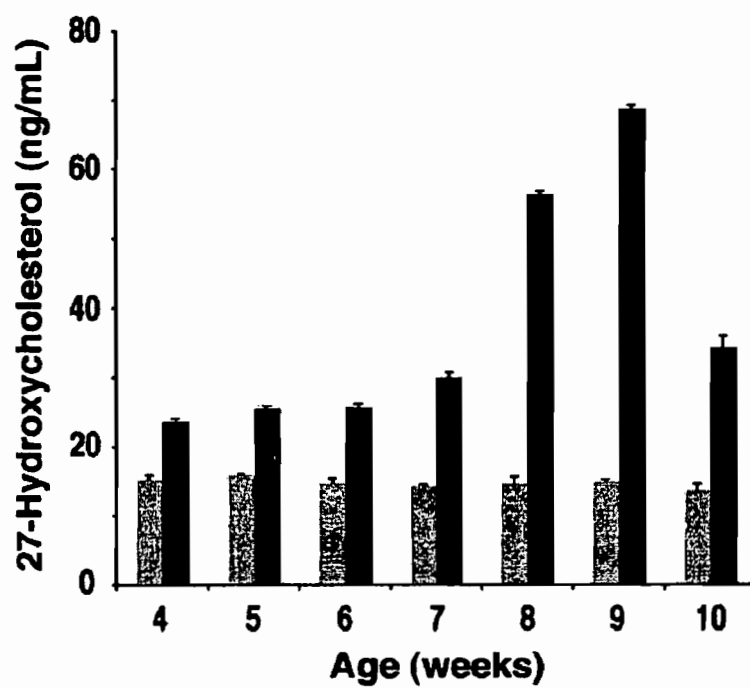


FIG. 9G

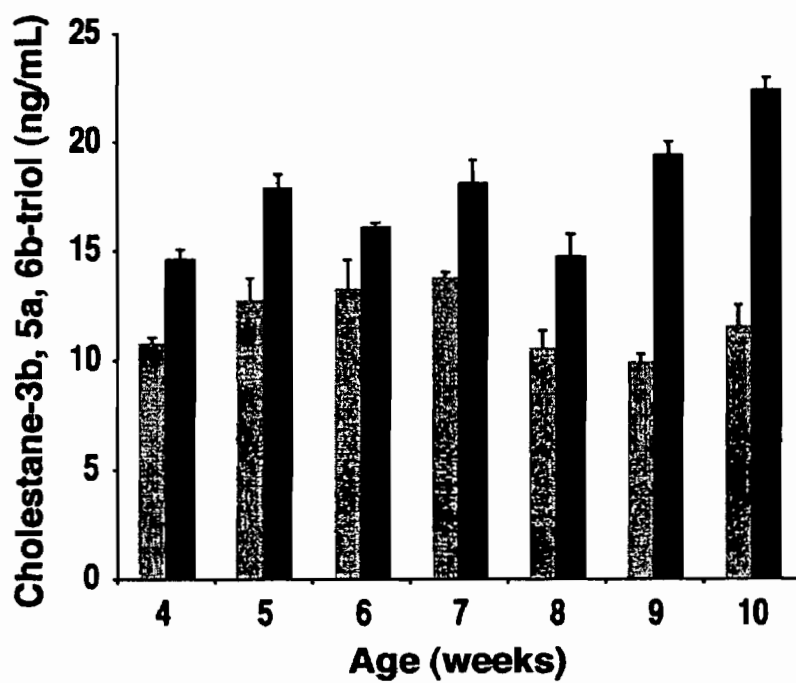


FIG. 9H

BIOMARKERS FOR NIEMANN-PICK C DISEASE AND RELATED DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/071,074, filed on Apr. 11, 2008. The disclosure of the application above is incorporated herein by reference in its entirety.

GOVERNMENT RIGHTS

[0002] This disclosure was made with government support under Grant No. P50 HLO83762 from the National Institutes of Health. The Government has certain rights in the invention.

FIELD

[0003] The present disclosure relates to biomarkers for disorders involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, Niemann-Pick C(NPC) disease, lysosomal storage diseases, cholesterol trafficking diseases, and neurodegenerative diseases.

INTRODUCTION

[0004] The statements in this section merely provide background information related to the present disclosure and may not constitute prior art.

[0005] Niemann-Pick type C(NPC) disease is an autosomal recessive lysosomal storage and neurodegenerative disorder. It can involve accumulation of cholesterol and other lipids in the viscera and the central nervous system, and patterned Purkinje cell death in the cerebellum. NPC disease is described, for example, on the On-Line Mendelian Inheritance in Man ("OMIM") website (www.ncbi.nlm.nih.gov/sites/entrez?db=omim), at OMIM number 257220. NPC presents a highly variable clinical phenotype. In childhood-onset NPC, the patients typically appear normal for 1 or 2 years with neurological symptoms, such as ataxia, grand mal seizures, loss of previously learned speech, spasticity, and seizures, appearing at 2 to 4 years. There are also prenatal and adult-onset forms of the disease.

[0006] In NPC disease, two genetic complementation groups, NPC1 and NPC2, have been identified. Mutations in the NPC1 gene cause ~95% of the cases, the rest being caused by NPC2 mutations. NPC2 is a soluble, glycosylated protein that is present in the lumen of the late endosome. "Loss-of-function of the NPC1 gene in mice yields marked impairment in both esterification of low-density lipoprotein (LDL) cholesterol and mobilization of newly hydrolyzed LDL cholesterol to the plasma membrane, resulting in lysosomal sequestration of LDL cholesterol, delayed down-regulation of the LDL receptor and de novo cholesterol biosynthesis, and impaired ATP-binding cassette transporter (ABCA1)-mediated cholesterol efflux. Associated with these lipid trafficking defects, NPC1 mutants exhibit cellular oxidative stress, leading to increased production of non-enzymatic cholesterol auto-oxidation products.

[0007] Information regarding the biochemical and histopathological defects associated with NPC has come through the use of two murine models which share many of the clinical abnormalities observed in humans with NPC: elevated levels of sphingomyelin and unesterified cholesterol in liver and spleen, presence of foamy macrophages, neuronal vacuoles, focal axonal swelling, and decreased Purkinje cell num-

ber. The two murine NPC models, C57B1Ks/J *spm* and BALB/c *npc^{nih}*, arose as spontaneous mutations, were determined allelic by cross breeding, and have been independently localized to mouse chromosome 18 in a region syntenic to the human NPC1 locus. Confirmation that the two mouse loci belong to the same complementation group as the human NPC1 locus was determined using heterokaryon fusions of human NPC1 fibroblasts to mouse mutant cell lines and by DNA-mediated complementation using a yeast artificial chromosome (YAC) from the human NPC1 region. Combined, these studies indicate that the same gene is altered in the two mouse NPC models (*spm* and *npcnih*) and that the orthologous gene in the mouse models is defective at the human NPC1 locus.

[0008] Despite recent progress in characterizing the biochemical and genetic defects in NPC disease, the mean time to diagnosis from initial presentation is approximately five years. The delay in diagnosis is largely due to the lack of both newborn screening and disease biomarkers, as well as the lack of widely available diagnostic tests. In addition, the absence of biomarkers that correlate with disease severity has hampered evaluation of the efficacy of therapeutic approaches to NPC disease.

DRAWINGS

[0009] The drawings described herein are for illustration purposes only and are not intended to limit the scope of the present disclosure in any way.

[0010] FIG. 1A shows the chemical structure of 24(S) hydroxycholesterol (herein referred to as "24HC")

[0011] FIG. 1B shows the chemical structure of 7-ketocholesterol (herein referred to as "7-keto").

[0012] FIG. 1C shows the chemical structure of cholestane-3 β , 5 α , 6 β -triol (herein referred to as "triol").

[0013] FIG. 2 shows a gas chromatogram/mass spectrum (GC/MS) of oxysterols in plasma samples obtained from confirmed NPC patients and control non-NPC afflicted subjects. The arrows indicate oxysterol biomarker identification. The Y-axis is the measure of relative abundance of sampled oxysterols measured against a known internal standard from which a calculation of the concentration of the oxysterol in the biological sample can be made. The X-axis is the retention time of each of the ion species eluting from the GC column in minutes. The table below the control and NPC samples summarizes the relative abundance of each oxysterol measured using the identification and testing methods of the present disclosure in the NPC disease group and the control group.

[0014] FIG. 3A shows a graph depicting plasma levels of the oxysterol 7-keto obtained from control non-afflicted subjects ("CONTROL"), confirmed NPC subjects ("NPC"), and heterozygote but not NPC-afflicted subjects having a NPC1+/-genotype ("HET"). The concentration of the oxysterol 7-keto is expressed as nanograms per milliliter (ng/mL) of plasma.

[0015] FIG. 3B shows a graph depicting plasma levels of the oxysterol triol obtained from control non-afflicted subjects ("CONTROL"), confirmed NPC subjects ("NPC"), and obligate or confirmed heterozygote non-NPC afflicted subjects having a NPC1+/-genotype ("H="). The concentration of the oxysterol triol is expressed as nanograms per milliliter (ng/mL) of plasma.

[0016] FIG. 4 shows a graph depicting levels of the oxysterol triol from cerebrospinal fluid (CSF) samples obtained from control non-afflicted subjects ("CONTROL") and con-

firmed NPC subjects ("NPC"). The concentration of the oxysterol triol is expressed as nanograms per milliliter (ng/mL) of plasma.

[0017] FIG. 5 shows a Pearson Correlation coefficient for the correlation between disease severity and plasma levels of 24-HC.

[0018] FIG. 6A shows a bar graph depicting the concentration of triol normalized to the concentration of triol from NPC plasma samples treated with butylated hydroxytoluene (BHT). Treatments include: NPC plasma samples treated with BHT ("BHT"), no BHT ("No BHT"), at 4° C. with BHT ("4 BHT"), at 4° C. without BHT ("4 No BHT"), at room temperature (20-21° C.) with BHT ("RT BHT"), and at room temperature (20-21° C.) without BHT ("RT No BHT").

[0019] FIG. 6B shows a bar graph depicting the relative concentration of 7-keto normalized to the concentration of 7-keto from NPC plasma samples treated with butylated hydroxytoluene (BHT). Treatments include: NPC plasma samples treated overnight with BHT ("BHT"), no BHT ("No BHT"), at 4° C. with BHT ("4 BHT"), at 4° C. without BHT ("4 No BHT"), at room temperature (20-21° C.) with BHT ("RT BHT"), and at room temperature (20-21° C.) without BHT ("RT No BHT").

[0020] FIG. 7A shows a bar graph depicting the relative amount of triol from NPC subjects normalized to amounts of triol in plasma samples obtained between the hours of 7:30 am-8:00 am ("0730-0800"). Other timed intervals include between 4:00 pm and 5:30 ("1600-1730") and between 11:00 pm and 11:30 pm ("2300-2330"). Results are indicative of averaged values over 5 different measurements for each time point.

[0021] FIG. 7B shows a bar graph depicting the relative amount of triol from NPC subjects normalized to amounts of triol in plasma samples obtained between the hours of 7:30 am-8:00 am ("0730-0800"). Other timed intervals include between 4:00 pm and 5:30 ("1600-1730") and between 11:00 pm and 11:30 pm ("2300-2330"). Results are indicative of averaged values over 5 different measurements for each time point.

[0022] FIG. 8A shows a graph depicting plasma levels of the oxysterol 7-keto obtained from control non-afflicted subjects ("CONTROL"), confirmed NPC subjects ("NPC") and from subjects having other lysosomal storage diseases, including those with known CNS involvement: infantile neuronal ceroid lipofuscinosis ("INCL"), GM1 gangliosidosis ("GM-1"), GM-2 gangliosidosis ("GM-2") (Tay-Sachs Disease), Gaucher's disease ("GD") and hepatosplenomegaly ("HSM"). The concentration of the oxysterol 7-keto and triol are expressed as nanograms per milliliter (ng/mL) of plasma.

[0023] FIG. 8B shows a graph depicting plasma levels of the oxysterol triol obtained from control non-afflicted subjects ("CONTROL"), confirmed NPC subjects ("NPC") and from subjects having other lysosomal storage diseases, including those with known CNS involvement: infantile neuronal ceroid lipofuscinosis ("INCL"), GM1 gangliosidosis ("GM-1"), GM-2 gangliosidosis ("GM-2") (Tay-Sachs Disease), Gaucher's disease ("GD") and hepatosplenomegaly ("HSM"). The concentration of the oxysterol triol is expressed as nanograms per milliliter (ng/mL) of plasma.

[0024] FIG. 9 shows bar graphs of oxysterols 24-hydroxycholesterol ("24-HC"), 25-hydroxycholesterol ("25-HC"), 27-hydroxycholesterol ("27-HC"), cholestane-3 β ,5 α ,6 β -triol ("triol"), 40-hydroxycholesterol ("4 β -HC"), 7 α -hydroxycholesterol ("7 α -HC"), 7 β -hydroxycholesterol

("7 β -HC"), and 7-ketocholesterol ("7-keto") isolated from wild-type and NPC1-/- null mice (NPC afflicted mice) as a function of age (weeks). Methods for isolating, identifying and quantifying the various oxysterols were in accordance with the present disclosure.

SUMMARY

[0025] The present disclosure relates to the finding that one or more of a set of oxysterols may be used as a biomarker for screening, diagnosing, or monitoring disorders involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, Niemann-Pick C(NPC) disease, lysosomal storage disease, and neurodegenerative disease. Oxysterols are commonly referred to as oxidized derivatives of cholesterol, typically generated non-enzymatically, enzymatically as a normal part of cholesterol metabolism, or absorbed through dietary intake. Oxysterols typically have a hydroxyl-, epoxy- or a keto-group on the cholesterol molecule. In some embodiments of the present disclosure, the oxysterol can be any one or more of 24(S)-hydroxycholesterol ("24-HC"), 25-hydroxycholesterol (25-HC), 7-ketocholesterol ("7-keto") and cholestane-3 β , 5 α , 6 β -triol ("triol").

[0026] An oxysterol may be present in a subject at a level that is elevated compared to the level of the oxysterol in a population not afflicted with a disorder involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, NPC disease, lysosomal storage diseases, cholesterol trafficking diseases, and neurodegenerative diseases. Certain oxysterols can be present at levels below those found in control populations, and/or at levels that vary over time. Levels of oxysterols can be used for screening, diagnosing, and/or monitoring disorders involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, NPC disease, lysosomal storage diseases, cholesterol trafficking diseases, and neurodegenerative diseases.

[0027] The present disclosure provides methods for identifying a subject having, or at risk for developing, NPC disease by detecting an increased concentration of one or more oxysterols in a biological sample collected from the subject. The present disclosure provides methods for screening or diagnosing subjects (including infants and neonatal subjects) for NPC disease. Also disclosed are methods for monitoring the progression, remission, and clinical status of NPC disease and for evaluating the efficacy of therapeutic treatment of NPC disease.

DESCRIPTION OF VARIOUS EMBODIMENTS

[0028] The following description is merely exemplary in nature and is not intended to limit the present disclosure, application, claims, compounds, or uses.

A. Oxysterols: Biomarkers for NPC Disease and Other Disorders.

[0029] An oxysterol may be present in a subject at a level that is elevated compared to the level of the oxysterol in subjects not afflicted with a disorder involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, NPC disease, lysosomal storage disease, cholesterol trafficking diseases, and neurodegenerative diseases. An oxysterol level can correlate with, and serve as a biomarker for, the presence, progression, or risk of developing one or more such disease states. Those skilled in the art will appreciate

ciate that some oxysterol levels may be higher in patients with early onset of NPC disease as compared with later onset forms of the disease.

[0030] In some embodiments, an elevated oxysterol useful as an indicator of disease or risk of developing disease can be any one or more of 24(S)-hydroxycholesterol ("24-HC"), 25-hydroxycholesterol (25-HC), 7-ketocholesterol ("7-keto"), and cholestane-3 β , 5 α , 6 β -triol ("triol"). Biomarkers can also include other oxysterol species that are abnormally elevated in disorder involving accumulation of one or more oxysterols such as cytotoxic oxysterol accumulation, NPC disease, lysosomal storage disease, cholesterol trafficking diseases, and neurodegenerative diseases. Oxysterols that correlate with NPC disease are shown in FIGS. 1A, 1B and 1C.

[0031] Without being bound by theory, and without limiting the scope of the disclosure or claims, it is presently hypothesized that the presence of elevated levels of oxysterols in peripheral and neural cells occurs as a result of autooxidation or normal enzymatic degradation of cholesterol, as a product of oxidative stress, and through dietary intake. With respect to 24-HC, it is presently hypothesized that this particular metabolite, also known as cerebrosterol, is produced as a result of 24(S)-hydroxylation of cholesterol in the cerebral cortex, hippocampus, dentate gyrus and thalamus.

[0032] The production of 24-HC is presently hypothesized to occur as a natural mechanism for cholesterol release from the brain, and the exchange of 24-HC from the brain to the circulation is thought to be a continuous age-dependent flux of about 4 mg/day. Other hydroxylases in the neuron may also be responsible for the conversion of cholesterol to 7-keto. Evidence presently suggests that the increased levels of the oxysterols 24-HC, 7-keto and triol in the plasma and cerebrospinal fluid might correlate with reactive species generation and 24-HC, 7-keto and triol indicative of lipid trafficking defects in neurons and other peripheral tissues, for example, the liver, spleen, kidney and in leukocytes. It is therefore presently hypothesized that elevated oxysterol concentrations in body fluids (e.g., blood, plasma, serum, cerebrospinal fluid, cell membrane and the like) and tissues correlates with and is indicative of lipid trafficking defects in neurons, and in particular, indicative of NPC disease.

B. Screening and Diagnosing Subjects Using Oxysterol Biomarkers.

[0033] In some embodiments of the present disclosure, a method for identifying a subject having, or at risk for, developing NPC disease is provided. The method may comprise quantifying or determining the concentration of one or more oxysterols in a biological sample obtained from the subject. The concentration of the one or more oxysterols in the tested biological sample collected from the subject can be compared with a reference value. Detection and quantification of elevated concentration(s) of one or more oxysterol(s) in the biological sample as compared to a reference value (which may be a predetermined value) can presumptively identify the subject as having, or at risk for developing, NPC disease.

[0034] In some embodiments, the present disclosure provides a method for identifying a subject having, or at risk of developing, NPC disease by an increased concentration of one or more oxysterols in a biological sample collected from the subject. According to some of these embodiments, steps for identifying a subject having NPC disease comprise: (a)

obtaining a biological sample from the subject; (b) quantifying the concentration of oxysterols comprising 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol or combinations thereof in the biological sample; and (c) comparing the concentration of the oxysterol present in the biological sample to a reference value of the oxysterol obtained from a control population, wherein if the concentration of the oxysterol from the subject is higher than the reference value, the subject can be identified as having, or at risk for developing, NPC disease. Diagnosis of NPC disease may also be determining using the methods of these embodiments.

[0035] In some embodiments, a biomarker can be any oxysterol that is elevated in NPC subjects and that can be reproducibly identified as elevated to a statistically significant degree when compared to a non-affected control population. Alternatively or additionally, a non-afflicted control population can also include one or more subject groups with wild-type, NPC1-/-, NPC1+/-, NPC2-/-, and NPC2+/- genotypes not having any symptoms or effects of NPC disease. The superscript "minus sign" can denote a mutation within the NPC1 or NPC2 genomic regions.

[0036] Illustrative examples of oxysterols useful as biomarkers for NPC disease include 24-HC, 7-keto and triol oxysterol, the chemical structures of which are shown in FIGS. 1A-1C. In some embodiments of the present disclosure, 24-HC, 7-keto and triol oxysterol concentrations from biological samples may be used to identify, screen, diagnose, and/or monitor NPC disease in subjects, including subjects suspected of having the disease and/or subjects diagnosed with the disease.

[0037] In some embodiments, elevated 7-keto or triol levels, or combinations thereof, can differentiate NPC subjects from subjects with other lysosomal storage diseases, including lysosomal storage diseases with CNS involvement. For example, as shown in FIG. 8A, levels of 7-keto are statistically greater in samples from NPC subjects than in samples at least from subjects afflicted with infantile neuronal ceroid lipofuscinosis (INCL), GM1 gangliosidosis (GM-1), GM-2 gangliosidosis (GM-2) (Tay-Sachs Disease), and hepatosplenomegaly (HSM). FIG. 8B shows that FIG. 8A, levels of triol are statistically greater in samples from NPC subjects than in samples at least from subjects afflicted with infantile neuronal ceroid lipofuscinosis (INCL), GM1 gangliosidosis (GM-1), GM-2 gangliosidosis (GM-2) (Tay-Sachs Disease), Gaucher's disease (GD), and hepatosplenomegaly (HSM). The present disclosure provides methods that can distinguish subjects with NPC disease from subjects with other lysosomal storage diseases based on levels of 7-keto and triol. For example, the combination of elevated 7-keto and triol levels can differentiate NPC subjects from subjects with other lysosomal storage diseases to a statistically significant degree.

[0038] 1. Reference Values.

[0039] A reference value represents a threshold value of oxysterol concentration to which a subject's measured oxysterol levels may be compared. The reference level can be selected based on the intended purpose of the test or assay being performed on the subject. For example, a reference level may be determined for purposes of identifying subjects affected by NPC while the same or different reference level may be used for monitoring the progression or severity of disease in a subject, or for screening a particular population of subjects for the disease, for example, neonatal subjects.

[0040] The selection of an appropriate reference level for a given test or assay is within the level of skill in the art. The

choice of the reference value is not absolute. For example, a relatively low value may advantageously be used to reduce the incidence of false negatives, but may also increase the likelihood of false positives. Accordingly, as for other screening techniques, the reference value may be based on a number of factors, including but not limited to cost, the benefit of early diagnosis and treatment, the invasiveness of follow-up diagnostic methods for individuals that have false positive results, the intended purpose of the test or assay, the limits of accurate detection of oxysterol levels in the particular sample type, the prevalence and average or mean oxysterol levels of the relevant population, the desired level of statistical significance of the results, the level of quantification desired in terms of disease risk, presence, progression, or severity, and other factors that are routinely considered in designing screening assays.

[0041] In some embodiments, the reference or control level for each oxysterol can be based on known concentrations of each oxysterol in healthy and affected populations. The reference or control levels may be set as appropriate for the subject being screened. For example, oxysterol levels in a sample from a neonatal subject can be compared with oxysterol levels in a healthy and/or affected neonatal population. In other embodiments, the oxysterol levels identified in a subject can be compared with a matched unselected, population. In some embodiments, the subject can be compared with a matched population of unaffected (i.e., healthy) subjects and/or a matched population of affected subjects. In some embodiments, the control population can be a matched control population wherein the control population is matched to the subject by gender and/or age.

[0042] As a non-limiting example of the selection of a reference value, subjects that have one or more of the oxysterol biomarkers selected from 24-HC, 7-keto and triol having values above about the 70th percentile, 75th percentile, 80th percentile, 90th percentile, 95th percentile, 96th percentile, 97th percentile, 98th percentile, 99th percentile, or higher, as compared with an appropriate control population may be presumptively identified as affected with NPC disease.

[0043] Alternatively or additionally, subjects having more than about 2, 3, 4, 5, 8, 10 or 20 fold higher oxysterol biomarker concentration than the average (alternatively, mean or median) value for an appropriate unaffected population as measured under similar or identical conditions may be presumptively identified as affected with NPC disease. In some cases, the diagnosis or identification of NPC corresponds to an oxysterol concentration that is above the reference value, even if the identified concentration of oxysterol in the biological sample of the NPC identified subject is not at least 2 fold higher than the reference value.

[0044] An appropriate reference value may be determined in a variety of ways and at a variety of times. In some embodiments, the reference value can be a predetermined value, based for example, on prior tests and assays on healthy or affected subjects ranging from six individuals to greater than a thousand. In other embodiments, the reference value can be determined during the course of the assay. For example, samples from known unaffected and/or affected subjects may be run concurrently with the test samples and a reference value determined therefrom. In some embodiments, test samples from a mixed population can be analyzed, and the reference value can be determined based on the distribution of the results using statistical methods known in the art.

[0045] 2. Subjects.

[0046] In general, the methods for identifying, screening or monitoring the progression and/or treatment efficacy disclosed herein have experimental, veterinary and medical applications. Accordingly, subjects may be humans, primates, simians, canines, felines, equines, bovines, ovines, caprines, porcines, lagomorphs, rodents, avians, and the like. Subjects according to the present disclosure can be human neonatal (i.e., from the time of birth to about one week post-natal), infant, juvenile, adolescent or adult subjects. In some embodiments, neonatal subjects can be screened for the presence or risk of NPC disease. As used herein, "neonatal" subjects can include premature infants, as that term is used in the art.

[0047] The subjects may be part of a general population, e.g., for a broad-based screening assay. Alternatively, the subject may be one that may have an enlarged liver or spleen, or is suspected of having a lysosomal cholesterol storage or trafficking disorder by one or more clinical symptoms. The subject may also be experimentally determined to be accumulating one or more oxysterols or present other clinical symptoms (e.g., elevated expression of one or more oxysterols in one of blood, plasma, serum, cerebrospinal fluid and cell membranes such as red blood cell membranes). In other embodiments, subjects have already been diagnosed as having NPC disease, for example, by detection of accumulation of one or more of 24-HC, 7-keto and triol, and the clinical condition of the patient and/or the efficacy of the treatment may be monitored.

[0048] 3. Biological Samples.

[0049] As used herein, a "biological sample" can include any body fluid, tissue, or matter from the subject, for example and without limitation, whole blood, plasma, serum, red blood cells, cord blood, biopsy samples, cerebrospinal fluid, sputum, amniotic fluid, or other tissue, cell (including in vitro cultured cells), fluid, or other biological material in which oxysterol concentration or accumulation may be detected and quantified. The suitability of a particular body fluid, tissue, or matter for use in the methods described herein may be determined by testing a sample of the fluid, tissue, or matter obtained from a subject suspected of having, diagnosed with, or screened positively for a lysosomal cholesterol storage or trafficking disease (e.g., cholesterol storage diseases) including cytotoxic oxysterol accumulation (NPC).

[0050] Methods for extracting or obtaining a biological sample of each of the subjects described above are well known in the art of medical and veterinary science, for example, phlebotomy, heel stick of an neonatal subject or infant, or a tissue biopsy. The biological sample may be obtained by surgical methods or biopsy. The biological sample may be obtained by relatively non-invasive methods, which are less traumatic to the subject, and more suitable for a broad-based screening assay. In some embodiments, the biological sample can be a body fluid sample. Illustrative body fluid samples, include, but are not limited to, plasma, sera, blood (including cord blood and maternal blood), sputum, amniotic fluid, cerebral spinal fluid, cellular membrane samples (for example red blood cell membranes), and the like.

[0051] Alternatively or additionally, the biological sample can be a cell or tissue sample, including cultured cells (e.g., fibroblasts) or tissues, and conditioned medium or effusions collected from cells or tissues. Exemplary cells or tissues can include, hematopoietic cells (including red blood cells, leu-

cocytes, lymphocytes and the like), neural cells (including neurons, microglial cells and astrocytes), muscle (including skeletal, smooth, cardiac and diaphragm), liver, kidney, lung, skin, foreskin, umbilical cells or tissue, and the like.

[0052] In some embodiments, the biological sample can be provided on a solid medium, e.g., a filter paper, swab, cotton, and the like. In some embodiments, the biological sample can be a dried blood or plasma sample from a neonatal subject, e.g., dried blood or plasma spots on neonatal screening cards (e.g., "Guthrie" cards).

[0053] 4. Evaluation.

[0054] Subjects may be presumptively identified as having, or at risk for developing, NPC disease by methods described herein. In some embodiments, the concentration of one or more oxysterols in a biological sample obtained from a subject can be quantified or determined. The concentration of the one or more oxysterols in the tested biological sample collected from the subject can be compared with a reference value. Elevated concentration(s) of one or more oxysterol(s) in the biological sample as compared to a reference value (which may be a predetermined value) can presumptively identify the subject as having, or at risk for developing, NPC disease.

[0055] In some embodiments, additional or alternative diagnostic testing can be carried out to confirm the diagnosis in these subjects. Typically, such alternative or additional methodologies (e.g., restriction fragment length polymorphism ("RFLP"), polymerase chain reaction ("PCR") and other genetic tests on tissue biopsies) are more costly, time-consuming, and invasive than the screening methods disclosed herein. For example, subjects having one or more oxysterol levels above a reference value may be presumptively identified as affected with NPC disease, and selected for additional diagnostic testing to confirm this diagnosis, determine the severity of the disease, assess whether the subject may be affected with another neurodegenerative disorder or lysosomal cholesterol storage disorder, or may be a healthy subject giving a false positive result in the screening assay.

[0056] Other tests for confirming the identification of a subject as having NPC disease using the methods of the present disclosure can include one of three commonly used tests. First, a skin biopsy can be obtained from the presumptively identified NPC disease afflicted subject. The skin fibroblasts are cultured and then stained with filipin to detect lysosomal accumulation of unesterified cholesterol. This assay may be qualitative but not quantitative. Second, an assay which determines the rate of cholesterol esterification can be measured in the cultures skin fibroblasts. Low rates of esterification, as compared to reference cell lines, are indicative of NPC disease. Third, the gold standard is sequence analysis of the NPC1 and NPC2 genes. This method detects >95% of clinically suspected NPC cases. Because the entire genomic region is not sequenced, a small percentage of NPC cases with novel intronic mutations or regulatory mutations within non-coding regions may escape detection.

[0057] Methods discussed below regarding monitoring course, severity, or clinical status, determining the efficacy of treatment, and neonatal screening may also be useful for screening and diagnosing subjects using oxysterol biomarkers.

C. Monitoring Course, Severity, or Clinical Status Using Oxysterol Biomarkers.

[0058] The present disclosure provides methods for monitoring the clinical course of a subject that has already been

positively diagnosed and/or is being simultaneously diagnosed as affected with a disorder involving the accumulation of an oxysterol, (for example at least one of 24-HC, 7-keto and triol), as this term is described above. The present disclosure provides that elevated oxysterol concentrations in biological samples (in particular, plasma, blood and sera) from affected subjects correlate with the clinical state of the affected subject.

[0059] Methods of the present disclosure can be used for monitoring the progression and/or stabilizing of NPC disease. In some embodiments, the increase in one or more oxysterols such as 7-keto and triol described herein correlates with NPC disease severity. Additionally or alternatively, the progression and/or remission of NPC disease can be measured by monitoring the concentration of one or more oxysterols, for example, 24-HC, 7-keto, and/or triol. Oxysterol concentrations may be elevated prior to the worsening of the disease in the affected subject, and thus may be used as an early indicator of stabilization.

[0060] In some embodiments, the level of 24-HC can be used to monitor the progression of NPC disease. The progress of NPC disease has been found to be inversely correlated with the concentrations of 24-HC after an initial elevation of 24-HC concentration above a 24-HC reference value obtained from a non-NPC afflicted control population. As an example, the clinical status of a subject and/or the severity of the NPC disease can be monitored in a subject or patient by measuring the increase in one or more oxysterol species and the decrease of the oxysterol 24-HC.

[0061] Reference values, subjects, biological samples, and evaluation discussed above for screening and diagnosis of NPC disease are also applicable to the embodiments relating to monitoring course, severity, or clinical status. Methods discussed herein regarding screening and diagnosis, determining the efficacy of treatment, neonatal screening, and differentiating NPC from other lysosomal storage diseases may also be useful for monitoring course, severity, or clinical status using oxysterol biomarkers.

D. Determining the Efficacy of Treatment Using Oxysterol Biomarkers.

[0062] The clinical condition of the subject may be monitored to determine the efficacy of a treatment regime, e.g., enzyme replacement therapy, gene therapy, pharmaceutical intervention (for example treatments with one or more of: statins or other cholesterol synthesis inhibitors, cycloheximide, liver X receptor (LXR) agonists, orphan nuclear receptor PXR ligands, neurosteroids, N-butyldioxyojirimycin (NB-DNJ) (miglustat), curcumin, chemical chaperones, antibiotics, salsalate, salicylic acid, RXR ligands, sphingolipid synthesis inhibitors (myriocin), KCl, EGTA, calcium channel inhibitors, nifedipine, verapamil, antioxidants (e.g., N-acetyl cysteine), vitamin E, vitamin C, auroitricarboxylic acid, flavonoids, cyclodextrins, estrogens, propyl gallate, glutathione, caspase inhibitors, MAP kinase inhibitors, peroxisome proliferator-activated receptor (PPARs) ligands, 15d-PGJ2, WY14643, indomethacin, glucocorticoids, dexamethasone, hydrocortisone, PI-3 kinase inhibitors, NMDA open channel blockers) and/or dietary therapy. For example, if the level of one or more oxysterol biomarkers suggests that the current therapeutic regime may not be effective, or not efficacious, it may be determined to initiate an altered course of treatment. Alternatively or additionally, the

condition of the subject may be monitored to determine whether to commence or re-initiate treatment of the subject.

[0063] The present disclosure provides methods for evaluating the efficacy of treatment by monitoring the concentration of one or more oxysterols, for example, 24-HC, 7-keto and triol during the course of treatment. In some embodiments, treatment efficacy can be generally correlated with a decrease in the concentration of 7-keto and/or triol oxysterol species. Specific method steps can include (a) obtaining a biological sample from a subject at a time (T_0) prior to or while being treated; (b) obtaining a biological sample from the subject at a time (T_1) subsequent to time (T_0); (b) quantifying the concentration of an oxysterol comprising 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol or combinations thereof in the biological samples obtained at (T_0) and (T_1); and (c) comparing the concentration of the oxysterol present in the biological samples obtained at T_0 and T_1 ; wherein if the concentration of the oxysterol 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_1) is greater than the oxysterol concentration of 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_0) or if the concentration of the oxysterol 24-hydroxycholesterol in the biological sample obtained at time (T_1) is less than the oxysterol concentration of 24-hydroxycholesterol in the biological sample obtained at time (T_0), then the treatment can be said to be not efficacious.

[0064] A decrease in the oxysterol concentration, for example 7-keto and/or triol, obtained from a subject or patient diagnosed with NPC disease as compared to an earlier measured 7-keto and/or triol oxysterol concentration indicates that the treatment is effective. Treatment efficacy can also be determined by measuring the concentration of 24-HC during the treatment period. In the initial stages of NPC disease there can be an increase of 24-HC over healthy controls, as the disease progresses and/or increases in severity, the concentration of 24-HC in the subject or patient decreases. In a subject or patient diagnosed NPC, if the treatment provides an increase or stabilization of 24-HC levels in an NPC patient during the course of treatment over an earlier measured concentration of 24-HC, the treatment can be said to be effective.

[0065] In some embodiments, the present disclosure provides a method to determine the efficacy of treatment for Niemann-Pick C disease in a subject. The method includes: (a) obtaining a biological sample from a subject at a time (T_0) concurrent with or prior to the commencement of treatment; (b) obtaining a biological sample from the subject at a time (T_1) subsequent to the commencement of treatment; (c) quantifying the concentration of an oxysterol comprising 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol or combinations thereof in the biological samples obtained at (T_0) and (T_1); and (d) comparing the concentration of the oxysterol present in the biological samples obtained at T_0 and T_1 . If the concentration of the oxysterol 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_1) is greater than the oxysterol concentration of 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_0) or if the concentration of the oxysterol 24-hydroxycholesterol in the biological sample obtained at time (T_1) is less than the oxysterol concentration of 24-hydroxycholesterol in the biological sample obtained at time (T_0) then the treatment is not efficacious.

[0066] The time lapsing between (T_0) and (T_1) can be determined by those of ordinary skill in the art, in particular, the subject's medical professional. While not wishing to be bound by theory, it is believed that altered expression of oxysterol biomarkers 24-HC, 7-keto and triol can be detected and measured as soon as 1 week. Therefore, the method for determining the efficacy of a treatment for Niemann-Pick C disease in a subject can include taking a biological sample at time 0 (T_0) before, or concurrently with the commencement of treatment for NPC disease. Then the efficacy of the treatment can be determined by quantifying the concentration of one or more oxysterols using the methods described herein at a time (T_0) which may be at least 1 week, or at least 2 weeks, or at least 3 weeks, or at least 4 weeks, or at least 8 weeks or at least 3 months, or at least 6 months after the commencement of treatment.

[0067] Reference values, subjects, biological samples, and evaluation discussed above for screening and diagnosis of NPC disease are also applicable to the embodiments relating to determining the efficacy of treatment. Methods discussed herein regarding screening and diagnosis, monitoring course, severity, or clinical status, neonatal screening, and differentiating NPC from other lysosomal storage diseases may also be useful for screening and diagnosing subjects using oxysterol biomarkers.

E. Neonatal Screening Using Oxysterol Biomarkers.

[0068] Methods disclosed herein may be advantageously employed as part of a neonatal screening program to identify affected neonatal subjects, for example, in the neonatal intensive care unit so as to permit early medical intervention. Many neonatal screening programs rely on a unique method of specimen collection, in which blood from a heel prick is absorbed onto a neonatal screening card (e.g., a cotton-fiber filter paper). Several neonatal diseases are screened in these subjects during their first weeks of life, including phenylalanine hydroxylase deficiency, which causes phenylketonuria ("PKU"), and branched-chain ketoacid dehydrogenase deficiency, which causes maple syrup urine disease ("MSUD"). Such screening programs are effective and cause no or little discomfort to these delicate patients.

[0069] Methods of the present disclosure can be used to identify neonatal subjects that have elevated levels of an oxysterol biomarker above a reference value—e.g., a value of 24-HC and/or 7-keto and/or triol concentration above a reference value of each of the oxysterol concentrations from non-NPC afflicted controls, indicative of a metabolic defect in NPC disease.

[0070] The neonatal screening methods disclosed herein, including high throughput screening assays may further advantageously be performed concurrently or in parallel (i.e., from the same sample but not necessarily in the same assay) with other neonatal screening assays, for example, on neonatal blood samples or dried blood spots on neonatal screening cards. In some embodiments, a neonatal screening program can be based on quantifying or measuring oxysterols from other biological samples, as described above. For example, 24-HC, 7-keto and triol can be measured in blood (e.g., cord blood), plasma, serum, or in the membrane of the neonatal blood cells, for example red blood cells. In some embodiments, one blood draw from a neonatal subject can provide sufficient biological sample to validate the quantity of one or

more oxysterols present in different biological samples, for example, plasma and red blood cells using the same or different testing methods.

[0071] Accordingly, in some embodiments of the present disclosure, a method for screening a neonatal subject for a disorder involving accumulation of one or more oxysterols comprising the step of quantifying or determining the concentration of oxysterol in a biological sample taken from the neonatal subject, wherein the detection of the oxysterol in the biological sample a concentration that is greater than a reference value of the same oxysterol identifies the neonatal subject as affected with the disorder.

[0072] In some embodiments, a method of screening a neonatal subject for NPC disease comprises the step of quantifying or determining the concentration of oxysterol in a blood sample taken from the neonatal subject, wherein the detection of the oxysterol in the biological sample at a concentration that is greater than a reference value identifies the neonatal subject as affected with NPC disease.

[0073] The methods can include the steps: (a) obtaining a biological sample from the neonatal subject; (b) quantifying the concentration of an oxysterol comprising 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol or combinations thereof in the biological sample; (c) providing a reference value of the oxysterol from a non-affected neonatal control population, wherein the reference value can be the concentration of the oxysterol obtained from the same type of biological sample as obtained from the subject; and (d) comparing the concentration of the oxysterol of the neonatal subject to the reference value, wherein if the concentration of the oxysterol from the neonatal subject is higher than the reference value, the neonatal subject can be identified as having, or at risk for developing, Niemann-Pick C disease.

[0074] In still further embodiments, methods as applied to diagnosing, monitoring of disease severity and determining the efficacy of treatment described above can also be applied to neonatal subjects.

[0075] In still further embodiments, described in more detail below, methods for quantifying an oxysterol concentration in a biological sample involving chemical derivitization of the oxysterol and tandem mass spectrometry are utilized as part of a neonatal screening program for NPC disease using oxysterols 24-HC, 7-keto and triol as biomarkers for the presence of the disease.

[0076] As described above, methods of neonatal screening may be at least partially automated. For example, once a sample is loaded onto an HPLC column or into a mass spectrometer, the data can be captured and analyzed using an automated system.

[0077] Reference values, subjects, biological samples, and evaluation discussed above for screening and diagnosis of NPC disease are also applicable to the embodiments relating to neonatal screening. Methods discussed herein regarding screening and diagnosis, monitoring course, severity, or clinical status, determining the efficacy of treatment, and differentiating NPC from other lysosomal storage diseases may also be useful for neonatal screening using oxysterol biomarkers.

F. Methods for Quantifying Oxysterol Concentration in Biological Samples

[0078] In some embodiments, the methods for quantifying oxysterol concentration on a biological sample can be simple, rapid, accurate, relatively non-invasive (e.g., non-surgical),

sensitive, and preferably minimize interfering signals from molecules other than the oxysterols 24-HC, 7-keto and triol. The method for quantifying the concentration of the oxysterol can include: (a) adding a known amount of an oxysterol internal standard to a biological sample; (b) extracting the oxysterols from the biological sample; and (c) quantifying the extracted oxysterols using a chromatography procedure.

[0079] As used herein, a chromatography procedure or combination of chromatography procedures can be used to quantify the oxysterol concentration in the collected biological sample. Methods for isolating sterols, including cholesterol and oxidized cholesterol are known in the art. In some embodiments, the quantification step of the method of identifying a subject with NPC disease can include: determining the relative concentration of the oxysterol and internal standard in the biological sample by correlating the area under the curve obtained for the known amount of oxysterol internal standard with the area under the curve obtained for the one or more oxysterols. Such determination can be accomplished when the isolated and/or derivatized oxysterols are passed through a chromatography procedure operable to derive the relative abundance of each oxysterol being identified relative to the known amount of oxysterol internal standard. The relative quantities of each of the oxysterols and oxysterol internal standard separated and/or isolated during the chromatography procedure can be routinely determined using computers and other processing devices that can be integrated with the detection means used in the chromatography equipment, for example, infra red, visible light diffraction, UV detection, mass spectroscopy, mass ionization and other methods used to detect lipids, sterols and/or oxysterols passing through a chromatography column or component.

[0080] In some embodiments, the oxysterol quantification methodology may be compatible with existing screening assays and may be adaptable to automation and high throughput screening. Methods useful to determine the concentration of one or more oxysterols in a biological sample indicative of NPC and other lysosomal cholesterol storage and trafficking diseases (e.g., cholesterol storage diseases), may be carried out using any suitable methodology or combination of methodologies that detects the presence or absence of oxysterols, and preferably, methodologies which determine the concentration of the oxysterol. Illustrative methods include, but are not limited to, chromatographic methods (e.g., high performance liquid chromatography ("HPLC")), thin layer chromatography ("TLC"), liquid chromatography-mass spectrometry ("LC-MS"); gas chromatography-mass spectrometry ("GC-MS"), time-of-flight mass spectrometry ("TOF-MS"), tandem mass spectrometry ("TMS"), matrix assisted laser desorption ionization-mass spectrometry ("MALDI-MS"), electrospray ionization-tandem mass spectrometry ("ESI-TMS") and combinations of these mass spectrometry techniques with or without sterol derivatization. Schroepfer Jr., G. J., "Oxysterols: *Modulators of Cholesterol Metabolism and Other Processes*", (2000), *Physiol. Rev.* 80(1): 362-521, provides additional assays and methods which may be useful in oxysterol quantification and identification. In some embodiments, HPLC, GC/MS, TOF-MS, and ESI-TMS can be used to determine the concentration of oxysterols in a biological sample. Further examples of quantification of oxysterol concentration from in vivo biological samples are described herein in the Examples section.

[0081] Jiang, X., et al. "Characterization of oxysterols by electrospray ionization tandem mass spectrometry after one-

step derivatization with dimethylglycine" Rapid Commun. Mass Spectrom., (2007); 21:141-152, describes high throughput screening methods for the detection identification and quantification of oxysterols using ESI-TMS. Such high throughput screening methods are useful in some embodiments of the present disclosure.

[0082] In some embodiments, the present disclosure provides methods that can be completely manual, alternatively and preferably, they are partially or completely automated. Screening programs to determine the concentration of oxysterols in a large number of biological samples, for example, a neonatal screening regime will generally be at least partially automated to facilitate high throughput of samples. The data can be captured and analyzed using an automated system. Other illustrative examples of high throughput methods can include arrays or micro-arrays of spotted biological samples (e.g., blood, plasma, serum, red blood cells and the like) on substrates which can be analyzed simultaneously. Such arrays or microarrays can contain greater than about 10, 50, 100, 200, 300, 500, 800, 1000, 2000, 5000 samples or more.

[0083] In biological samples in which the concentration of the oxysterol can be low relative to the limits of detection of the technique, a derivatization step may be used prior to the step of detecting (alternatively, quantifying) oxidized cholesterol in the biological sample. In some embodiments, the derivatization step can also be performed after the oxysterols have been isolated or concentrated to yield higher quantities of oxysterols for quantification. The derivatization of oxysterols in the biological sample facilitates proper identification using the chromatography and mass spectrometry methods such as HPLC, LC-MS, GC-MS, TOF-MS, and ESI-TMS and may also be used to separate the oxysterol analyte from contaminants or interfering substances. Jiang, et al. *supra* provides additional protocols for the derivatization of oxysterol species in mixtures using ESI-TMS which may be useful in the present disclosure.

[0084] Referring to FIG. 2, the table below each mass spectra illustrates the relative concentration of each oxysterol which can be determined by calculating the area under the curve of each tested oxysterol in control and NPC affected representative samples relative to known amounts of an oxysterol internal standard, for example, D5-27-hydroxycholesterol. Such calculations can be automated and processed by the chromatographic software used. Relative concentrations of oxysterols found in various biological samples such as plasma (FIGS. 3A & 3B) and cerebrospinal fluid (FIG. 4) from NPC subjects and healthy control subjects using the methods described herein, illustrate the selectivity and specificity of the oxysterols 24-HC, 7-keto and triol as exquisite biomarkers for NPC disease.

G. Methods for Quantifying An Oxysterol In A Biological Sample Using Tandem Mass Spectrometry (TMS)

[0085] In some embodiments, the present disclosure further provides a method for quantifying or determining the concentration of oxysterols in a biological sample by ESI-TMS. In some embodiments, the oxysterols present in the biological sample (blood, plasma, serum or cell membrane) will need to be extracted and isolated from other substances; including substances that may interfere with the detection and/or quantification of oxysterol biomarkers. Methods for extracting oxysterols from complex mixtures are well known in the art. Conventionally, extraction of lipids from complex biological samples commences with a two-phase extraction

with chloroform and methanol. In some embodiments, the organic phase of the two-phase extraction containing the oxysterols may be further purified using chromatography steps such as HPLC or passage through aminopropyl and silica columns to isolate a neutral sterol fraction prior to derivatization.

[0086] Derivatization of the oxysterols in the sample may be performed by any method known in the art, including derivatization using N,N-dimethylglycine, Girard P reagent and Pyridine:Hexamethyldisilazane:Trimethylchlorosilane in ratios of 5:2:1. In some embodiments, oxysterols can be saponified under mild conditions to avoid artificial oxysterol generation and derivatized with bis-(trimethyl-silyl)-trifluoroacetamide (BSTFA) and pyridine into trimethylsilyl ethers.

[0087] In some embodiments, matrix assisted laser desorption ionization—time of flight mass spectrometry ("MALDI-TOF MS"), GC-MS, LC-MS or ESI-TMS can be used to carry out the inventive methods described herein. Jiang, et al. *supra* provides additional protocols for the preparation, derivatization and identification of oxysterol species in mixtures using ESI-TMS which may be useful in the present disclosure.

[0088] Any suitable MS methodology known in the art may be employed, including, but not limited to GC-MS, LC-MS, Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometric ("LC/APCI-MS"), ESI-MS, MALDI-MS and MALDI-TOF MS. Electrospray ionization, ion traps and ion cyclotron resonance equipped mass spectrometers can also be employed.

[0089] Further disclosed herein is an example of an ESI-TMS protocol for screening for NPC disease using one or more oxysterols as biomarkers to identify a subject as having NPC or to be used as a screening method, for example, a neonatal screening method before the onset of clinical symptoms.

[0090] For the selective detection of compounds of a similar structural type, either a precursor ion scan function to identify the molecular species that fragment to a common product ion, or a constant neutral loss scan function to identify ions that lose a common fragment, or a multiple reaction monitoring where selected precursor and product ions only are detected can be employed. Addition of appropriate oxysterol internal standards, such as stable analogs, to the biological matrix before work-up and analysis facilitates accurate quantification of the target oxysterols.

[0091] An oxysterol internal standard can generally be added to the sample prior to manipulations, so that the standard can be subjected to the same conditions as the analyte. Any suitable internal standard may be used. For example, oxidized cholesterol homologs in which one of the carbons, for example at position 27 as in D₅-27-hydroxycholesterol, is hydroxylated can be used. The internal standard can be added to the sample in a known quantity. The ratio of signals produced by 24-HC, 7-keto, triol and other oxysterols that are elevated in NPC subjects and the internal standard will allow the starting quantity of the oxysterol biomarkers in the sample to be determined by use of a calibration curve. The calibration curve can be a plot of the signal ratio (oxysterol biomarkers of NPC to internal standard) against different known concentrations of 24-HC, 7-keto, triol standards, using the same fixed quantity of internal standard. In some embodiments, peak identification can be confirmed by relative retention time and mass spectral comparison with one or more authenticated

oxysterol standards, as well as with the HP MS Chemstation NBS Mass Spectral Data Library of compounds.

[0092] In some embodiments of the present disclosure, a method for quantifying or determining oxysterol biomarkers in a biological sample comprises: (1) collecting a biological sample; (2) adding a known quantity of a suitable oxysterol standard to the sample; (3) extracting the oxysterols from the sample using a two-phase extraction medium; (4) purifying the oxysterol in the sample by normal phase chromatography, followed by elution from the column with a suitable solvent; (5) derivatization of the oxysterol and (6) quantification of the oxysterol biomarker using MS. A D₅-27-hydroxycholesterol standard can be used as an internal standard for the MS analysis.

[0093] The foregoing methodology can be employed in inventive screening and testing methodologies described above. As further described above, the methods may be partially or completely automated.

[0094] TMS based methodologies are particularly suitable for quantifying or determining oxysterols 24-HC, 7-keto and triol as biomarkers for NPC disease in dried blood spots from neonatal screening cards. According to this embodiment, the method above further comprises a step of extracting the lipid component from the dried blood spot using a suitable solvent (e.g., an organic solvent or aqueous/organic mixture).

[0095] Thus, in some embodiments, the present disclosure provides a method of screening a neonatal subject for NPC disease, comprising: (1) providing a blood sample, comprising a dried blood spot on a solid absorbent substrate (e.g., a filter paper); (2) adding a known quantity of a suitable oxysterol standard to the sample; (3) extracting the oxysterols from the sample using a two-phase extraction medium; (4) purifying the oxysterol in the sample by normal phase chromatography, followed by elution from the column with a suitable solvent; (5) derivatization of the oxysterol and (6) quantification of the oxysterol biomarker using MS. A D₅-27-hydroxycholesterol standard can be used as an internal standard for the MS analysis; and (7) presumptively identifying those subjects as affected with NPC disease based on oxysterol concentrations in the biological sample that are greater than a reference value (as described above).

EXAMPLES

[0096] Aspects of the present disclosure may be further understood in light of the following examples, which should not be construed as limiting the scope of the present disclosure or the claims in any way.

Example 1

Isolation of Oxysterols from NPC Patient Samples

[0097] 200 pmol of d₅-27-hydroxycholesterol (27-HC), 50 µg butylated hydroxytoluene (BHT) and a magnetic flea bar are added to a 10 mL centrifuge tube. The tube is placed under a gentle stream of argon. 250 µL of plasma from a subject and 2.5 mL 0.35 M KOH is added to the tube. The tube is sealed tightly with a Teflon-lined screw cap, vortexed, and allowed to incubate for 2 hr at room temperature while stirring. After incubation, the flea bar is removed and the tube is centrifuged at 3000 rpm for 5 min to pellet the protein. The liquid phase of plasma mixture is removed and added to a 10 mL centrifuge tube placed under argon stream. The tube is then sealed tightly. The pH of liquid phase is lowered to approximately 7.0-8.0 using a 1:10 dilution of 85% H₃PO₄. While vortexing

the centrifuge tube, 200 µL of H₃PO₄ is added dropwise to the plasma mixture until desired pH is achieved. 1.54 mL of 150 mM NaCl and 4.5 mL CHCl₃ is added to the plasma mixture. The tube is sealed with a Teflon screw cap, vortexed, and centrifuged at 3400 rpm for 5 min. The lower organic phase is extracted, transferred to a new 10 mL centrifuge tube, and placed under argon stream. 2.0 mL of CHCl₃ is added to the remaining aqueous phase of the first extraction, vortexed, and then centrifuged. The organic phases are collected from the centrifuge tubes and transferred to clean tubes under argon. The extraction steps above are then repeated. The pooled organic phases are evaporated under argon in a clean tube and sealed tightly with a Teflon-lined cap. The samples are then ready for oxysterol purification.

Example 2

Oxysterol Purification Using Aminopropyl and Silica Columns

[0098] The extracted lipid samples are resuspended in 250-300 µL CHCl₃ and then vortexed. One or more aminopropyl columns (Sep-Pak Vac RC 500 mg NH₂ Cartridges, part no. WAT054515) are primed with 4 mL hexane and are loaded with the lipid sample in CHCl₃. The sample drips into the column using gravity. The neutral fraction is eluted with 4 mL CHCl₃/isopropyl (2:1 v/v) and then dried under argon. The neutral fraction is resuspended in 1 mL toluene and the fraction is added to a silica column (Isolute 100 mg SI 10 mL XL cartridges, part no. 460-0010-G) primed with 4 mL hexane. The neutral fraction volume is pulled through the silica column (without drying out) using vacuum. The column is washed with 8 mL of 0.5% isopropyl in hexane, followed by elution of the neutral fraction with 2 mL of 30% isopropyl in hexane. The eluted fraction is collected and dried under argon. After silica column purification, the sample is resuspended in 250 µL of acetonitrile and transferred to a low retention/silanized microcentrifuge tube. The tube containing the eluted fraction is again washed with 250 µL acetonitrile and the resuspension added to a second microfuge tube. The eluted fraction containing the oxysterol component is then dried and sealed under argon.

Example 3

Derivatization of Oxysterols from Plasma Samples

[0099] To derivatize the oxysterols, a solution of 5:2:1 (v:v:v) of Pyridine: Hexamethyldisilazane: Trimethylchlorosilane is mixed in a centrifuge tube, vortexed, and the solution is centrifuged at 3400 rpm for 2 min. 100 µL of the derivatization solution is added to each of the samples prepared above containing the oxysterol component in eppendorf tubes. The eppendorf tubes are then sealed tightly, vortexed, and the derivatization solution and samples are incubated at 60° C. for 30 min. The reaction product containing the derivatized oxysterols is then dried down under a stream of argon. The derivatized oxysterols are resuspended in 100 µL heptane and the resuspension is then vortexed, centrifuged and transferred to an autosampler vial.

Example 4

Analysis of Oxysterol Profile by Gas Chromatography-Mass Spectrometry

[0100] The samples containing the derivatized oxysterols are run on an Agilent Technologies 5975B inert XL MSD

with an Agilent Technologies 6890N Network GC System equipped with an Agilent Technologies 7683 Autosampler and an Agilent Technologies 7683B injector. A J&W Scientific 25 m model DB-1 column with an internal diameter of 0.2 mm and film thickness of 0.33 μ m is used to separate the oxysterol species. 2.0 μ L of sample is injected into the inlet, which is kept at 250° C. with a pressure of 28.98 psi. A split ratio of 10:1 is used, with a split flow of 10.6 mL/min and a total flow of 15 mL/min within the inlet. The oven starts at 180° C. prior to ramping up to 250° C. at a rate of 20° C./min followed by an additional ramp up to 300° C. at a rate of 5° C./min where it stays for 15 min, allowing for an overall run time of 28.5 min. Initial flow of gasses through the column is set at 1.0 mL/min allowing for a nominal initial pressure of 28.04 psi and average velocity of 42 cm/sec. The MSD transfer line is kept at 280° C. throughout the sample run. The MS quadrupole is set to 150° C. and the MS source is set to 230° C. The MSD is set to monitor for 461.4, 544.4, 472.4, 456.4, and 413.4 ions at dwell times of 61 ms. FIG. 2 shows a gas chromatogram/mass spectrum (GC/MS) of oxysterols in plasma samples obtained from confirmed NPC patients and control non-NPC afflicted subjects.

Example 5

Oxysterol Concentration in Control and NPC Confirmed Subjects from Plasma Samples

[0101] Thirty-three plasma samples were obtained from subjects with NPC disease enrolled in the natural history study at the National Institutes of Health. The plasma samples were collected in “purple-top” tubes containing K₃-EDTA and BHT. The tubes were immediately centrifuged and the plasma was isolated, stored in 1 ml aliquots and frozen at -80° C. The plasma samples were thawed immediately prior to analysis and the samples were processed as described above. The NPC plasma samples were analyzed for levels of d5-27-HC (internal standard), 27-HC, 24-HC (24-hydroxycholesterol), 7-ketocholesterol (7-keto), cholestane-3 β ,5 α ,6 β -triol (triol), and 7 α ,27-HC (see FIG. 2.). The results are presented in Table 1 below:

TABLE 1

Oxysterol quantification in control and NPC confirmed subjects from plasma samples using GC-MS.			
Oxysterol	NPC (ng/mL)	Control (ng/mL)	p value
7 α ,27	9.79	9.95	0.875
7-keto	455.14	106.06	2.98E-12
27-HC	93.20	117.41	0.132
24-HC	69.49	33.08	2.63E-09
triol	174.41	8.88	5.46E-17

[0102] A two-sample t-test assuming equal variance was used to test for significance between NPC patients and normal controls for 24-HC, 7 α ,27 HC, 27-HC, and 7-keto. A two-sample t-test assuming unequal variance was used for triol. Three of the oxysterols (24-HC, 7-keto and triol) were significantly elevated in NPC subjects, as compared to normal controls, and thus represent plasma biomarkers for diagnosis of NPC disease.

[0103] The relationship between the oxysterol biomarkers and disease severity in the NPC subjects was also examined. Among the selected oxysterols, the concentration of 24-HC revealed a statistically significant inverse correlation between

plasma levels of 24-HC and clinical disease severity (see FIG. 5). The Pearson correlation coefficient obtained was 0.67, with an R² value of 0.45 and a p value=0.0022. The concentration of 24-HC in a biological sample may be a useful biomarker for following NPC subjects longitudinally, and may provide a quantitative biomarker to follow efficacy of therapeutics interventions in slowing the progression of neurodegeneration in NPC subjects.

[0104] The stability of the oxysterols triol and 7-keto prepared in accordance with the methods of the present disclosure are shown in FIGS. 7A and 7B. Patient's having confirmed NPC disease were used to obtain plasma biological samples. The plasma biological samples were processed as described in Examples 1-4 except that some of the samples were not treated with butylated hydroxytoluene (BHT, a known antioxidant that prevents further oxidation of cholesterol), and either treated at room temperature or at 4° C. with and without BHT. The results show that the methods of the present disclosure can be performed at 4° C. or at room temperature and with and without BHT without adversely affecting the ability to isolate and quantify the presence of the oxysterols triol and 7-keto in the methods steps outlined in Example 1-4.

[0105] In addition to testing the stability of the oxysterols when isolated and determined using the methods of the present disclosure, the time of biological sampling was also measured to determine whether there is a difference in the in vivo production by the subject of the oxysterols being identified. As shown in FIGS. 7A and 7B, there is no statistically beneficial or detrimental time to take a biological sample from the subject to be identified or screened for NPC disease. The data appears to indicate that the production of oxysterols triol and 7-keto is fairly constant throughout a 24 hour day.

Example 5

Specificity of the Oxysterols 7-Keto and Triol to Identify NPC Disease from Other Lysosomal Storage and Trafficking Diseases

[0106] Oxysterols 7-keto and triol from a variety of human subjects having a lysosomal storage or trafficking disease including those with known CNS involvement: infantile neuronal ceroid lipofuscinosis (INCL), GM1 gangliosidosis (GM-1), GM-2 gangliosidosis (GM-2) (Tay-Sachs Disease), Gaucher's disease (GD) and hepatosplenomegaly (HSM). As shown in FIGS. 6A and 6B, the combination of elevated 7-keto and triol levels was able to differentiate NPC subjects from subjects with other LSDs. The concentration of the oxysterol 7-keto and triol are expressed as nanograms per milliliter (ng/mL) of plasma. The results depicted in FIGS. 8A and 8B for the oxysterols 7-keto and triol respectively, demonstrate that for identifying and screening purposes, (e.g., in human subjects), the oxysterols 7-keto and triol can be used as selective biomarkers to identify and screen NPC subjects from other subjects having a lysosomal storage or trafficking disease.

Example 6

Determination of Oxysterols in NPC and Wild-Type Mice

[0107] The present methods for quantifying the presence of oxysterols from NPC afflicted subjects, including NPC1-/- mice and wild-type non-afflicted NPC mice were performed

to determine the expression of various oxysterols in these population. Measurements of various plasma oxysterols in the NPC1-deficient and wild type mice from ages 4-10 weeks were performed. Note that for these oxysterols, the divergence is generally greatest after 7 weeks when the mice are most symptomatic. Methods for isolating and quantifying the levels of oxysterols in the wild-type and NPC1 null mice are described herein.

[0108] As shown in FIG. 9, significant elevations in plasma 25-hydroxycholesterol (25-HC) and cholestane-3 β ,5 α ,6 β -triol (triol) in NPC1 mice as compared to wild-type mice were observed at all time points. Even more striking was the separation in the plasma oxysterol levels (4 β -hydroxycholesterol (4 β -HC), 7 α -hydroxycholesterol (7 α -HC), 7 β -hydroxycholesterol (7 β -HC), and 7-keto), between wild-type and NPC1 mice beginning at 7-8 weeks of age, when the NPC1 mice begin losing weight and are overtly symptomatic. The elevated plasma oxysterols in the NPC1 null mice were corroborated by increased oxysterol levels in cerebellar and liver tissues. Remarkably, elevated triol levels in cerebellar tissue were detected as early as postnatal day 8 in the NPC1 mice, when the mice are asymptomatic yet display neuronal cholesterol storage. Taken together, these findings indicate that plasma levels of non-enzymatically generated oxysterols, including 25-HC, triol, 4 β -HC, 7 α -HC, 7 β -HC, and 7-keto can be used to distinguish between wild-type and NPC1 disease, and may vary with disease progression. These oxysterols can be used as biomarkers for the identification and monitoring of NPC disease and its progression.

[0109] The foregoing description of the embodiments has been provided for purposes of illustration and description. It is not intended to be exhaustive or to limit the scope of the disclosure or claims. Individual elements or features of a particular embodiment are generally not limited to that particular embodiment, but, where applicable, are interchangeable and can be used in a selected embodiment, even if not specifically shown or described. The same may also be varied in many ways. Such variations are not to be regarded as a departure from the scope of the disclosure or claims, and all such modifications are intended to be included within the scope of the disclosure and claims.

What is claimed is:

1. A method for determining whether a subject is afflicted with Niemann-Pick C disease, the method comprising:

- (a) obtaining a biological sample from the subject;
- (b) determining the concentration in the biological sample of at least one oxysterol selected from 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol, and combinations thereof; and
- (c) comparing the concentration of the at least one oxysterol present in the biological sample to a reference value of the at least one oxysterol obtained from a control population, wherein if the concentration of the at least one oxysterol from the subject is greater than the reference value, the subject is identified as afflicted with Niemann-Pick C disease.

2. The method according to claim 1, wherein determining the concentration of the at least one oxysterol comprises:

- (a) adding a known amount of an oxysterol internal standard to the biological sample;
- (b) extracting the at least one oxysterol from the biological sample; and
- (c) quantifying the extracted at least one oxysterol using a chromatography procedure.

3. The method of claim 1, wherein the control population comprises subjects matched with the subject in either one or both of age and sex, and either subjects afflicted with Niemann-Pick C disease or subjects not afflicted with Niemann-Pick C disease.

4. The method of claim 3, wherein the reference value comprises an oxysterol concentration expressed as one or more of an average, mean, or median of a non-Niemann-Pick C afflicted control group.

5. The method of claim 1, wherein the subject is identified as affected with Niemann-Pick C disease when the concentration of the at least one oxysterol in the subject biological sample is more than about 2, 3, 4, 5, 8, 10 or 20 fold greater than the reference value, and wherein the reference value is an average or median oxysterol concentration in the same biological sample type from a healthy control population matched with the subject in at least one of sex or age.

6. A method for screening a neonatal subject for Niemann-Pick C disease, the method comprising:

- (a) obtaining a biological sample from the neonatal subject;
- (b) determining the concentration in the biological sample of at least one oxysterol selected from 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol, and combinations thereof;
- (c) providing a reference value of the oxysterol from a neonatal control population, wherein the reference value is the concentration of the at least one oxysterol obtained from the same type of biological sample as obtained from the subject; and
- (d) comparing the concentration of the oxysterol of the neonatal subject to the reference value, wherein if the concentration of the oxysterol from the neonatal subject is higher than the reference value, the neonatal subject is identified as afflicted with Niemann-Pick C disease.

7. The method according to claim 6, wherein quantifying the concentration of the oxysterol comprises:

- (a) providing a neonatal screening card spotted with the biological sample from the neonatal subject;
- (b) spotting to the neonatal screening card a known amount of an oxysterol internal standard;
- (c) extracting the at least one oxysterol and the oxysterol internal standard from the neonatal screening card; and
- (d) determining the concentrations of the extracted at least one oxysterol and the internal standard using a chromatography procedure.

8. The method of claim 6, wherein the reference value is an oxysterol concentration expressed as one or more of an average, mean, or median of a non-Niemann-Pick C afflicted control group.

9. The method of claim 6, wherein the neonatal subject is identified as affected with Niemann-Pick C disease when the concentration of oxysterol in the subject biological sample is more than about 2, 3, 4, 5, 8, 10 or 20 fold higher than the average or median oxysterol concentration found in the same biological sample from sex-matched controls of non-NPC afflicted neonates.

10. A method for determining the status of Niemann-Pick C disease in a subject, the method comprising:

- (a) obtaining at least one biological sample from the subject;
- (b) quantifying at least one oxysterol selected from 24-hydroxycholesterol, 7-ketocholesterol, cholestane-3 β , 5 α ,

6 β -triol, and combinations thereof, in the biological sample to determine a quantification value;

- (c) comparing the quantification value of at least one oxysterol in the biological sample to a reference value of the same at least one oxysterol obtained from the subject or from a control population, wherein the magnitude of a difference between the quantification value of the at least one oxysterol from the subject and the reference value is representative of the status of Niemann-Pick C disease in the subject.

11. The method of claim **10** wherein the quantification value of the at least one oxysterol comprises the concentration of 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol, or both, and wherein the concentration of 7-ketocholesterol, cholestane-3 β , 5 α , 6 β -triol, or both, is greater than the reference value.

12. The method according to claim **10**, wherein quantifying the at least one oxysterol comprises:

- (a) adding a known amount of an oxysterol internal standard to the biological sample;
- (b) extracting the at least one oxysterol from the biological sample; and
- (c) quantifying the extracted at least one oxysterol using a chromatography procedure.

13. The method according to claim **12**, wherein quantifying the extracted at least one oxysterol comprises determining the relative concentration of the at least one oxysterol and the internal standard in the biological sample by correlating the area under the curve obtained for the known amount of oxysterol internal standard with the area under the curve obtained for the at least one oxysterol.

14. The method of claim **10** wherein the quantification value of the at least one oxysterol comprises the concentration of 24-hydroxycholesterol, and wherein the concentration of 24-hydroxycholesterol is lower than the reference value.

15. The method of claim **10** wherein the quantification value of the at least one oxysterol comprises the concentration of 24-hydroxycholesterol, and wherein the concentration of 24-hydroxycholesterol is lower than the reference value.

16. The method of claim **10** wherein the quantification value comprises the concentration of 24-hydroxycholesterol and the concentration of either or both of 7-ketocholesterol and cholestane-3 β , 5 α , 6 β -triol, and the reference value comprises the concentration of 24-hydroxycholesterol and the concentration of either or both of 7-ketocholesterol and cholestane-3 β , 5 α , 6 β -triol,

wherein the concentration of 24-hydroxycholesterol from the subject is less than the reference value for 24-hydroxycholesterol,

and wherein the concentration of 7-ketocholesterol is greater than the reference value for 7-ketocholesterol, the concentration of cholestane-3 β , 5 α , 6 β -triol is greater than the reference value for cholestane-3 β , 5 α , 6 β -triol, or both.

17. The method of claim **10** wherein:

- (a) a first biological sample is obtained from the subject at a time (T_0);
- (b) a second biological sample is obtained from the subject at a time (T_1) subsequent to time (T_0);

- (c) quantifying the at least one oxysterol comprises determining the concentration of the at least one oxysterol in the biological samples obtained at (T_0) and (T_1); and
- (d) comparing the concentration of the at least one oxysterol present in the first biological sample to the concentration of the same at least one oxysterol present in the first biological sample;

wherein if the concentration of the at least one oxysterol in the first biological sample is greater than the oxysterol concentration of the same at least one oxysterol in the second biological sample then the severity of Niemann-Pick C disease in the subject is determined to have increased from time (T_0) to time (T_1).

18. The method of claim **17** wherein the quantification value comprises the concentration of 24-hydroxycholesterol and the concentration of either or both of 7-ketocholesterol and cholestane-3 β , 5 α , 6 β -triol, and the reference value comprises the concentration of 24-hydroxycholesterol and the concentration of either or both of 7-ketocholesterol and cholestane-3 β , 5 α , 6 β -triol,

wherein the concentration of 24-hydroxycholesterol from the subject is less than the reference value for 24-hydroxycholesterol,

and wherein the concentration of 7-ketocholesterol is greater than the reference value for 7-ketocholesterol, the concentration of cholestane-3 β , 5 α , 6 β -triol is greater than the reference value for cholestane-3 β , 5 α , 6 β -triol, or both.

19. The method of claim **10** wherein:

- (a) a first biological sample is obtained from the subject at a time (T_0) concurrent with, prior to, or after the commencement of a treatment;
- (b) a second biological sample is obtained from the subject at a time (T_1) subsequent to time (T_0);
- (c) quantifying the at least one oxysterol comprises determining the concentration of the at least one oxysterol in the biological samples obtained at (T_0) and (T_1); and
- (d) comparing the concentration of the at least one oxysterol present in the first biological sample to the concentration of the at least one oxysterol present in the first biological sample;

wherein if the concentration of the oxysterol 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_1) is greater than the oxysterol concentration of 7-ketocholesterol or cholestane-3 β , 5 α , 6 β -triol in the biological sample obtained at time (T_0), or if the concentration of the oxysterol 24-hydroxycholesterol in the biological sample obtained at time (T_1) is less than the oxysterol concentration of 24-hydroxycholesterol in the biological sample obtained at time (T_0) then the treatment is determined to be not efficacious.

20. The method of claim **17**, wherein the biological sample obtained from the subject at time (T_1) is at least 1 week, or at least 2 weeks, or at least 3 weeks, or at least 4 weeks, or at least 8 weeks or at least 3 months, or at least 6 months after the commencement of treatment.

* * * * *

Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease

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Niemann-Pick type C1 (NPC1) disease is a rare progressive neurodegenerative disorder characterized by endolysosomal accumulation of unesterified or free cholesterol. The recognition that NPC1 disease is associated with oxidative stress raised the possibility that oxidative attack of the excess free cholesterol might lead to increased non-enzymatic formation of cholesterol oxidation products in vivo. To test whether cholesterol oxidation products could serve as disease biomarkers, we measured these metabolites in the plasma and tissues of the *Npc1*^{-/-} mouse model. We found several non-enzymatically-formed cholesterol oxidation products that were elevated in *Npc1*^{-/-} mice and were associated with disease progression. To study whether these oxysterols are associated with human NPC disease, we measured a plasma oxysterols in 25 NPC1 subjects, 25 age-matched controls and 17 obligate heterozygotes. Cholesterol oxidation products were increased in the plasma of all human NPC1 subjects studied, and delineated an oxysterol profile specific for NPC1 disease. The NPC1 oxysterol profile also correlated with age of disease onset and disease severity. These cholesterol oxidation products provide the first robust blood-based biochemical markers for NPC1 disease, and may prove useful as diagnostic tools for early detection of NPC1 disease and as outcome measures for clinical trials.

CEREBROSPINAL FLUID BIOMARKERS AND β -AMYLOID METABOLISM IN NIEMANN-PICK TYPE C DISEASE

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OBJECTIVES

The lysosomal storage disorder Niemann-Pick type C disease (NPC) shows some intriguing similarities with Alzheimer's disease (AD), including neurofibrillary tangles. Further, although NPC patients generally lack β -amyloid (A β) plaques found in AD, NPC-like cells accumulate intracellular A β . Experimental studies have found that lipids may affect A β metabolism, but an effect of high brain lipid load on A β metabolism has been difficult to prove *in vivo*. To pin-point this we examined cerebrospinal fluid (CSF) from NPC patients.

METHODS

Thirty-eight NPC patients and 15 controls were included. CSF was analyzed for A β 38, A β 40, A β 42, α -cleaved soluble (sAPP- α), β -cleaved soluble (sAPP- β), total-tau (T-tau) and phospho-tau (P-tau).

RESULTS

Compared to controls, NPC patients had significantly higher A β 42 (359 vs. 87 ng/L, $P < 0.001$) and T-tau (234 vs. 79 ng/L, $P < 0.001$). Also, ratios of A β 42:A β 38 and A β 42:A β 40 were higher (0.93 vs. 0.60; 0.08 vs. 0.06, $P < 0.001$). Controls and NPC patients had similar sAPP- α and sAPP- β levels. A β 42 correlated to disease severity ($R = -0.499$, $P = 0.001$). Eighteen patients were on miglustat treatment at start of the study and these had lower A β 1-42 (243 vs 277 ng/L, $P = 0.048$) and T-tau (170 vs. 348 ng/L, $P = 0.033$) than untreated patients. CSF T-tau decreased after treatment start in five patients (955 vs. 382 ng/L, $P = 0.043$).

CONCLUSIONS

A β release is increased in NPC, with a production shift towards A β 42. CSF A β and CSF T-tau may be useful as NPC biomarkers. This study provides the first *in-vivo* evidence that high lipid load in human brain affects A β production.

Neuronal lipid accumulation increases γ -secretase-dependent release of β -amyloid

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Abstract

Background Niemann-Pick type C disease (NPC) is an inherited storage disorder characterized by accumulation of cholesterol and glycosphingolipids in endosomes and lysosomes. NPC shows some intriguing similarities with Alzheimer's disease (AD), including neurofibrillary tangles. Although NPC patients generally lack β -amyloid ($A\beta$) plaques, NPC-like cells accumulate intracellular $A\beta$. Cholesterol and other lipids affect γ -secretase-dependent $A\beta$ metabolism *in vitro*, but this has been difficult to prove *in vivo*. To assess the effect of lipid accumulation on γ -secretase-mediated $A\beta$ release in humans, we examined $A\beta$ in cerebrospinal fluid (CSF) from NPC patients.

Methods Thirty-eight patients and 14 controls were included. CSF was analyzed for $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, $A\beta_{1-42}$, α -cleaved soluble (sAPP- α), β -cleaved soluble (sAPP- β), total-tau (T-tau) and phospho-tau (P-tau).

Findings Patients had significantly higher $A\beta_{42}$ (359 vs. 87 ng/L, $P<0.001$), T-tau (234 vs. 79 ng/L, $P<0.001$), and ratios of $A\beta_{42}:A\beta_{38}$ (0.93 vs. 0.60, $P<0.001$) and $A\beta_{42}:A\beta_{40}$ (0.08 vs. 0.06, $P<0.001$). sAPP- α and sAPP- β levels were similar. Eighteen patients were on miglustat treatment at the start of the study. These had lower $A\beta_{1-42}$ (243 vs 277 ng/L, $P=0.048$) and T-tau (170 vs. 348 ng/L, $P=0.033$) than untreated patients. CSF T-tau decreased after treatment in five patients (955 vs. 382 ng/L, $P=0.043$).

Interpretation $A\beta$ release is increased in NPC, with a production shift towards $A\beta_{42}$. Normal sAPP- β levels in patients suggest augmented activity of γ -secretase rather than β -secretase. CSF T-tau may be useful as a biomarker in NPC treatment. This study provides the first *in vivo* evidence that lipid accumulation affects γ -secretase-dependent $A\beta$ metabolism.

Introduction

β -Amyloid ($A\beta$) is the main component of extracellular plaques in Alzheimer's disease (AD), and abnormal $A\beta$ metabolism is widely recognized as a core pathological event in AD.¹ $A\beta$ metabolism has been linked to lipid homeostasis (reviewed in²⁻⁵). Hypercholesterolemia is a risk factor for AD in epidemiological settings⁶ and cholesterol-lowering agents reduce $A\beta$ in experimental studies.^{7, 8} Furthermore, the $\epsilon 4$ allele of *APOE*, the main cholesterol carrier in the central nervous system, is the major genetic risk factor for sporadic AD.⁹ $A\beta$ is released from amyloid- β precursor protein (APP), a transmembrane protein, through orchestrated cleavages by the enzymes β -secretase and γ -secretase. Several experimental studies suggest that increased lipid content in membranes facilitates γ -secretase activity^{7, 10-12} while the influence of lipids on β -secretase activity is disputed.¹³ β -Secretase cleavage releases the extracellular sAPP- β fragment. α -Secretase cuts APP within the $A\beta$ domain, precluding $A\beta$ formation and resulting in the release of sAPP- α . The putative α -secretase ADAM10 is absent from lipid rafts, providing a link between low lipid content and non-amyloidogenic APP processing.¹⁴ However, evidence from humans of the effects of cellular lipid homeostasis on amyloid metabolism in the central nervous system is lacking. Also, despite early positive results with statin treatment,¹⁵⁻¹⁹ a recent Cochrane report found no evidence that statins prevent AD.²⁰ To evaluate the effects of altered lipid homeostasis *in vivo* we examined $A\beta$ in cerebrospinal fluid (CSF) from patients with Niemann-Pick type C disease (NPC).

NPC is an autosomal recessive lysosomal storage disorder characterized by accumulation of different lipid species, including unesterified cholesterol and glycosphingolipids in late endosomes and lysosomes.²¹⁻²³ The impaired intracellular transport of cholesterol disrupts normal cholesterol homeostasis,^{24, 25} but the lipid accumulation is complex, and it is not clear

which species is the primary offending metabolite.²⁶ The disease incidence is estimated to 1/120,000–150,000²⁷ and the clinical spectrum is broad, with onset of symptoms ranging from foetal onset with ascites to progressive dementia in adults.²⁸⁻³⁰ Progressive neurological impairment is the major clinical problem. Ninety-five percent of cases are caused by mutation of the *NPC1* gene, which encodes an integral membrane protein that functions in intracellular cholesterol transport.²⁴ Remaining cases are caused by mutation of the *NPC2* gene, which encodes a lysosomal cholesterol binding protein.³¹ There is no definitive therapy for NPC but substrate reduction therapy using miglustat (N-butyldeoxynojirimycin, Zavesca®), an inhibitor of glucosylceramide synthase, improved neurological symptoms after one year of therapy in a randomized trial.³² The long-term efficacy of miglustat is unknown and disease monitoring is difficult. Although one small pilot-study showed useful results from measurement of the brain choline/creatine ratio,³³ there is a great need for improved biomarkers to monitor NPC disease progression.³⁴

NPC has some intriguing similarities with Alzheimer's disease (AD), including intraneuronal tangles containing phosphorylated tau (P-tau).^{35, 36} Also, both NPC and AD show accelerated neurological deterioration in the presence of *APOE* ε4.^{37, 38} Finally, the diseases share endosomal alterations and NPC-like cells accumulate intracellular Aβ^{39, 40} although autopsy generally does not reveal Alzheimer-type extracellular Aβ in NPC patients.^{36, 37}

The aims of this study were I) to assess the effects of altered neuronal lipid homeostasis on Aβ metabolism *in vivo* in humans, and II) to evaluate the use of CSF biomarkers for Aβ metabolism and axonal degeneration in NPC patients.

Methods

Study population

NPC1 patients included in this study were enrolled in an ongoing longitudinal observational trial at the National Institutes of Health between August 2006 and April, 2009. The study was approved by the NICHD Institutional Review Board and informed consent, and when appropriate assent, was obtained for all subjects. Patients under one year of age were excluded, due to known effects of age on CSF biomarkers for A β metabolism and axonal degeneration.⁴¹ Thirty-eight patients were included, of which 22 underwent a single evaluation and 16 were seen at 12 month intervals. Patient severity was scored as described by Yanjanin et al.⁴² This phenotyping index ascertains primarily neurological signs and symptoms in nine major (ambulation, cognition, eye movement, fine motor, hearing, memory, seizures, speech, and swallowing) and eight minor (auditory brainstem response, behaviour, gelastic cataplexy, hyperreflexia, incontinence, narcolepsy, psychiatric and respiratory problems) domains. The total possible score ranges from 0 to 61, with a higher score indicating more severe clinical impairment. The diagnosis was established by biochemical testing and mutation analysis. *APOE* genotyping was performed according to standard procedures. At baseline, 18/38 (47%) patients were on off-label miglustat use (usage without indication approved by the United States Food and Drug Administration). This is representative of miglustat use in the USA during the study period, and miglustat use was primarily determined by availability of insurance coverage. Investigators with this study neither provided nor prescribed miglustat; however, the NICHD IRB specifically approved following patients who were prescribed miglustat by other physicians in this observational trial. Control CSF was collected from 14 gender and age-matched patients who were undergoing CSF collection for another clinical indication (12 for acute lymphatic leukemia, one for pseudotumour and one for seizures). No control subject had a fever above 38.5 °C.

For samples with available data, glucose was normal, protein was slightly elevated in one sample, and cultures were negative. White blood and red blood cell counts were normal in all samples. Demographic data is available in table 1.

CSF sampling

All CSF samples were collected in the morning by lumbar puncture in the L4/L5 interspace, after an age-appropriate overnight fast. The lumbar puncture was done under anaesthesia and concurrent with MRI and ABR testing. CSF was collected in a polystyrene tube, and immediately transported to a local laboratory where it was aliquoted into polypropylene tubes. Samples were frozen on dry ice and stored at -80 °C prior to assay. Samples were coded prior to sending to the Clinical Neurochemistry Laboratory in Mölndal, Sweden.

CSF biomarkers of amyloid metabolism and neuronal cell damage

CSF levels of A β ₁₋₄₂, the axonal damage marker T-tau and tau phosphorylated at threonine 181 (P-tau) were determined using xMAP technology, as previously described.^{43, 44} CSF sAPP- α and sAPP- β levels were determined using the MSD® sAPP α /sAPP β Multiplex Assay as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA).⁴⁵ This assay employs the 6E10 antibody to capture sAPP- α and a neoepitope-specific antibody to capture sAPP- β . Both isoforms are detected by SULFO-TAG™-labeled anti-APP antibody p2-1. CSF A β _{x-38}, A β _{x-40} and A β _{x-42} were measured using the MSD® Human/Rodent (4G8) Abeta Triplex Assay as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA). This assay employs C-terminal specific antibodies to specifically capture A β _{x-38}, A β _{x-40} and A β _{x-42}. All isoforms are detected by SULFO-TAG™-labeled 4G8 detection antibody. Intra-assay CVs were <5% for all analyses and kits, except for one kit of A β ₃₈ (CV

11.7%), one kit of sAPP- β (CV 10.9%) and one kit of P-tau (CV 5.13%). A β_{42} measured by MSD correlated to A β_{1-42} measured by Luminex in the total study population ($R=0.938$, $P<0.001$) and in the sub-groups of patients ($R=0.898$, $P<0.001$) and controls ($R=0.933$, $P<0.001$). When not otherwise stated, results for A β_{1-42} were similar to those for A β_{42} . All biochemical analyses were performed at the Clinical Neurochemistry Laboratory in Mölndal, Sweden, by experienced and certified laboratory technicians who were blinded to diagnoses and clinical data. Two internal control samples (aliquots of pooled CSF) were run on each plate, and strict acceptance criteria were used for approval of each assay.

Statistical analyses

Statistical calculations were performed using SPSS 17.0 (SPSS Inc, Chicago, USA). As the distribution of quantitative measures was significantly skewed as determined by the Shapiro-Wilk test of normality, statistical tests involving these variables were conducted using the non-parametric Kruskal-Wallis test for multiple comparisons followed by Mann-Whitney U test for pair-wise comparisons. Chi-square statistics with Fisher's exact test were used for group comparisons of dichotomized data. Wilcoxon Signed Ranks test was used for pair-wise comparisons of two related samples of quantitative data. The Spearman correlation coefficient was used for analyses of correlation between variable levels in different study groups. Quantitative variables are presented as median (range). The significance level threshold was set to $P<0.05$.

Results

Biomarkers in NPC patients and controls

NPC patients had significantly higher A β 38, A β 40 and A β 42 than controls (Fig 1, panel A-C). Also, ratios of A β 42:A β 40 and A β 42:A β 38 were higher in NPC (Fig 1, panel D-E). T-tau was higher in NPC patients, but the groups did not differ in P-tau (Table 2). Controls and NPC patients had similar levels of sAPP- α and sAPP- β . Correlations between A β 38, A β 40, A β 42, sAPP- α , sAPP- β and T-tau were seen in controls and NPC (Table 3). The only consistent difference in correlations between controls and patients was for P-tau, where correlations with other biomarkers were seen only in patients. T-tau and P-tau correlated inversely with disease duration ($R=-0.509$, $P=0.001$; $R=-0.619$, $P<0.001$) and age ($R=-0.461$, $P=0.004$; $R=-0.540$, $P<0.001$) in patients. No *APOE*-dependent differences were seen on CSF biomarker levels in patients (*APOE* $\epsilon 4/\epsilon 3$, $N=5$; *APOE* $\epsilon 2/\epsilon 3$, $N=2$; *APOE* $\epsilon 3/\epsilon 3$, $N=26$).

Miglustat treatment

At baseline, 18 patients were on off-label miglustat treatment (age 7.9 [2.9-17.2] years). These had significantly lower A β 1-42 (243 [106-351] vs. 277 [172-373] ng/L, $P=0.048$) and T-tau (170 [51-627] vs. 348 [59-1271] ng/L, $P=0.033$) than the 20 patients without treatment (age 9.1 [1.9-51.3] years, $P=0.76$ vs. treated patients). Also, sAPP- α and sAPP- β were lower in treated patients (326 [164-801] vs. 502 [158-971] ng/mL, $P=0.015$; 81 [34-264] vs. 140 [33-285] ng/mL, $P=0.028$).

For five patients we had serial values where miglustat therapy was initiated within one week after baseline samples were collected. There was a significant drop in T-tau (955 [338-1271] vs. 382 [187-736] ng/L, $P=0.043$) at the following CSF sampling (median time between samplings 357 days [196-633]), but no changes in amyloid biomarkers. No changes in biomarker levels were seen for patients with consecutive samplings without preceding start of treatment ($N=6$, median time between samplings 284 days [183-644], $P=0.522$ vs. patients

with start of treatment, figure 2). Patients put on treatment during the study tended to be younger than patients with consecutive samplings without preceding start of treatment (6.08 [3.75-11.42] vs. 19.17 [5.5-51.25] years) but the difference was not statistically significant ($P=0.052$).

Biomarkers and clinical severity

We compared baseline biomarker levels in patients with high and low disease severity score. Patients with high score (above the median value 13.5, $N=19$) had lower $A\beta_{38}$ (318 [48-1873] vs. 500 [202-1370] ng/L, $P=0.027$), $A\beta_{40}$ (4221 [1208-9719] vs. 5495 [2955-10637] ng/L, $P=0.018$), $A\beta_{42}$ (307 [78-1122] ng/L vs. 487 [231-1045] ng/L, $P=0.012$) and sAPP- β (109 [33-265] vs. 151 [68-285] ng/mL, $P=0.027$, see supplementary figure 1). When subgrouping by miglustat treatment, differences were seen only in untreated patients ($A\beta_{38}$: 338 [129-1873] vs. 573 [321-1370] ng/L, $P=0.016$; $A\beta_{40}$: 4105 [2312-9719] vs. 6256 [4698-10637] ng/L, $P=0.004$; $A\beta_{42}$: 314 [152-1122] vs. 535 [311-1045] ng/L, $P=0.007$; P-tau: 22 [10-30] vs. 31 [21-54] ng/L, $P=0.004$).

Discussion

Previous data on altered lipid homeostasis and amyloid processing are contradictory. Clinical correlations between hypercholesterolemia and AD are difficult to interpret, since cholesterol does not cross the blood-brain-barrier and nearly all brain cholesterol is synthesized *in situ*.⁴⁶ Here we present the first evidence of increased $A\beta$ release to CSF in patients with altered neuronal lipid homeostasis. The increased $A\beta$ release was most likely caused by increased neuronal γ -secretase activity. APP is cleaved by either presenilin 1 or presenilin 2 of the γ -

secretase enzyme complex, encoded by the genes *PSEN1* and *PSEN2*, respectively.⁴⁷ Experimental studies have located γ -secretase activity to late secretory, endosomal and synaptic pathways.⁴⁸ Due to its ability to cut APP at different positions, γ -secretase may yield A β peptides ranging in length from 37 to 49 amino acid residues, and recent data suggests even shorter γ -secretase dependent isoforms.⁴⁹ The influence of lipids on γ -secretase activity is complex. It has been proposed that the cleavage specificity might be influenced by membrane thickness,⁵⁰ and different lipid species have different effects on γ -secretase activity *in vitro*.⁵¹ In the present study, NPC patients had increased A β 42:A β 40 and A β 42:A β 38 ratios, demonstrating that there was not only an increase in γ -secretase activity, but also a shift of the cleavage site activity towards production of A β 42.

Significant data support that BACE1 is the main β -secretase.^{52, 53} BACE1 is a transmembrane protease with a low pH optimum found in the acidic intracellular endosomes and trans-golgi. Upon maturation, BACE1 is S-palmitoylated on residues located at the junction of the transmembrane and cytosolic domains, facilitating targeting to lipid rafts^{54, 55} which might enhance BACE1 processing of APP.^{56, 57} However, one recent study showed that non-palmitoylated BACE1 was equally efficient in APP processing.¹³ We found similar levels of sAPP- β in NPC patients and controls, arguing against increased activity of β -secretase. Likewise, decreased activity of α -secretase was unlikely, since levels of sAPP- α were similar in patients and controls. Finally, the normal sAPP-levels argue against increased production or intra-cellular transport of APP in NPC, but protein expression studies would elucidate this further.

In autopsy studies, only NPC patients homozygous for *APOE* ϵ 4 showed amyloid plaques, and these were diffuse, while dense core plaques were absent.^{36, 37} This lack of AD-type

dense core A β plaques is surprising considering the increased production of A β 42. NPC patients in autopsy studies might be too young to present dense core plaque pathology,^{36, 58} but patients up to 40 years of age have been examined, corresponding well to age at autopsy in familial AD where dense core plaques are readily detected.⁵⁹⁻⁶³ In this study, *APOE* genotype did not affect biomarker levels, although only five patients carried the *APOE* ϵ 4 allele and these individuals were quite young (age 3.3-12.7 years). Amyloid markers did not correlate with disease duration or age, arguing against amyloid accumulation even in later stages. If NPC patients indeed do not aggregate A β , increased A β 42 production might be insufficient for plaque formation. Since the relative occurrence of different A β isoforms affects fibrillation, more detailed investigations of amyloid peptides in NPC are needed.⁶⁴⁻⁶⁶ The role of A β in NPC neurodegeneration is unclear. Experiments have shown intracellular A β accumulation in NPC endosomes³⁹ and such accumulation might be toxic.^{67, 68} Patients on miglustat treatment at baseline had slightly lower CSF A β , but treatment initiation during the study did not affect amyloid biomarker levels. Correlations between amyloid markers and the clinical disease severity score suggest that CSF A β may be used to evaluate disease activity. It is not clear why more severely affected patients had lower CSF A β , but advanced neurodegeneration could hypothetically compromise the ability to release A β , since synaptic activity is required for A β release.⁶⁹ Extended longitudinal studies are needed to clarify this. Trials with amyloid-targeting drugs may unveil the precise role of amyloid in NPC neurodegeneration.

Since this study was designed as an observational study, miglustat therapy was not formally randomized. Given the limited number of patients for whom pre- and post-therapy values are available, caution is warranted. However, miglustat therapy resulted in a decrease of CSF T-tau, most likely representing a dynamic decrease in axonal degeneration.⁷⁰ Similarly, CSF T-

tau was reduced in antibody responders in the AN1792 AD trial with immunization against A β , interpreted as a possible reduction of cellular degeneration.⁷¹ Thus, CSF T-tau is a promising biomarker for treatment effects on axonal degeneration, exemplifying the use of CSF biomarkers in small pilot-studies for detection of biochemical drug effects within the central nervous system.

Contributors

NM, KB, HZ and FP designed the study. SB, NY and FP established the clinical protocol, managed patients and collected samples. RF performed genotyping. NM and KB analyzed the data and performed the statistical analysis. NM, KB, HZ and JM interpreted the data. NM wrote the manuscript. All authors revised the manuscript.

Conflicts of interest

HZ has participated in an advisory board for GlaxoSmithKline. KB has participated in an advisory board for Innogenetics. The other authors have no disclosures.

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Figure legends

Fig 1. CSF levels of A β 38, A β 40 and A β 42 (panel A-C), and ratios of A β 42 to A β 40 and A β 38 (panel D-E) in controls and NPC patients.

Fig 2. Changes in levels of T-tau (panel A) and A β 42 (panel B) between two consecutive CSF samplings in patients where miglustat treatment was initiated in-between samplings, and patients without treatment.

Supplementary figures legends

Supp fig 1. CSF levels of A β 38, A β 40, A β 42 and sAPP- β in NPC patients with disease severity score over and under the median value 13.5.

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Table 1 Demographics

Parameter	Controls (N=14)	NPC (N=38)	P
Age (years)	8.9 (1.4-20.3)	7.9 (1.9-51.3)	0.665
Women	9 (64%)	20 (53%)	0.539
Age at first symptom (years)	NA	0.85 (0-39)	NA
Duration of NPC (years)	NA	6.84 (1.55-24.08)	NA
Disease severity score	NA	13.5 (1-40)	NA

Data presented as median (range). NPC, Niemann-Pick type C disease.

Table 2 CSF parameters in controls and NPC

CSF parameter	Controls (N=14)	NPC (N=38)	P
sAPP- α (ng/mL)	360 (160-653)	432 (158-971)	0.27
sAPP- β (ng/mL)	99 (34-209)	120 (33-285)	0.38
T-tau (ng/L)	79 (23-186)	234 (51-1271)	<0.001
P-tau (ng/L)	24 (7-50)	26 (9-54)	0.40

Data presented as median (range). CSF, cerebrospinal fluid; NPC, Niemann-Pick type C

disease; sAPP, soluble amyloid precursor protein; T-tau, total tau; P-tau, phosphorylated tau.

Table 3 Correlations between CSF parameters in controls and NPC

Correlation	Controls	NPC
A β 38 vs A β 40	R=0.935, P<0.001	R=0.974, P<0.001
A β 38 vs A β 42	R=0.849, P<0.001	R=0.976, P<0.001
A β 40 vs A β 42	R=0.934, P<0.001	R=0.977, P<0.001
A β 38 vs sAPP- α	R=0.653, P=0.011	R=0.760, P<0.001
A β 38 vs sAPP- β	R=0.598, P=0.024	R=0.803, P<0.001
A β 40 vs sAPP- α	R=0.569, P=0.034	R=0.712, P<0.001
A β 40 vs sAPP- β	R=0.503, P=0.067	R=0.768, P<0.001
A β 42 vs sAPP- α	R=0.429, P=0.126	R=0.737, P<0.001
A β 42 vs sAPP- β	R=0.393, P=0.164	R=0.794, P<0.001
T-tau vs A β 38	R=0.713, P=0.004	R=0.416, P=0.009
T-tau vs A β 40	R=0.723, P=0.003	R=0.521, P=0.001
T-tau vs A β 42	R=0.745, P=0.002	R=0.474, P=0.003
T-tau vs sAPP- α	R=0.319, P=0.267	R=0.209, P=0.209
T-tau vs sAPP- β	R=0.226, P=0.436	R=0.281, P=0.087
P-tau vs A β 38	R=0.215, P=0.460	R=0.528, P=0.001
P-tau vs A β 40	R=0.117, P=0.691	R=0.589, P=0.001
P-tau vs A β 42	R=-0.007, P=0.982	R=0.578, P=0.001
P-tau vs sAPP- α	R=0.491, P=0.074	R=0.155, P=0.353
P-tau vs sAPP- β	R=0.306, P=0.287	R=0.194, P=0.243
P-tau vs T-tau	R=0.324, P=0.259	R=0.616, P<0.001

Data presented as Spearman's correlation coefficients, with significance levels. CSF,

cerebrospinal fluid; NPC, Niemann-Pick type C disease; sAPP, soluble amyloid precursor

protein; T-tau, total tau; P-tau, phosphorylated tau.

Figure 1

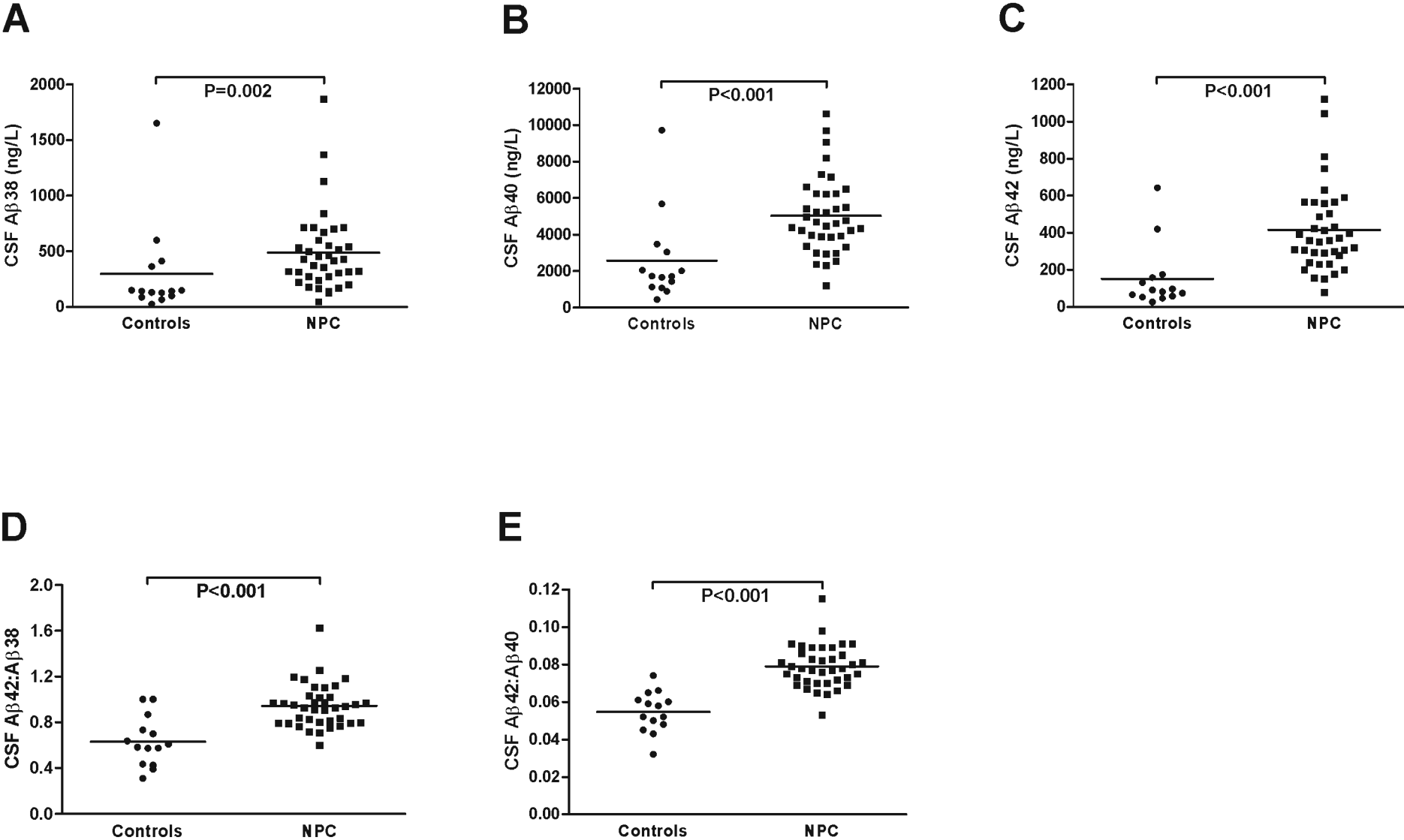
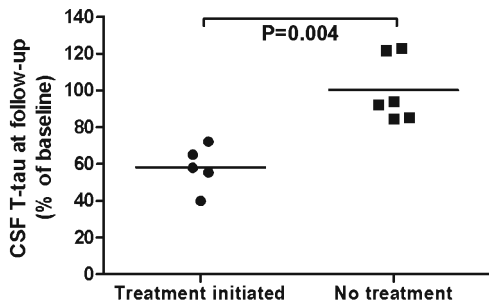
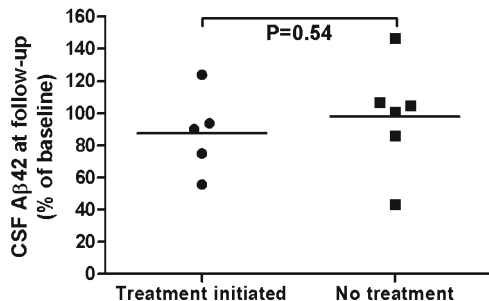


Figure 2

A



B



Cholesterol depletion inhibits the generation of β -amyloid in hippocampal neurons

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ABSTRACT The amyloid precursor protein (APP) plays a crucial role in the pathogenesis of Alzheimer's disease. During intracellular transport APP undergoes a series of proteolytic cleavages that lead to the release either of an amyloidogenic fragment called β -amyloid ($A\beta$) or of a nonamyloidogenic secreted form consisting of the ectodomain of APP (APP_{sec}). It is $A\beta$ that accumulates in the brain lesions that are thought to cause the disease. By reducing the cellular cholesterol level of living hippocampal neurons by 70% with lovastatin and methyl- β -cyclodextrin, we show that the formation of $A\beta$ is completely inhibited while the generation of APP_{sec} is unperturbed. This inhibition of $A\beta$ formation is accompanied by increased solubility in the detergent Triton X-100 and is fully reversible by the readdition of cholesterol to previously depleted cells. Our results show that cholesterol is required for $A\beta$ formation to occur and imply a link between cholesterol, $A\beta$, and Alzheimer's disease.

The amyloid precursor protein (APP) is a transmembrane protein containing a large N-terminal ectodomain and a small C-terminal cytoplasmic domain (1). Although its physiological function remains unknown, several findings suggest a crucial role for APP in the pathogenesis of Alzheimer's disease (2, 3). During intracellular transport APP undergoes a series of proteolytic cleavages by as yet unidentified proteases, which lead to the release either of an amyloidogenic fragment called β -amyloid ($A\beta$) or of a nonamyloidogenic secreted form consisting of the ectodomain of APP (APP_{sec}) (4). $A\beta$ generation occurs in two steps (5). The first cleavage occurring in the luminal domain of APP (β -cleavage) generates a 10-kDa fragment that is further cleaved within the transmembrane domain (γ -cleavage) to produce $A\beta$. The generation of APP_{sec} by α -cleavage in the luminal domain leaves an 8-kDa transmembrane fragment in the cell membrane, which subsequently is a substrate for γ -cleavage producing nonamyloidogenic p3. It is $A\beta$ that accumulates in the brain lesions that are thought to cause the disease (5).

Cholesterol metabolism and Alzheimer's disease are genetically linked. The *apoE4* allele of the apolipoprotein E gene is associated with higher cholesterol levels (6) and has been shown to increase the risk of developing the disease (7). Also, atherosclerosis for which hypercholesterolemia is considered a risk factor is associated with Alzheimer's disease (8). These genetic links between cholesterol metabolism and Alzheimer's disease lead us to address the question whether APP processing is cholesterol dependent. Another possible link between cholesterol and APP processing is the finding that a fraction of APP is Triton X-100 insoluble in neurons and present in a

low-density membrane fraction consisting of sphingolipid-cholesterol rafts (9). It could be this pool of APP that would be susceptible to β -cleavage generating $A\beta$. If this were the case then cholesterol depletion could affect generation of $A\beta$. To study the influence of cholesterol on APP metabolism we used primary cultures of rat hippocampal neurons infected with recombinant Semliki Forest virus (SFV) carrying APP (10). This system has been used successfully to study the intracellular transport and processing of human APP (11–13). Infection of mature and polarized neurons with recombinant SFV allows expression of relatively high amounts of APP without disturbing the polar organization and the viability of the cells within the time course of the experimental window chosen (3–7 hr after infection).

Cholesterol depletion of the cultured neurons was achieved by a combination of lovastatin treatment and methyl- β -cyclodextrin extraction. Lovastatin in the presence of low amounts of mevalonate prevents the new synthesis of cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (14). β -Cyclodextrins have been shown to selectively and rapidly extract cholesterol from the plasma membrane, in preference to other lipids (15–17). By combining lovastatin and methyl- β -cyclodextrin we previously have shown that total cellular cholesterol levels can be reduced by 60–70% without significantly affecting cell viability and integrity (18). Under these conditions we observed a dramatic reduction of transport of influenza virus hemagglutinin from the trans-Golgi network to the cell surface of nonpolarized baby hamster kidney cells as well as to the apical surface of polarized Madin–Darby canine kidney cells. The transport of the vesicular stomatitis virus glycoprotein to the basolateral surface was not affected under these conditions. These data provided us with direct evidence for the functional significance of cholesterol and sphingolipid-cholesterol rafts as sorting platforms for inclusion of protein cargo destined for delivery to the apical membrane (9).

We now have applied the cholesterol depletion conditions previously established for baby hamster kidney and Madin–Darby canine kidney cells to hippocampal neurons, and we show that these conditions lead to a 70% reduction of cellular cholesterol levels while not affecting neuronal viability. Under these conditions $A\beta$ formation is inhibited while the generation of APP_{sec} is unperturbed. Our results show that cholesterol is required for $A\beta$ formation to occur and imply a link between cholesterol, $A\beta$, and Alzheimer's disease.

MATERIALS AND METHODS

Cell Culture. Hippocampal neurons were prepared from 18-day-old fetal rats as described (19). After the hippocampi

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Abbreviations: APP, amyloid precursor protein; $A\beta$, β -amyloid fragment of APP; APP_{sec} , nonamyloidogenic secreted form consisting of the ectodomain of APP; DIGs, detergent-insoluble low-density glycosphingolipid-enriched complexes; SFV, Semliki Forest virus.

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were dissected and dissociated, the cells were plated on either polylysine-coated dishes or on glass coverslips and kept in MEM supplemented with 10% horse serum. The cells were maintained under serum-free conditions in MEM with N2-supplement (maintenance medium) at 5% CO₂ and 36.5°C. The proliferation of nonneuronal cells was prevented by adding 5 mM cytosine arabinonucleoside. After 5–7 days in culture, 4 μ M lovastatin (MSD Sharp & Dohme, Haar, Germany) and 0.25 mM mevalonate (Sigma) were added for 4 days. Control cells were left untreated.

Antibodies. Polyclonal antibodies used were antibody FdAPP directed against APP695 (20), antibody B12/4 directed against the 20 C-terminal amino acids of APP (12), and antibody B7/6 recognizing amino acids 1–16 of human A β synthetic peptide 1–40 (12).

Cholesterol Depletion and Metabolic Labeling of Infected Neurons. Recombinant SFV encoding human APP695 was prepared as previously described (10, 21). Neurons were infected for 1 hr at 37°C and 5% CO₂ with recombinant SFV. The virus solution was replaced by maintenance medium containing lovastatin/mevalonate, and the cells were incubated for an additional 2 hr. After a treatment for 5–20 min with 5 mM methyl- β -cyclodextrin (Sigma) in methionine-free labeling medium (MEM with 1/10 N2-supplement), the cells were labeled for 2.5 hr with 150 μ Ci of [³⁵S]methionine (Amersham; 1 Ci = 37 GBq). Control cells were processed in parallel in the absence of lovastatin/mevalonate and methyl- β -cyclodextrin.

Cholesterol–Methyl- β -Cyclodextrin Inclusion Complexes. Cholesterol–methyl- β -cyclodextrin inclusion complexes were prepared as described (22). These complexes containing 0.3 mM complexed cholesterol were added together with 2 μ g/ml free cholesterol for 15 min to neurons grown, infected, and treated with methyl- β -cyclodextrin as described above.

Flotation of Triton X-100 Extracts. Lovastatin-treated neurons grown, infected, and treated with methyl- β -cyclodextrin as described above were pulse-labeled for 20 min and then chased or not for 100 min in maintenance medium. The cells were extracted for 30 min on ice with 1% Triton X-100 in TEX (150 mM NaCl/50 mM Tris-HCl, pH 7.4/2 mM EDTA/2 mM DTT containing 25 μ g/ml each of chymostatin, leupeptin, antipain, and pepstatin A). The extracts then were mixed with an equal volume of OptiPrep (Nycomed, Oslo), and overlaid in a TLS 55 centrifugation tube (Beckman) with a step gradient of 30%, 25%, and 3% OptiPrep in TEX. After a centrifugation for 3 hr at 4°C and 50,000 rpm in a TL-100 ultracentrifuge (Beckman), fractions were collected and APP was immunoprecipitated.

Immunoprecipitation and Quantitation. After metabolic labeling the culture medium was collected and cell extracts were prepared in 2% Nonidet P-40/0.2% SDS/5 mM EDTA, supplemented with protease inhibitors. Immunoprecipitates were recovered on protein A-Sepharose (Boehringer Mannheim), and were analyzed on 10–20% Tris-Tricine polyacrylamide gels (NOVEX, San Diego). Radioactivity in the individual bands was determined by using a PhosphorImager (Molecular Dynamics).

Quantitation of Cholesterol Depletion. [1 α ,2 α (N)-³H]Cholesterol (6.25 μ Ci; Amersham) was added for 8 hr to neurons grown on glass coverslips in the presence of lovastatin/mevalonate. After an equilibration for 16 hr with the cellular cholesterol pool, the cells were treated for 20 min with 5 mM methyl- β -cyclodextrin. [³H]Cholesterol released into the medium and remaining in the cells, respectively, was determined by scintillation counting. Control cells were processed in parallel in the absence of lovastatin/mevalonate and methyl- β -cyclodextrin.

Staining of Hippocampal Neurons with Filipin. Neurons grown on glass coverslips in the presence of lovastatin/mevalonate were treated for 20 min with 5 mM methyl- β -

cyclodextrin. After fixation on ice with 4% paraformaldehyde, they were stained with 125 μ g/ml filipin (Sigma) in PBS. Digital images were taken with a Zeiss Axioskop microscope equipped with a three-chip color camera (Photonic Sciences, Millham, U.K.).

RESULTS AND DISCUSSION

Cholesterol depletion of the cultured neurons was achieved by a combination of lovastatin treatment and methyl- β -cyclodextrin extraction as described (18). Lovastatin in the presence of low amounts of mevalonate inhibits cholesterol biosynthesis (14). Methyl- β -cyclodextrin specifically removes cellular cholesterol (15–17). We measured the extent of cholesterol removal using filipin, a fluorescent polyene antibiotic that forms complexes with cholesterol that can be visualized by fluorescence microscopy (23). After 4 days of treatment with 4 μ M lovastatin, followed by extraction of the cells for 20 min with 5 mM methyl- β -cyclodextrin a dramatic reduction of the filipin staining as compared with control cells was seen (Fig. 1). Lovastatin treatment alone did not lead to a significant decrease of staining. However, lovastatin and methyl- β -cyclodextrin treated neurons labeled with [³H]cholesterol were depleted of \approx 70% of the cellular [³H]cholesterol as compared with control cells.

We used these conditions for the subsequent experiments. Prolonging the lovastatin treatment or increasing the concentration of methyl- β -cyclodextrin or the length of treatment started to affect the viability of the neuronal cultures.

To monitor the amount of APP_{sec} being secreted in cholesterol-depleted cells, hippocampal neurons, treated for 4 days with lovastatin, were infected for 1 hr with SFV carrying the

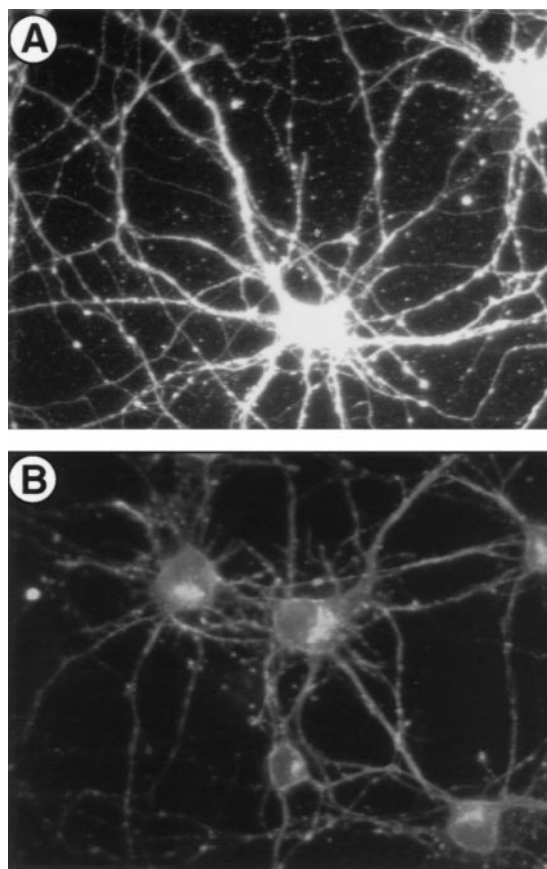


FIG. 1. Staining of cholesterol-depleted hippocampal neurons with filipin. (A) Untreated neurons stained with filipin. (B) Neurons stained with filipin after treatment for 4 days with lovastatin/mevalonate and for 20 min with 5 mM methyl- β -cyclodextrin.

gene for human APP. Two hr after infection, methyl- β -cyclodextrin was added for 20 min and the cells were subsequently labeled for 2.5 hr with [35 S]methionine. APP was immunoprecipitated from cell lysates and from the culture media using a polyclonal anti-APP antibody. A prominent 115-kDa band (full-length APP) was revealed by SDS/PAGE in similar amounts in both cholesterol-depleted and in control cells (Fig. 2). The immunoprecipitation demonstrated that a 110-kDa fragment (APP_{sec}) was secreted by the neurons and that the amount secreted was not affected by cholesterol depletion (Fig. 2) as quantified by PhosphorImager analysis. The ratio of full-length APP to APP_{sec} being 7.4 ± 4.2 and 5.9 ± 1.6 in cholesterol-depleted and control cells (mean of 10 experiments), respectively. These data indicate that the generation of APP_{sec} that is the main processed form of APP in neurons and other cells (5) is not significantly affected by reduction of cellular cholesterol levels.

Next we compared A β secretion in the same cholesterol-depleted and control neurons that we studied for APP_{sec} formation. A striking inhibition of A β generation was seen (Fig. 3A). Even a shorter treatment with methyl- β -cyclodextrin (5 min) was sufficient to reduce A β secretion. The lovastatin treatment protocol alone did not cause a significant difference in A β secretion, suggesting that fairly large amounts of cholesterol need to be removed before effects on A β secretion can be observed. This is in agreement with the observation that the same lovastatin treatment protocol reduced the total cellular cholesterol levels by only $\approx 10\%$ in baby hamster kidney cells and had no effect on biosynthetic transport in these cells (18).

To demonstrate that the inhibition of the production of A β was due to cholesterol depletion and not to any other effects of methyl- β -cyclodextrin we performed two control experiments (Figs. 3B and C). First we used a cholesterol-methyl- β -cyclodextrin complex instead of methyl- β -cyclodextrin alone. Under these conditions there was no reduction of A β formation. Second, we attempted to replete the cholesterol-depleted cells with cholesterol by adding back the cholesterol-methyl- β -cyclodextrin complex to the neurons after the 20 min extraction of cholesterol by methyl- β -cyclodextrin. The repletion incubation was for 15 min. Under these conditions the production of A β was fully restored. These data convincingly demonstrate that cholesterol depletion by a combination of lovastatin and methyl- β -cyclodextrin causes inhibition of A β secretion in hippocampal neurons. On the other hand, cholesterol depletion of COS cells, stably transfected with human APP695 did not lead to a significant decrease of A β production (data not shown).

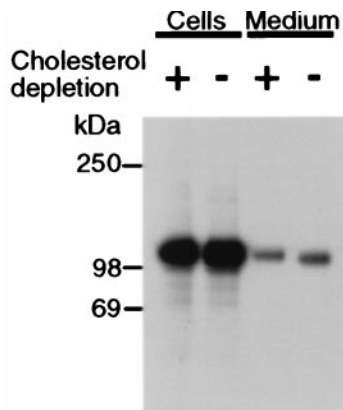


FIG. 2. Cholesterol depletion does not change APP biosynthesis and secretion. Hippocampal neurons were grown for 4 days in the presence (+) or absence (–) of lovastatin/mevalonate. After infection with SFV/APP, cells were treated (+) or not (–) for 20 min with 5 mM methyl- β -cyclodextrin, and metabolically labeled for 2.5 hr. Cellular and secreted APP were recovered by immunoprecipitation.

Next we analyzed which A β cleavage is inhibited. A β generation occurs in two steps (5). The first cleavage (β -cleavage) generates a 10-kDa fragment from APP that is further cleaved within the transmembrane domain (γ -cleavage) to produce A β . The generation of APP_{sec} by α -cleavage leaves an 8-kDa transmembrane fragment in the cell membrane. When antibodies recognizing the C-terminal end of APP were used to immunoprecipitate APP fragments from the cell lysates of cholesterol-depleted and control neurons a dramatic inhibition of the β -cleavage was detected by SDS/PAGE, while the production of the fragment generated by α -cleavage was unperturbed (Fig. 3D). Under these conditions there was also no change in the production of nonamyloidogenic p3 that is generated by α - and γ -cleavage (data not shown). Thus our combined results suggest that cholesterol-depletion interferes with the β -cleavage but not with α -cleavage that produces APP_{sec} (5). The γ -cleavage is probably inhibited indirectly because it requires prior β -cleavage (24). We conclude that depletion of cholesterol affects amyloidogenic processing while allowing nonamyloidogenic cleavage to proceed.

One reason to believe that APP-processing might be cholesterol-dependent comes from the genetic links between cholesterol metabolism and Alzheimer's disease. The apoE4 allele is associated with higher cholesterol levels (6) and increases the risk of developing the disease (7). Also, atherosclerosis for which hypercholesterolemia is considered a risk factor is associated with Alzheimer's disease (8). All these findings led us to test the hypothesis that A β production might be dependent on the cholesterol level in neurons.

However, the major reason why we initiated these studies was the observation that a fraction of APP in neurons is insoluble in Triton X-100 at 4°C (25). This is a property shared by glycosyl-phosphatidylinositol-anchored proteins and transmembrane proteins associating with sphingolipid-cholesterol rafts (9). These proteins are in detergent-insoluble low-density glycosphingolipid-enriched complexes (DIGs). Recent studies demonstrated that cholesterol plays an essential role in raft assembly (9): cholesterol depletion abolishes the association of proteins with rafts (18, 26, 27). Therefore we wanted to check the relation between APP insolubility in Triton X-100 and cholesterol-depletion. After infection with recombinant SFV carrying APP we extracted cholesterol-depleted and control neurons with detergent. The extract was mixed with an equal volume of OptiPrep and overlaid with 30%, 25%, and 3% OptiPrep. After centrifugation a fraction of the cellular APP was found to float to lower density in the extract from control neurons (Fig. 4). DIG association was greatly reduced in cholesterol-depleted cells. Thus under conditions in which A β formation is inhibited APP does not associate with DIGs. Moreover the pulse-chase experiment (Fig. 4) demonstrated that APP does not associate with DIGs immediately after synthesis, but presumably after the protein has entered the Golgi complex as is the case for glycosyl-phosphatidylinositol-anchored proteins (28) and for influenza virus hemagglutinin (29, 30).

This finding raises the question of how cholesterol depletion affects A β formation. One possibility is that reduction in membrane cholesterol changes the intracellular transport of APP so that the protein does not reach the cellular site(s) where β -cleavage takes place. Influenza virus hemagglutinin that is routed apically in epithelial cells is missorted to the basolateral surface in cholesterol-depleted Madin-Darby canine kidney cells (18). Alternatively, the protease responsible for β -cleavage is active only in intact rafts that depend on cholesterol for integrity. Interestingly, conversion of the glycosyl-phosphatidylinositol-anchored prion protein to the protease-resistant form is reduced by cholesterol depletion (31), suggesting that the raft environment is important for the generation of the disease-causing form of prions. One factor

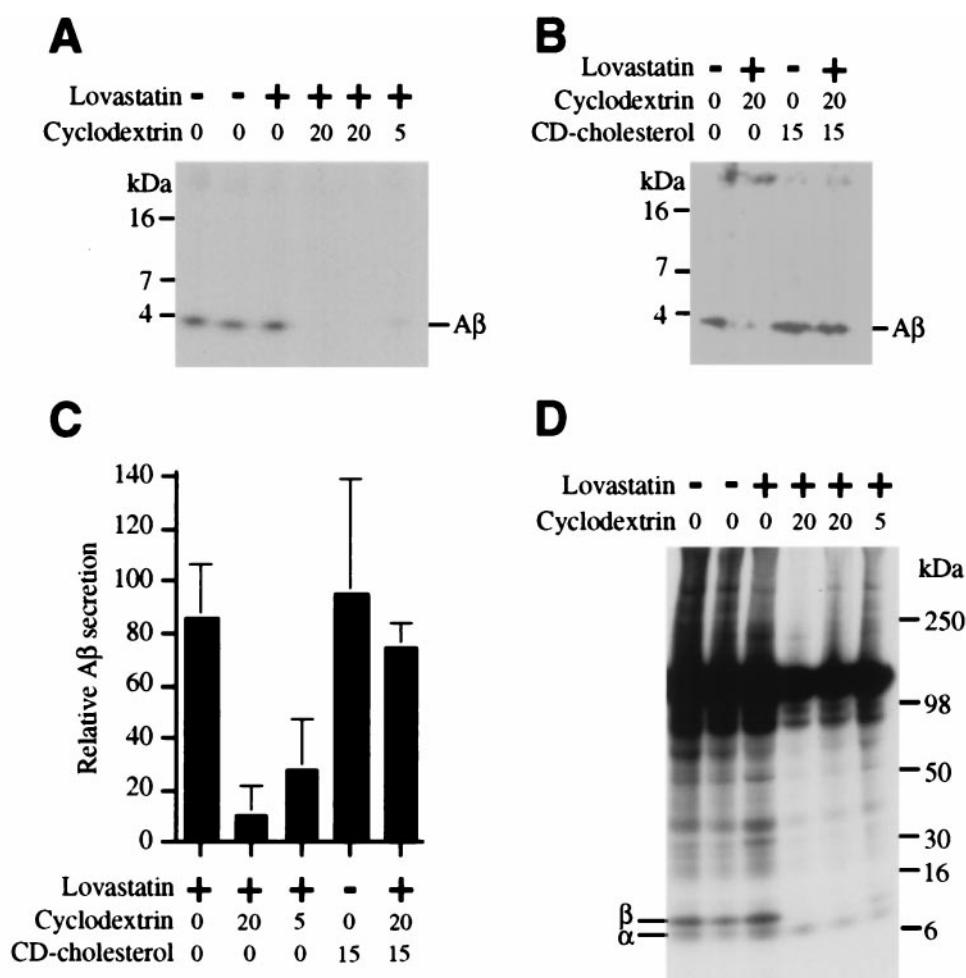


FIG. 3. Cholesterol depletion inhibits production and secretion of Aβ. Hippocampal neurons were grown for 4 days in the presence (+) or absence (–) of lovastatin/mevalonate and after infection with SFV/APP were treated with 5 mM methyl-β-cyclodextrin for the indicated times (min) and metabolically labeled for 2.5 hr. Cholesterol was added back for the indicated times (min) as a cholesterol-methyl-β-cyclodextrin inclusion complex (CD-cholesterol). (A) Aβ secreted from control and cholesterol-depleted cells (immunoprecipitated using antibody B7/6). (B) Aβ secreted from cholesterol-depleted/repleted cells. (C) Aβ secretion in cholesterol-depleted cells as compared with untreated control cells (mean of 3–11 experiments). (D) Immunoprecipitation of APP and its proteolytic fragments from cell homogenates using antibody B12/4. Fragments generated by α- and β-cleavage are indicated.

influencing raft association of APP could be the binding of the Aβ fragment to the raft lipid ganglioside, GM1 (32). These

intriguing relationships raise the hopes of new strategies to influence the progression of Alzheimer's disease.

We thank Bianca Hellias for preparation of hippocampal neurons, Gerd Multhaup for antibodies, and Renate Luedicke from MSD Sharp & Dohme for the gift of lovastatin. This work was supported by the Roche Research Foundation, the Deutsche Forschungsgemeinschaft, and the Training and Mobility of Researchers program of the European Community.

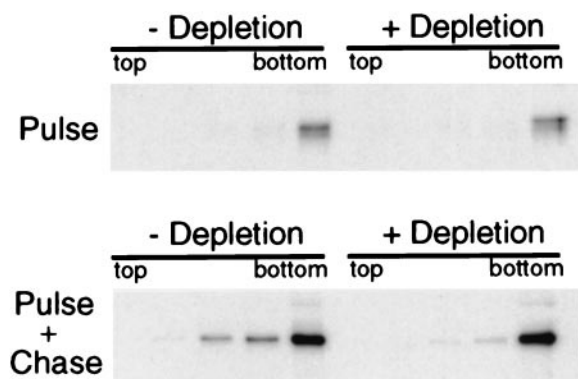


FIG. 4. Cholesterol depletion reduces association of APP with detergent-insoluble low-density DIGs. Hippocampal neurons treated or not for 4 days with lovastatin/mevalonate and 20 min with 5 mM methyl-β-cyclodextrin were extracted on ice with 1% Triton X-100 immediately after pulse labeling for 20 min or after an additional chase for 100 min. After flotation in an OptiPrep step-gradient, APP was immunoprecipitated from the collected fractions.

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2-Hydroxypropyl-β-cyclodextrin does not cross the blood-brain barrier (BBB) in +/- or *Npc1*^{-/-} mice

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Introduction

Cyclodextrins are complex carbohydrates. 2-Hydroxypropyl-[¹⁴C]-propyl-β-cyclodextrin is composed of seven glycosidic residues complexed into a ring structure. Cyclodextrins have the property of being able to form inclusion complexes with smaller molecules within the hydrophobic cavity formed by the sugar ring. (Dodziuk 2006). Cyclodextrins readily complex with the steroid cholesterol and have been demonstrated to deplete intracellular cholesterol from macrophages in tissue culture (Kilsdonk et al. 1995). It was shown by Griffin et al. (2004) that allopregnenolone complexed with CD delayed the onset of neurodegeneration and reduced the storage of GM2 and GM3 gangliosides in *Npc1*^{-/-} mice. A later publication by Davidson et al. (2009) showed that cyclodextrin treatment alone was effective in reducing neurodegeneration and ganglioside storage and prolonged life in *Npc1*^{-/-} mice. It was naturally assumed that CD was able to enter the brain complex with, and remove, cholesterol from brain cells and be cleared from the CNS by bulk flow and clearance of cerebrospinal fluid to blood whence it could be eliminated by the kidney. However other carbohydrates, for which no transporter exists at the blood-brain barrier (BBB), for example sucrose (342.30 Da) and inulin (~6000 Da) remain almost exclusively in the vascular compartment and do not enter brain (Smith 1988). It was therefore thought imperative to investigate the interaction of CD (MW 1300 Da) with the BBB.

Methods

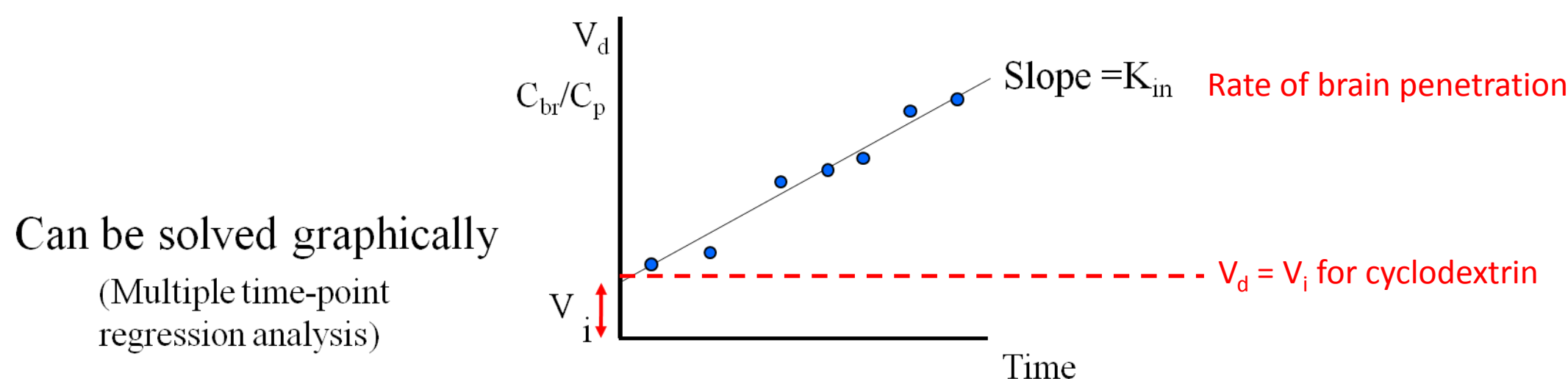
Brain uptake of 2-Hydroxypropyl-[¹⁴C]-propyl-β-cyclodextrin (CD - specific activity 6.95mCi/g) was determined in +/- (BalbC) and *Npc1*^{-/-} mice using two methods. In situ brain perfusion and multi-time-point regression analysis flowing intraperitoneal administration. For in situ brain perfusion a cannula was inserted into the aorta via the left ventricle, the descending aorta tied off and the upper part of the animal perfused at a rate of 10ml/min with a buffered saline solution containing tracer [¹⁴C]-CD at a radioactive concentration of 0.3μCi/ml. After two or four minutes of perfusion the experiment was terminated by decapitation and the brain removed from the skull. Brain regions were then manually dissected out and their contained radioactivity determined by scintillation counting. Under these conditions a volume of distribution in brain (*V_d*) is given by the expression *V_d* = *C_{br}*/*C_p*, where *C_{br}* is the CD radioactive concentration in brain, *C_p* the radioactive concentration of CD in the perfusate. Units are ml/g⁻¹. For multi-time-point regression analysis animals were injected intraperitoneally with 10μCi of CD and the experiment terminated by decapitation after varied times up to 1 hour. The volume of distribution in brain at different experimental times, was then plotted against experimental time (min). The slope of the resultant line gives a unidirectional influx constant (*K_{in}*) and the intercept represents the an instantaneous volume of distribution (*V_i*) or the vascular volume (Begley 1999). All experiments were conducted under general anaesthesia using a mixture of medetomidine HCl 1mg/kg and ketamine HCl 75 mg/kg under the provisions of the Animals Scientific Procedures Act 1986 and Home Office Project Licence 70/6381.

FIGURE 1 Uptake of Solutes by the Brain

(1) Basic equation $\frac{C_{br}(T)}{C_p(T)} = K_{in} \int_0^T C_p(t) dt + V_i$

(2) Simplifies $\frac{C_{br}(T)}{C_p(T)} = K_{in}T + V_i$ When CNS exposure *C_p* is constant

The slope of a plot of *V_d* against *T*, gives the unidirectional influx constant, *K_{in}*. *K_{in}* represents brain uptake with time. The intercept of the plot on the abscissa gives the value *V_i* which represents an instantaneous volume of distribution at zero time. This varies from solute to solute. For an inert tracer such as sucrose, with in situ brain perfusion this value approximates to the total vascular volume (0.04-0.05ml/g), with i.p. or i.v. administration of sucrose this value approximates the plasma volume 0.02-0.025 ml/g. If the *V_i* is substantially greater than these values this would indicate significant binding of tracer at the BBB.



Results

FIGURE 2 Cyclodextrin in-situ brain perfusion in +/- and *Npc1*^{-/-} mice at 6-8 weeks of age

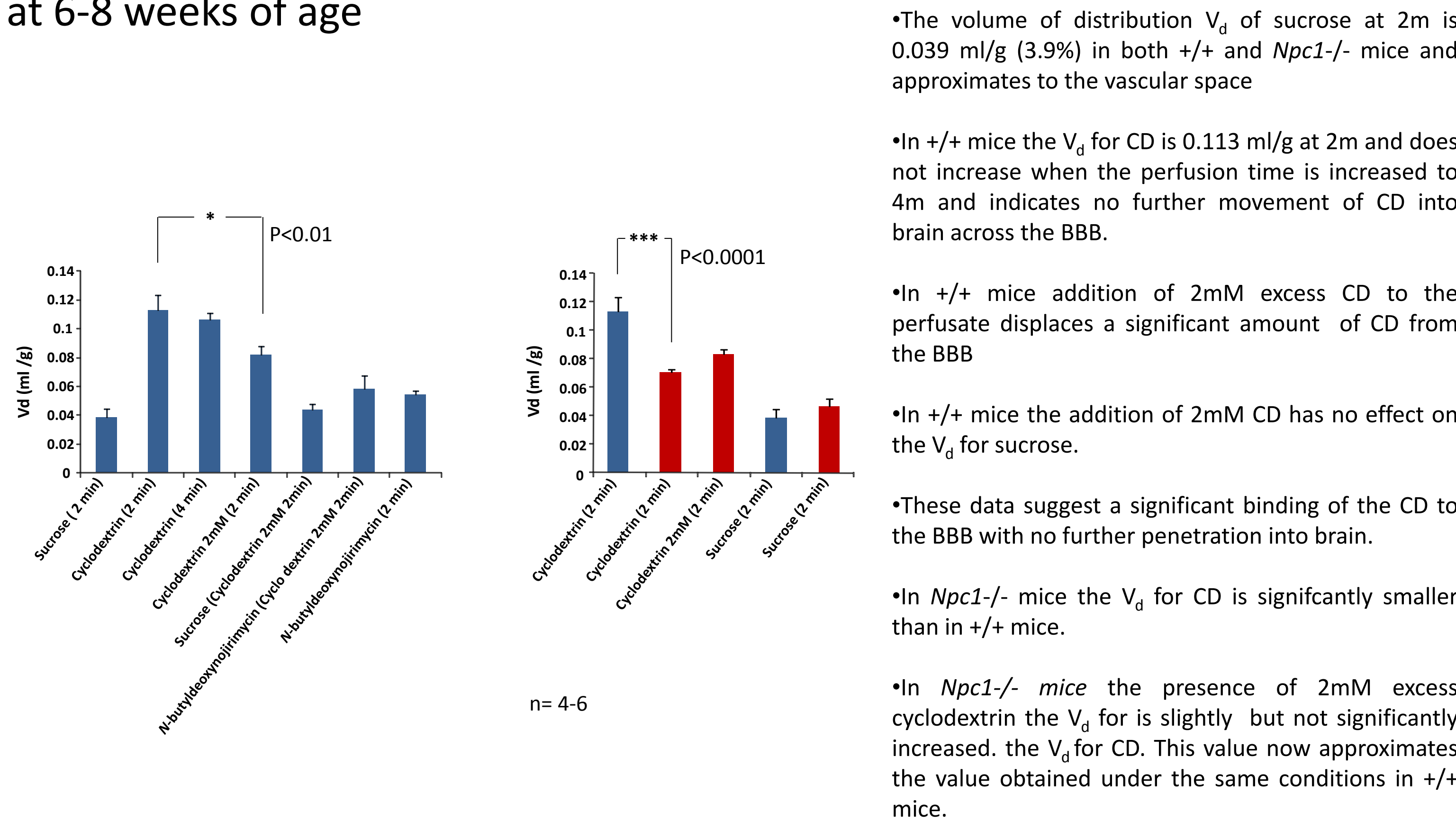


FIGURE 3 Plasma radioactivity of CD after tracer and high dose intraperitoneal administration in 6-8 week mice

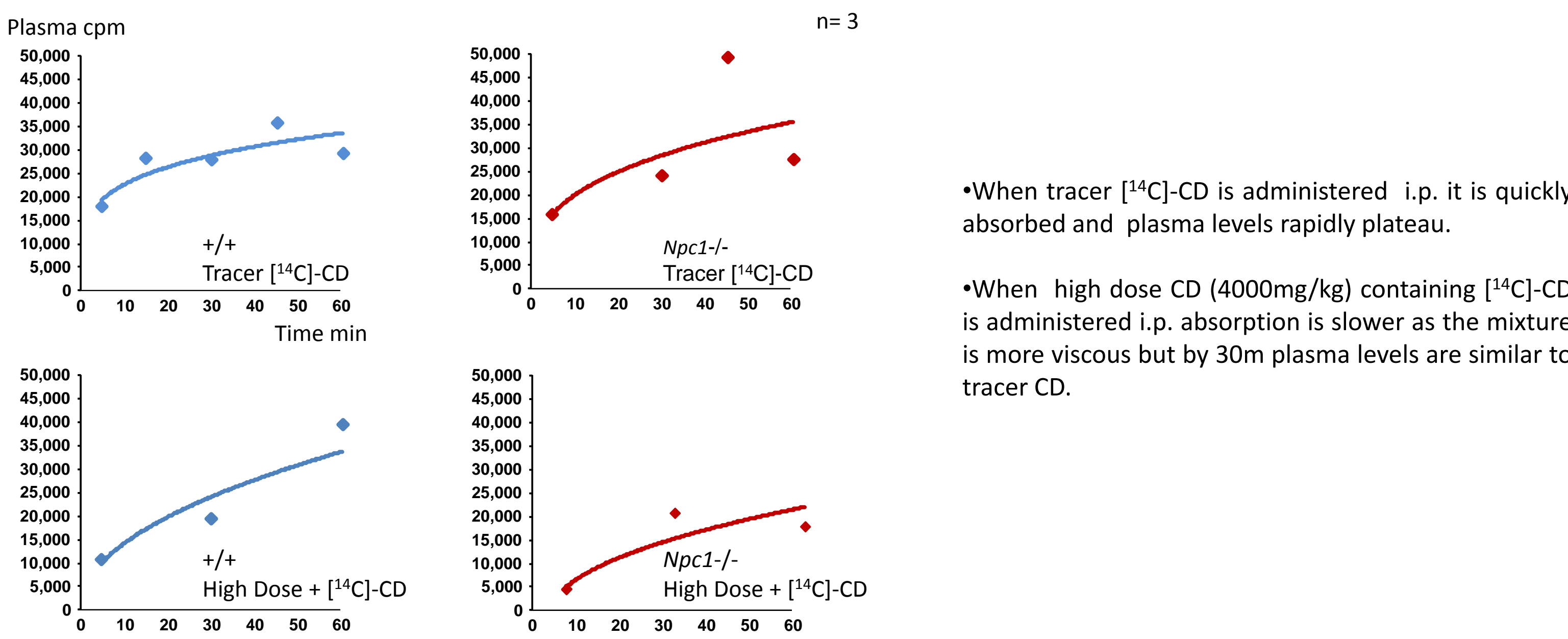
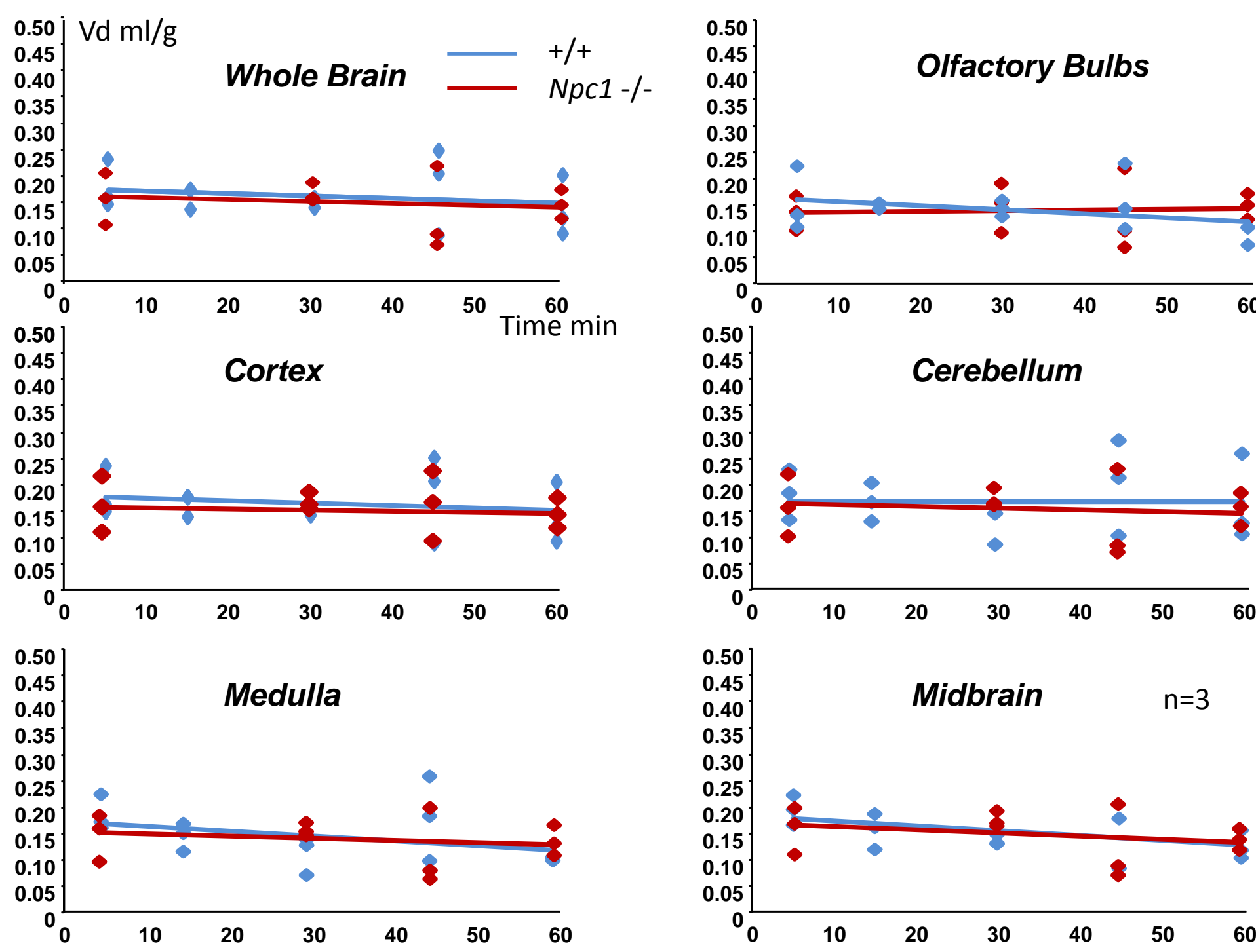


FIGURE 4 Multi-time-point regression plot of tracer [¹⁴C]-cyclodextrin in 6-8 week old mice

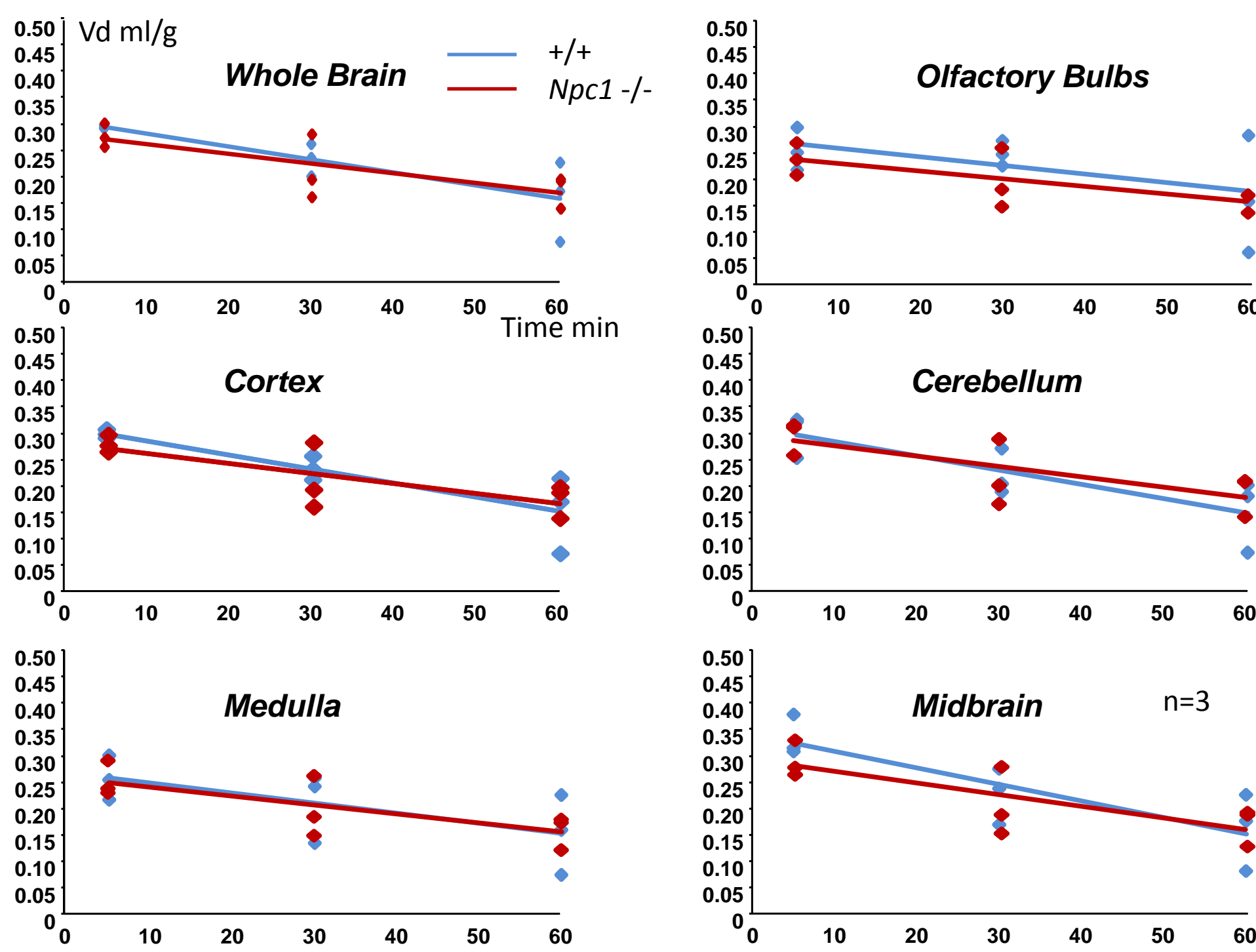


•In whole brain and the regions investigated there is no slope to the plot indicating no penetration with time of CD into the brain.

•The volume of distribution (*V_d*) for CD is very large 0.075–0.300 ml/g indicating a considerable binding of CD to the BBB.

•There is no difference in the data between +/- and *Npc1*^{-/-} mice.

FIGURE 5 Multi-time-point regression plot of high dose [¹⁴C]-cyclodextrin in 6-8 week old mice



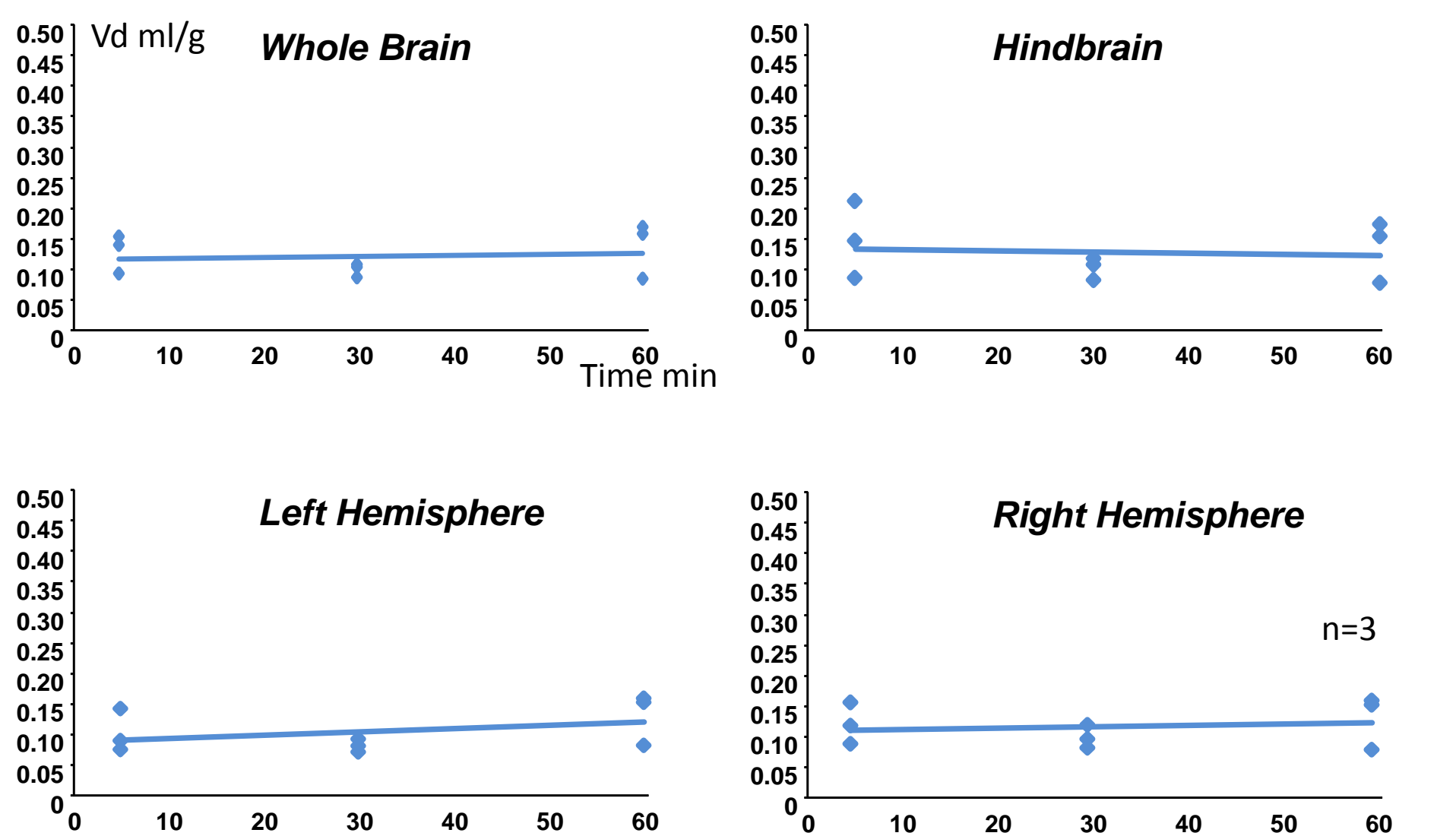
•In all brain regions there is an apparent negative slope to the plot. This is an pharmacokinetic artifact produced by the lower plasma values for CD at the shorter time points.

•The data still indicate no penetration of CD into the brain.

•The data still indicate a high level of binding of CD to the BBB.

•There is no difference in the data between +/- and *Npc1*^{-/-} mice.

FIGURE 6 Multi-time-point regression plot of tracer [¹⁴C]-CD in P7 +/- mice



•In 7 day old mice there is no evidence for brain penetration of CD.

•There is a similarly high binding of CD to the BBB.

•There is no difference in the BBB to CD between the 7day and 6-8 week old +/- animals.

Conclusions

•None of these data collected so far, indicate that CD enters the brain.

•CD does however interact with the BBB in a number of unexpected ways.

•There appears to be a high degree of binding to the BBB possibly an interaction with the plasma membrane glycolyx.

•This binding appears to be lower in the *Npc1*^{-/-} mice.

•With in situ perfusion 2mM CD displaces tracer CD from the BBB in +/- mice but increases tracer binding in *Npc1*^{-/-} mice.

•This may suggest two binding sites, one high affinity low capacity and one low affinity high capacity and that the ratio of binding sites may change in the mutant mice with more low affinity high capacity sites expressed.

•The high binding of CD to the BBB may be associated with its ability to remove cholesterol from the brain.

•The interaction of CD with the BBB needs to be further investigated and the mechanism of how CD can remove cholesterol from the brain without entering the CNS needs elucidation.

•There is good evidence that CD administration in NPC disease reduces neuropathology but the mechanism of action is still unclear.

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2-Hydroxypropyl- β -Cyclodextrin Raises Hearing Threshold in Normal Cats and in Cats With Niemann-Pick Type C Disease

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ABSTRACT: 2-hydroxypropyl- β -cyclodextrin (HP β CD) is a promising experimental therapy for Niemann-Pick type C disease that improved intracellular cholesterol transport, substantially reduced neurodegeneration and hepatic disease, and increased lifespan in *npc1*^{-/-} mice. On the basis of favorable treatment outcome in mice, HP β CD is being evaluated as a therapy in children with Niemann-Pick type C (NPC) disease. We evaluated the efficacy of HP β CD in the feline model of NPC disease and recognized a dose-dependent increase in hearing threshold associated with therapy as determined by brain stem auditory evoked response (BAER) testing. To further assess the effect of HP β CD on hearing threshold, normal cats were administered the drug s.c. at either 4000 mg/kg or 8000 mg/kg body weight, or intrathecally at a dose of 4000 mg/kg brain weight. HP β CD caused a significant increase in hearing threshold following one dose of 8000 mg/kg s.c. or 120 mg intrathecally, and the effect was maintained for at least 12 weeks. Repeated weekly s.c. administration of 4000 mg/kg HP β CD resulted in a similar increase in hearing threshold. These studies are the first to describe a specific negative effect of HP β CD on the auditory system and suggest the need for auditory testing in patients receiving similar doses of HP β CD. (*Pediatr Res* 68: 1–2, 2010)

Cyclodextrins are cyclic oligosaccharides with hydrophobic interiors used as formulation vehicles to increase the amount of drug, including hormones and vitamins, which can be solubilized in aqueous vehicles (1). 2-hydroxypropyl- β -cyclodextrin (HP β CD) was extensively studied in rodents, dogs, and monkeys where it was generally well tolerated at low doses (1,2). Daily i.v. administration of greater than 200 mg/kg caused reduced body weight, foamy macrophage infiltration of the lungs, elevations in hepatic enzymes, increased Kupffer cells in the liver, and renal cortical tubular vacuolization in rodents (1,3,4). All of these changes were reversible following cessation of HP β CD administration (1).

Niemann-Pick type C (NPC) disease is an incurable lysosomal storage disorder characterized by the intralysosomal accumulation of unesterified cholesterol, hepatosplenomegaly, progressive neurologic dysfunction, and early death (5,6). Weekly intraperitoneal administration of 1500 mg/kg of HP β CD to *npc1*^{-/-} mice resulted in improvement in hepatic disease with no effect on neurologic disease or lifespan (7). In contrast, the administration of a single s.c. dose of 4000 mg/kg of a 20% solution of HP β CD

to 7-d-old *npc1*^{-/-} mice reversed the defect in the lysosomal transport of cholesterol and significantly improved hepatic dysfunction, decreased neurodegeneration, and prolonged lifespan (8). Every other day s.c. administration of 4000 mg/kg of a 20% solution of HP β CD to *npc1*^{-/-} mice was the most effective treatment regimen at ameliorating clinical disease and increasing lifespan, and also significantly decreased neuronal cholesterol, ganglioside, and sphingosine accumulation, and decreased neuroinflammation (9). It was hypothesized that high doses of HP β CD were needed to ameliorate neurologic dysfunction because higher blood levels allowed more drug to cross the blood-brain barrier. An alternative hypothesis was that increased serum levels of HP β CD could bind enough circulating sterols to result in enhanced cholesterol egress from the CNS by an undefined mechanism (9). In each of these animal studies, no significant toxicity was observed after the administration of HP β CD except for increased macrophage infiltration of the lungs found at post-mortem examination (9). On the basis of these data from the murine model, HP β CD has been approved for use in a group of children with NPC disease by the Food and Drug Administration (FDA).

Naturally occurring NPC disease occurs in cats which have a mis-sense mutation in *NPC1* (2864G-C) with clinical, neuropathological, and biochemical abnormalities similar to those present in juvenile-onset patients making this model homologous to the most common form of the disease seen in human patients (10,11). Brain stem auditory evoked response testing (BAER) of cats with NPC disease showed a prolongation in central conduction time with no significant alteration in hearing threshold compared with wild type cats (11). While evaluating the efficacy of HP β CD to treat NPC disease in cats, we noted a significant elevation of hearing threshold in animals receiving repeated s.c. doses of 4000 mg/kg. To our knowledge, a negative effect of HP β CD on auditory function has not been evaluated in any species. This study investigated the effects of the s.c. and intrathecal administration of HP β CD treatment on the BAER of both normal cats and cats with NPC disease.

METHODS

Animals. Cats were raised in the animal colony of the School of Veterinary Medicine, University of Pennsylvania, under National Institutes of Health and USDA guidelines for the care and use of animals in research. The experi-

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mental protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

All animals examined were produced from the same line bred to produce cats with autosomal recessively inherited Niemann-Pick type C (NPC) disease. All cats were housed at 21°C with *ad libitum* food and water, 12-h light cycles, with 12–15 air changes per hour. Peripheral blood leukocytes from all cats were tested at 1 day of age for the *NPC1* mis-sense mutation using a PCR-based DNA test (10). Cats with two copies of the mis-sense mutation were classified as affected with NPC disease while cats with one or no copies of the mutation were classified as normal. Heterozygote to heterozygote breedings produced ~25% affected and ~75% normal offspring.

Study groups. Normal cats were placed in one of five study groups (Table 1). Group 1 ($n = 7$) were 6 months old and received no drug. Group 2 ($n = 3$) received one dose of 4000 mg/kg body weight HP β CD s.c. at 6 months of age. Group 3 ($n = 3$) received one dose of 8000 mg/kg body weight HP β CD s.c. at 6 months of age. Group 4 ($n = 3$) received one dose of 4000 mg/kg brain weight (120 mg for a 30-g brain weight) HP β CD intrathecally at 6 months of age. Intrathecal administration was achieved by anesthetizing cats with i.v. propofol (up to 6 mg/kg; Abbott Laboratories, Chicago, IL). A 20-gauge spinal needle was placed into the cerebellomedullary cistern and 1.0 mL of spinal fluid was removed. 0.6 mL of 20% HP β CD in saline was injected intrathecally over a 2-minute time period into the cerebellomedullary cistern. Group 5 ($n = 3$) received weekly 4000 mg/kg body weight HP β CD s.c. beginning at 8 weeks of age for a total of seven doses.

Cats affected with NPC disease were already involved in a clinical study evaluating the efficacy of HP β CD to treat disease. All cats were first administered the drug at 3 weeks of age, before the onset of clinical signs of disease, and continued to receive the drug weekly thereafter. Cats were placed into one of five groups (Table 2). Group 6 ($n = 8$) received no HP β CD and served as the control group for cats with NPC disease. Groups 7 ($n = 5$), 8 ($n = 2$), and 9 ($n = 5$) received a weekly dose of 1000 mg/kg HP β CD body weight s.c., 4000 mg/kg body weight HP β CD s.c., and 8000 mg/kg body weight HP β CD s.c., respectively. Group 10 ($n = 2$) received 4000 mg/kg brain weight (120 mg for a 30-g brain weight) HP β CD intrathecally every 2 wk (intrathecal administration methods described earlier).

HP β CD formulations. All HP β CD was administered in a 20% (wt/vol) solution dissolved in 0.9% sodium chloride. HP β CD was received from Sigma Chemical Co. and the powdered form (HP β CD-H107; Sigma Chemical Co. Aldrich, St. Louis, MO) was used in all s.c. administrations and the cell culture tested form (HP β CD-C0926; Sigma Chemical Co. Aldrich) was used for all intrathecal administrations. As a control for the saline injection, addition normal cats were injected one time s.c. ($n = 2$) and intrathecally ($n = 2$) with similar volumes of saline.

To control for possible differences between HP β CD available from Sigma Chemical Co., the product used in published mouse studies (8,9) and the FDA-approved formulation for use in patients (Trappsol–Pharm grade, Cyclodextrin Technologies Development, Inc, High Springs, FL), Trappsol was administered to four cats: one dose of 8000 mg/kg body weight s.c. ($n = 2$) and one dose of 4000 mg/kg brain weight intrathecally (120 mg for a 30-g brain weight; $n = 2$).

Brain stem auditory evoked response testing. All measurements of the BAER were obtained from cats given atropine sulfate (0.02 mg/kg; Butler Animal Health Supply, Dublin, OH) and then anesthetized with i.v. propofol (up to 6 mg/kg). The BAER data were recorded using 12 mm, 29-gauge subdermal needle electrodes and a Nicolet Viking Quest signal analyzer (Nicolet Biomedical, Madison, WI). The active electrode was placed over the osseous bulla of the stimulated ear, the reference electrode was situated over the vertex of the skull, and the ground electrode over the contralateral osseous bulla. All recording electrodes were placed s.c. Alternating rarefaction and condensation clicks were produced by connecting a square wave pulse (0.1 ms in duration) to a speaker (Model TIP-300; Nicolet Biomedical, Madison, WI). The stimuli were presented monaurally at a rate of 11.1 Hz using a 25-cm plastic tube of 1.5 mm diameter which was connected to the speaker at one end, with the other end laid in the unsealed ear canal (an open field stimulus). The stimulator delivered a 125 dB

Table 2. Summary of groups of cats with NPC disease treated with HP β CD

Group	No. of cats	Dose and method of administration of HP β CD
6	8	NA
7	5	1000 mg/kg body weight subcutaneously weekly
8	2	4000 mg/kg body weight subcutaneously weekly
9	5	8000 mg/kg body weight subcutaneously weekly
10	2	4000 mg/kg brain weight intrathecally every two weeks

NA, not applicable.

pSPL click to the recorded ear and delivered an 85 dB SPL white noise to the contralateral ear. The high pass filter on the amplifier was 20 Hz and the low pass cutoff was 3 kHz. A sensitivity of 1 μ V/cm was used to record the responses and the averaging epoch was 10 ms with a sampling resolution of 0.01 ms. One thousand evoked responses were averaged for each BAER response obtained. Central conduction time was defined as the time between the first and the fifth peak. Wave V/I amplitude was determined by dividing the amplitude of the fifth wave by the amplitude of the first wave and multiplying by 100; amplitude was measured from peak to trough and expressed as microvolts. A modified method of limit procedure was used to estimate threshold. When a clearly defined BAER was identified at the reference stimulus of 125 dB, the attenuator was then increased in 3 dB steps and a signal averaged response was sought at each step. If an evoked response was observed, the attenuator was then increased by another 3 dB and the BAER response again observed. This continued until a sound level was reached at which an averaged evoked response could not be identified.

In normal cats, BAER studies were performed every other week following the administration of HP β CD in groups 2, 3, and 4 for a total of 12 wk, and were performed every week in group 5 for a total of 12 wk. In cats with NPC disease, BAER studies were performed at 16 weeks of age.

Statistical methods. The mean and SD of the threshold, central conduction time, and wave V/I ratio estimates in each group were calculated to describe the data and an unpaired 2-tailed *t* test was used to compare data between various groups. Significance values of $p < 0.05$ (*) are given. Threshold differences between groups were considered statistically reliable if the probability of chance occurrence was 0.05 or less.

RESULTS

Normal cats. None of the normal animals that received either s.c. or intrathecal HP β CD injections showed evidence of loss of balance or ataxia at any point during the study. No clinical signs were attributable to HP β CD administration aside from pain at the s.c. injection site which was common in cats receiving weekly doses. Subjective evaluation of hearing was difficult to perform because normal, untreated colony-bred animals frequently do not respond repeatably to sound. Detailed behavioral testing was not performed.

A single s.c. dose of 4000 mg/kg HP β CD evoked waveforms the same as in cats which received no HP β CD (Fig. 1, groups 1 and 2). In contrast, a single s.c. dose of 8000 mg/kg HP β CD resulted in diminished wave form amplitude with changes severe enough to make specific waveforms difficult to identify (Fig. 1, group 3). Similarly, a single intrathecal dose of 120 mg HP β CD resulted in altered evoked responses characterized by reduced amplitude (Fig. 1, group 4). A single injection of intrathecal saline left the BAER unchanged in two cats (data not shown).

Hearing threshold, wave V/I amplitude, and central conduction time were measured for groups 1–5 (Table 3). Cats in groups 3 and 4 showed a significant increase in hearing threshold 2 weeks after injection compared with uninjected cats (group 1). The average click BAER threshold in control cats was 66 dB, whereas in groups 3 and 4 that were treated with 4000 mg/kg and 8000 mg/kg HP β CD, respectively, the

Table 1. Summary of groups of normal cats treated with HP β CD

Group	No. of cats	Dose and method of administration of HP β CD
1	7	NA
2	3	4000 mg/kg body weight subcutaneously once
3	3	8000 mg/kg body weight subcutaneously once
4	3	4000 mg/kg brain weight intrathecally once
5	3	4000 mg/kg body weight subcutaneously weekly for 7 doses

NA, not applicable.

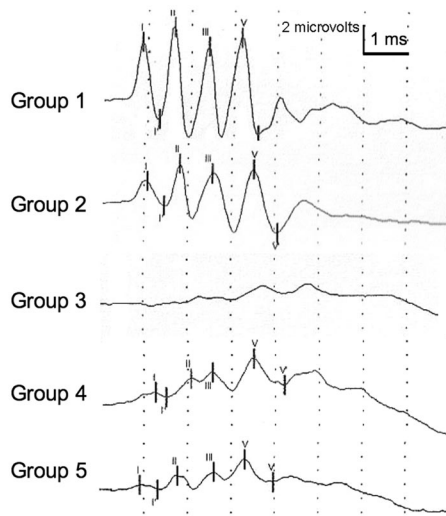


Figure 1. Representative brainstem auditory evoked responses for normal cats following the administration of HPβCD (group 2–5). Recordings for groups 2–4 were made 2 weeks after administering a single drug dose. The recording for Group 5 was made one week after the sixth weekly drug dose. A decrease in wave form amplitude was noted in groups 3, 4, and 5 compared with untreated cats (group 1). Changes in groups 3–5 were severe enough that waveforms could not always be reliably identified (an example is given for group 3).

Table 3. Hearing threshold, wave V/I amplitude, and central conduction time 2 weeks after administering a single dose of HPβCD to normal cats (groups 2–4) and 1 wk after the sixth dose (group 5)

	Hearing threshold (dB SPL)	Wave V/I amplitude	Central conduction time (ms)
Group 1 (<i>n</i> = 7)	66.4 ± 3.2	181 ± 16.7	2.41 ± 0.09
Group 2 (<i>n</i> = 3)	63.0 ± 5.2	212 ± 24.0	2.43 ± 0.02
Group 3 (<i>n</i> = 3)	79.0 ± 4.6*	545 ± 402	2.36 ± 0.02
Group 4 (<i>n</i> = 3)	81.0 ± 3.0*	525 ± 127	2.37 ± 0.05
Group 5 (six doses of HPβCD) (<i>n</i> = 3)	80.0 ± 1.7*	469 ± 423	2.38 ± 0.09

* *p* < 0.05.

threshold increased to 79 and 81 dB, respectively. This approximately ~3 dB difference between groups was statistically reliable (*p* < 0.05). Differences in wave V/I amplitude or in central conduction time were due to random sampling and were statistically insignificant among the groups. The BAER of cats in groups 2, 3, and 4 remained unchanged, neither improving nor worsening, during the 12-wk study following a single administration of HPβCD (data not shown).

Cats in group 5 received 7 weekly injections of 4000 mg/kg HPβCD s.c. Interestingly, although these cats showed no significant elevation in hearing threshold 2 weeks after the first injection (data not shown), repeated weekly injections of the same dose resulted in a progressive elevation of hearing threshold with the threshold on the fourth through seventh week significantly greater than the hearing threshold observed before injection (week 0) (Table 3; Fig. 2).

Cats with NPC disease. Affected cats began weekly s.c. therapy with HPβCD at 3 weeks of age in an attempt to ameliorate disease progression. At 16 wk of age, hearing thresholds were statistically the same in normal cats (group 1, 66.4 dB ± 3/2

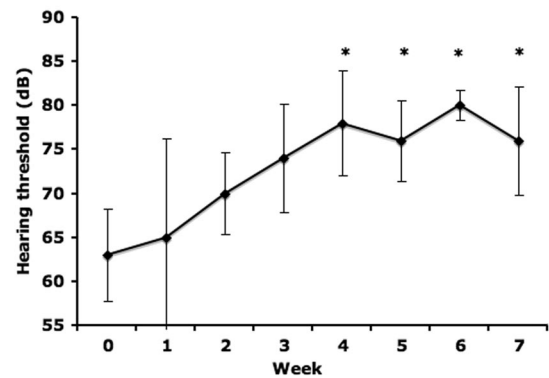


Figure 2. Weekly hearing thresholds following 4000 mg/kg weekly HPβCD administration (group 5; *n* = 3). The first dose was administered immediately after Week 0 threshold testing. Repeated HPβCD administration resulted in progressive elevation of the hearing threshold with a statistically significant (*p* < 0.05) increase from weeks 4 to 7 when compared with week 0.

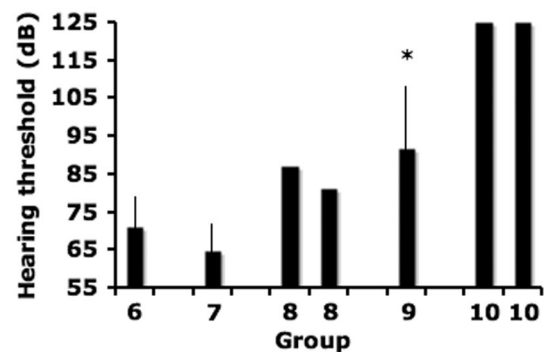


Figure 3. Cats with NPC disease cats showed no increase in hearing threshold after weekly administration of 1000 mg/kg HPβCD (group 7; *n* = 5) compared with untreated affected cats (group 6; *n* = 8). Both cats treated with weekly 4000 mg/kg (group 8; *n* = 2) showed an increase in hearing threshold and cats treated with weekly 8000 mg/kg (group 9; *n* = 5) had a statistically significant (*p* < 0.05) increase in threshold. No waveforms were evoked at the maximum stimulus intensity of 125 dB from two cats treated with every other week intrathecal HPβCD (group 10).

dB) and cats with NPC disease (group 6, 71.9 dB ± 7.9 dB). Interestingly, significant differences were absent between cats treated s.c. with 1000 mg/kg HPβCD (65 dB ± 7.5 dB; group 7) and untreated cats (group 6) (Fig. 3). However, both animals treated with weekly s.c. administered 4000 mg/kg (group 8) had higher hearing thresholds than any untreated cats with NPC disease. Similarly, cats treated with weekly s.c. administered 8000 mg/kg (91.8 dB ± 16.4 dB; group 9) had significantly greater hearing threshold compared with untreated cats with NPC disease. Finally, both cats given HPβCD intrathecally (every other week) had no click evoked waveforms even at the highest sound intensity of 125 dB. Where waveforms could be reliably discerned, the wave V/I amplitude and the central conduction time did not differ between untreated cats with NPC disease and cats treated with HPβCD.

Normal cats receiving trappsol. Trappsol was administered s.c. to two normal cats as a single dose of 8000 mg/kg body weight and intrathecally to two normal cats as a single dose of 4000 mg/kg brain weight (120 mg for a 30-g brain weight) in the same manner as the Sigma Chemical Co. product was

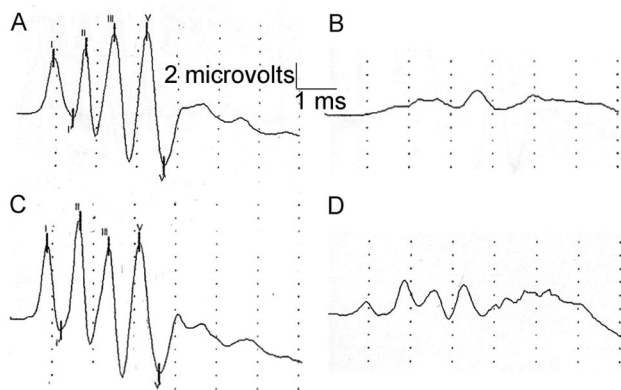


Figure 4. Normal cats treated with either intrathecal or s.c. Trappsol showed abnormal waveforms 1 week after administration. A, shows BAER tracing immediately before intrathecal injection of 120 mg Trappsol; B, shows BAER in same cat 1 week later. C, shows BAER tracing immediately before s.c. injection of 8000 mg/kg Trappsol; D, shows BAER in same cat 1 week later.

administered. BAER recordings were first made within 15 min of s.c. and intrathecal administration and no changes in the waveforms were noted (data not shown). Figure 4A and C show the BAER recordings before Trappsol administration in two cats. Figure 4B shows the BAER recording 1 week after the administration of HP β CD intrathecally, and Figure 4D shows the tracing obtained 1 week after the administration of s.c. HP β CD. The administration of Trappsol to normal cats resulted in changes to the BAER similar to that seen with the Sigma Chemical Co. product. Hearing threshold in two cats treated intrathecally increased from 69 dB to 72 dB before drug administration to 87 dB and 90 dB following administration. Hearing threshold in two cats treated s.c. increased from 69 dB and 72 dB before drug administration, to 87 dB in both cats following drug administration.

DISCUSSION

Niemann-Pick type C disease has an incidence of 1:150,000, with >250 disease-causing mutations identified (5). Natural history studies and therapy trials are difficult to perform on this disorder because of the relatively low incidence and the heterogeneity of disease in human patients. One particular mis-sense mutation represents >20% of mutant alleles and >50% of patients have a juvenile onset of neurologic disease. The feline model of NPC disease has a spontaneously occurring mis-sense mutation in *NPC1* (2864G-C) and exhibits clinical, neuropathological, and biochemical abnormalities similar to those present in juvenile-onset patients. Thus the cat model is homologous to the most common form of disease seen in human patients (10,12–17). The feline model has been useful for identifying the late endosomal/lysosomal accumulation of unesterified cholesterol and gangliosides (GM2 and GM3) (18), for evaluating the association of GM2 storage with meganeurite formation and abnormal dendritogenesis (19), for correlating neuroaxonal dystrophy with neurologic dysfunction (16), and for evaluating efficacy of experimental therapies (10,13). The onset and progression of neurologic dysfunction in the feline model has been well characterized (11,15). A regular onset of progressive cerebellar and vestibular dysfunction occurred in affected cats beginning with

intention tremors and ataxia at 6 weeks of age. This dysfunction progressed until cats could no longer maintain sternal recumbency at ~24 wk of age. Changes in hearing threshold were not found although a delay in central conduction time and a decrease in wave V/I amplitude ratio was observed in 16- and 24-wk old affected cats compared with wild type cats (11).

HP β CDs are cyclic oligosaccharides consisting of seven β -(1–4) glucopyranose units (7). HP β CDs have a hydrophilic exterior and a hydrophilic interior making them useful for increasing the aqueous solubility of hydrophobic molecules such as cholesterol, steroids, and vitamins (20). *In vitro* studies using β -cyclodextrins have shown a marked removal of cholesterol from cultured neuronal (21,22) and nonneuronal cell lines (23–25). HP β CDs were shown to cross the blood brain barrier in *in vitro* (25) and in *in vivo* with difficulty (7,26). However, β -cyclodextrins were safely administered intrathecally in rodent studies and used to improve the delivery to the brain of drugs including anesthetic agents, galanin-like peptide, and estradiol (27–29).

Recently, HP β CD was shown to release cholesterol from NPC-deficient lysosomes and allowed unesterified cholesterol to be available to the NPC cell. This resulted in the amelioration of disease and the prolongation of life in the murine model (8,9,30). However, high doses of HP β CD (at least 4000 mg/kg) however appeared necessary to retard the progression of neurologic disease. Studies in *npc1*^{-/-} mice showed that 1500 mg/kg HP β CD administered weekly caused a decrease in hepatic unesterified cholesterol concentrations without substantial effect on neurologic signs (7). Increasing the dose to either 4000 mg/kg weekly or every other day delayed clinical disease onset, increased survival time, corrected cholesterol metabolism, and improved biochemical and histologic disease (8,9). Because β -cyclodextrins do not easily penetrate the blood brain barrier (7,25), these studies suggested that parenteral administration of high doses of HP β CD are necessary to get sufficient amounts of HP β CD to cross the blood brain barrier and to have an effect on neurologic disease. Unfortunately, the pharmacokinetics of HP β CD are not well understood particularly in the nervous system. A plasma elimination half-life in rats was 0.4 h and in dogs was 0.8 h, although the concentration in cerebrospinal fluid after systemic administration was not described (1). Serum and cerebrospinal measurements of unlabeled HP β CD are technically difficult to perform, and these concentrations were not determined in the recent murine articles (8,9,30). Clearly, the kinetics of HP β CD in serum and spinal fluid will be necessary to clarify how HP β CDs effect neurologic dysfunction in NPC disease and to determine what dose is most efficacious while also limiting toxicity.

As a result of the dramatic improvement in clinical signs seen in the mouse model of NPC disease, HP β CD was given recent FDA approval for use in a small number of patients with NPC disease. This has increased the urgency to more fully characterize any dose-related potential toxic effects of the drug. In humans, i.v. administration of up to 3 g in healthy volunteers was well tolerated and doses of 16 g per day given with itraconazole did not result in hearing abnormalities (4). The authors could not find examples of doses of 1000 mg/kg and higher being used in human patients, although these are being proposed to treat patients with NPC disease. The authors are aware of no previous

study examining the effect of HP β CD on auditory function and yet we were able to determine an effect on hearing using a small number of normal cats and cats with NPC disease. Our data show that 1000 mg/kg had no effect on the BAER response when given weekly for 14 doses between the ages of 3 and 16 wk of age. Doses of 4000 mg/kg body weight resulted in an increase in hearing threshold only after repeated dosing and doses of 8000 mg/kg body weight resulted in significant increases in hearing threshold in both normal cats and cats with NPC disease following the administration of a single dose. Interestingly, the doses needed to negatively impact the BAER response were the same dose necessary to retard nervous system dysfunction in mice. Our preliminary data in cats affected with NPC disease suggest a similar requirement for doses equal to or >4000 mg/kg to positively affect neurologic disease (data not shown). One conclusion that suggested itself is that doses of >4000 mg/kg are necessary for HP β CD to cross the blood brain barrier and that the effect on hearing is related to the ability of the drug to enter the CNS. Our data on the effect of intrathecal administration of HP β CD on hearing threshold supported the conclusion that the drug had its negative effect on hearing only after it entered the spinal fluid. Importantly, whether the drug was given s.c. or intrathecally, the negative effect of HP β CD on the auditory system was not ameliorated up to 12 wk after the cessation of drug administration suggesting that the effects may be irreversible.

The increased hearing threshold with no change in central conduction time suggested that the damage from HP β CD occurred in the peripheral auditory pathway (cochlea or eighth nerve) and that potential mechanisms of action for the hearing loss observed include a direct effect on the stria vascularis and its role in maintaining the ionic environment of the inner ear fluid space, the transduction and motility mechanisms of inner and outer hair cells, and/or the excitation patterns in the auditory nerve discharges. Identifying the site of action of HP β CD within the peripheral auditory system will likely be the first step in overcoming toxicity of HP β CD applications. Otoacoustic emission testing would be a useful method for evaluating outer hair cell function in cats but was unavailable for these studies. Histopathology of the cochlea should be performed in the future to identify any pathologic changes.

In summary, hearing impairment following HP β CD administration appeared to be both dose dependent and long lasting and may be a limiting factor in the use of this drug at high doses to treat Niemann-Pick type C disease. Auditory testing is recommended for patients receiving doses of 4000 mg/kg HP β CD or greater to evaluate the effect on hearing threshold in these patients.

Acknowledgments. We acknowledge the critical review of the manuscript by Drs. Shel Steinberg and James Saunders. Trappsol was provided by Dr. Rick Stratton.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

1

* Final Report *

Result type: Auditory Evoked Potential
Result date: 15 April 2010 00:00
Result status: Auth (Verified)
Result title: chm chugani, h 10981
Performed by: ASANO MD, EISHI on 15 April 2010 16:29
Verified by: ASANO MD, EISHI on 21 April 2010 12:15
Encounter info: 715691481, CHILDRENSHOSP, Outpatient-Active, 04/10/10 - 04/11/10

*** Final Report ***

**CHILDREN'S HOSPITAL OF MICHIGAN
DEPARTMENT OF ELECTRONEURODIAGNOSTICS
OUTPATIENT**

NAME: HEMPEL, Addison
CHM: 890220951
FIN: 715691481
EEG:
BD: 01/23/2004

REFERRED BY: Harry T Chugani, M.D.

DATE OF PROCEDURE: 04/15/2010

PROCEDURE: Brainstem Auditory Evoked Potential

CLINICAL HISTORY: This is a 6-year-old female with Niemann-Pick disease to evaluate for auditory function.

MEDICATIONS: Zebesca, Kirkiman, and Keppra.

RECORDING DATA: Brain stem auditory evoked potentials were recorded with alternating clicks at 70 dBl, rate of 9.1 Hz, duration of 100 ms and filter setting of 150-3000 Hz. A total of 1000 sweeps were averaged each time and the study was repeated at least once for confirmation.

FINDINGS:	Left	Right
Wave I	2.48	2.52
Wave III	4.74	4.84
Wave V	7.36	6.56
Wave I-III	2.20	2.32

* Final Report *

Wave III-V	2.58	1.72
Wave I-V	4.84	4.04

Latency intensity plot revealed threshold for Wave V peak being at 25 dB_l on the left and right ear.

IMPRESSION: Normal brain stem auditory evoked potential response was observed bilaterally. Hearing threshold was also within normal limits.

INTERPRETED BY: Gireesh Velugubanti, M.D.

Eishi Asano, M.D.
Assistant Professor

"I personally viewed the images and performed the interpretation of this procedure."

Date Typed: 04/16/2010
Typed By: rmt:31 10981

Signature Line

This document has been electronically signed by: ASANO MD, EISHI

Completed Action List:

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- * Modify by VELUGUBANTI MD-Resident, GIREESH on 19 April 2010 08:36
- * Sign by VELUGUBANTI MD-Resident, GIREESH on 19 April 2010 08:36
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Result type: Auditory Evoked Potential
Result date: 15 April 2010 00:00
Result status: Auth (Verified)
Result title: chm chugani, h 11001
Performed by: ASANO MD, EISHI on 15 April 2010 16:34
Verified by: ASANO MD, EISHI on 21 April 2010 12:15
Encounter info: 715691473, CHILDRENSHOSP, Outpatient-Active, 04/10/10 - 04/11/10

*** Final Report ***

**CHILDREN'S HOSPITAL OF MICHIGAN
DEPARTMENT OF ELECTRONEURODIAGNOSTICS
OUTPATIENT**

NAME: HEMPEL, Cassidy
CHM: 890220992
FIN: 715691473
EEG:
BD: 01/23/2004

REFERRED BY: Harry T Chugani, M.D.

DATE OF PROCEDURE: 04/15/2010

PROCEDURE: Brainstem Auditory Evoked Potential

CLINICAL HISTORY: This is a 6-year-old female with a history of Niemann-Pick Type C being evaluated for auditory dysfunction.

MEDICATIONS: Keppra, Zebesca, Kirkiman

RECORDING DATA: Brain stem auditory evoked potentials were recorded with alternating clicks at 80 dbL, rate of 9.1 Hz, duration of 100 ms and filter setting of 150 to 3000 Hz. A total of 1000 sweeps were averaged each time and the study was repeated at least once for confirmation.

FINDINGS:	Left	Right
Wave I	2.38	2.70
Wave III	4.86	4.96
Wave V	7.44	7.78
Wave I-III		2.26
Wave III-V		2.82

* Final Report *

Wave I-V 5.06 5.08

Latency intensity plot revealed threshold for Wave V peak for being 10 dB_l on the left and 20 dB_l on the right.

IMPRESSION: Normal brain stem auditory evoked responses were observed bilaterally. Hearing threshold is also within normal limits.

INTERPRETED BY: Gireesh Velugubanti, M.D.

Eishi Asano, M.D.
Assistant Professor

"I personally viewed the images and performed the interpretation of this procedure."

Date Typed: 04/16/2010
Typed By: rmt:31 11001

Signature Line

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Completed Action List:

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* VERIFY by ASANO MD, EISHI on 21 April 2010 12:15

**CHILDREN'S HOSPITAL
& RESEARCH CENTER OAKLAND**

Department of Pediatric Hematology/ Oncology

747 Fifty Second Street, Oakland, CA 94609-1809 – Phone: 510-428-3000

FACSIMILE TRANSMITTAL SHEET

To:	Chris and Hugh Hempel	From:	Karen Barca
Attn:		Dept:	Hematology/ Oncology Administrative Coordinator, Oncology
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Phone:		Phone:	510 428-3885 Ext 2860
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CHILDREN'S HOSPITAL OAKLAND
Pediatric Audiology
510-428-3344
510-450-5631 Fax

Name: HEMPEL, ADDISON
B.D.: 01/23/04
M.R.: 893506
Evaluation Date: 12/09/09

AUDIOLOGIC EVALUATION

HISTORY: Addison, a 5 year 10 month old female, was seen today in our department for an initial audiologic evaluation. Addison is one of twins with a medical history of Niemann-Pick disease Type C, and continues on cyclodextrin infusions. She is followed in the Hematology-Oncology Department at Children's Hospital and Research Center at Oakland. She presents with developmental delays, neurologic problems, and learning problems. Reportedly, her last audiologic evaluation completed at NIH 1 1/2 - 2 years ago recorded normal hearing sensitivity in each ear.

PAIN ASSESSMENT: No pain reported.

TEST PROCEDURES: Visual reinforcement audiometry with the help of a test assistant. Addison was very sleepy throughout the test assessment. Reliability was poor as Addison was unable to condition to the auditory task.

TEST RESULTS: Audiologic testing was initiated in the soundfield environment and a speech detection threshold was observed down to 25 dBHL for at least one ear. Further behavioral audiologic information could not be obtained as Addison was unable to condition to the auditory task. Acoustic immittance testing recorded bilateral Type A tympanograms consistent with normal ear drum mobility in each ear. Transient evoked otoacoustic emission testing was performed as a cross check measurement. TEOAEs were present in both ears consistent with normal cochlear outer hair cell function, bilaterally.

SUMMARY and EDUCATION: Addison presents with normal hearing sensitivity to speech stimuli in at least one ear. TEOAEs were present in each ear, consistent with normal cochlear outer hair cell function, bilaterally. Addison PASSED the TEOAE hearing screening in each ear. Her current auditory status is adequate to support speech and language development.

RECOMMENDATIONS:

1. Addison return to the Audiology Department as needed; sooner as recommended by her physician for monitoring purposes.

If there are any questions regarding today's test results, please do not hesitate to contact me at (510) 428-3344.

Kelly Tremmel-Howell, Au.D., CCC-A
Pediatric Audiologist

cc: CHO Medical Records
Mr. and Mrs. Hempel, 14125 Shadow Bow Drive, Reno, Nevada, 89511
Caroline Hastings, M.D., CHO

*The pediatric medical center for Northern California
747 Fifty Second Street Oakland, CA 94609-1809*

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Run: 03/29/10-16:22 by BARCA, KAREN M.

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BAY AREA PEDIATRIC PULMONARY (BAPP)

Pulmonary Clinic Visit

Name: HEMPEL,ADDISON

B.D.: 01/23/04

M.R.: 893506

D.P.: 4264911

INS.: BLUE CROSS OF CALIFORNIA PPO

Date of Visit: 10/08/09

Time of Visit: 0923

Referring Physician: SUSAN SORENSEN, MD

() NKDA Allergies/Adverse Reactions:

Patient brought to visit by on 10/8/09 Office site: Reno

CC: trouble breathing and sleeping

Source: parents, record from PMD

HPI:

I met mother today and reviewed the history for Addie and her twin Cassie. Mother is very well informed regarding their illness and she took this opportunity to share much of the background information with me today.

Addie was a healthy infant toddler and one year old. In retrospect mother thinks she was a little clumsy, but nothing dramatic enough to warrant attention. At about 2 y/o she had a bad virus, flu like symptoms with fatigue and cough. She continued with these symptoms and gradually developed clear signs of ataxia and dementia. She also has splenomegaly which Mom believes was missed before the diagnosis due to many photos showing abdominal enlargement. She was referred to Stand LPHC and underwent a number of tests. They also had consultation by Dr. Caroline Hastings. Mom estimated that it took about 16 months to clarify her diagnosis of Nieman Pick C disease in 10/08. Additionally very recently she has been determined to have XMRV a newly discovered retrovirus. Researchers believe that the initial illness at 2 y/o was likely due to this virus.

Addie is now a 5 y/o with NPC and XMRV. Recently she and her sister were started onto a compassionate use treatment protocol with twice weekly infusions of cyclodextrin. The NIH first has them infused with very low dose. After a few months of stability they agreed to a gradually escalating dose of CD which is increased 100mg/k each infusion. Since the infusions were started she has improved with less ataxia, increased vocalization and shrinking of the spleen. Her lysosomes are decreasing in size. OHSU testing her urine and 70fold increase in cholesterol excretion is documented since starting cyclodextrin. Glutathione levels also assessed and decreased. She has elevated oxsterols, 7 keto chol and 6 triol chol (documented in alzheimers and heart disease). Carriers and patients have this finding. Amyloid beta levels in CSF are very high per NIH testing.

The parents have now met a number of parents with children who have or have died from NPC. Many have counseled them to get pulmonary assessments and assistance with the twins. Mom currently notes that Addie has difficulty with sleeping, waking intermittently through the night but that cough is not clearly the cause of this. She walks but is ataxic, she lost ability to talk, but now is making a lot more noise as she seems better. Her cough is weak and is not triggered normally. She drools and chews on bibs and her shirt. She is fed orally, picky eater, spits if bites are too big, better with softer foods. Her chewing and eating is much worse than her sister. Addie does not have a vertical eye gaze palsy. She does have Echolalia. Occasional new words this week "I like orchids". She may have absence seizures. Flickering tree shadows and lights can trigger these spells. Wakes up nights, occ night cough if sick. Generally don't cough even who very congested. No parasomias, pattern wake 5am 1-3 nap down for night about 9pm. Gets weak if laughs, not overt cataplexy, sensitive to smells but has much more gagging than her

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Children's Hospital Oakland

HEMPEL, ADDISON

MR#: 893506

sister and can vomit easily..

Other information from mother:

Parents involved to raise money and do research on both NPC and Now XMRV.

The new retrovirus XMRV has been found in about 10 million folks have this. It was discovered in UNR and is related to prostate cancer and may relate to chronic fatigue syndrome. Articles about this virus are breaking on Science any day. Mom and Dad and MGM have latent virus. Mom getting involved in the virus research and how it relates to NPC. When AIDS comes into the body it attaches to cholesterol. Researches think the XMRV does something similar and uses cholesterol machinery to infect a host.

The kids are on Cyclodextrin study. It is the active ingredient helping cat and mice in studies. This molecule can surround cholesterol. No one knows why it works on cats and mice. It has been shown to double the life span and organs normalize organ by decreasing cholesterol deposition in NPC animals. Pulmonary penetration for the drug is poor. No one is sure if lung disease is now recognized because of the longer life, or if alters macrophage function. UT Southwestern trying to nebulize to the lung to see if it will affect lung function. Additionally CD does not cross the blood brain barrier easily and perhaps inhalation will facilitate crossing this barrier.

Cyclodextrin also kills the AIDS virus - possibly removing the cholesterol from HIV so that medications work better. Research studies on CD use on monkeys is promising. CD also kills anthrax.

MEDICATIONS:

NAC 3000mg day, Mom gradually worked her way up per info from researchers
Cyclodextrin IV started at 80mg/k for 3 months, then increased 100mg/k/infusion and FDA agreed. At 2800mg/k twice weekly plan over 8 hours to get to max of 5000mg/k. Monitoring renal function with urine 5am and blood for Cr and Ccr on each infusion day.

Zovexca 100mg bid (for the XMRV)

Fish oil

Sesame oil

Coconut oil

Curcumin 1800mg once daily (makes mice live longer)

Ibuprofen 75mg bid

Vit D3 5000 IU (makes mice live longer) never got any baseline data

Vit B2 def and on B complex once daily

Brazil nut and mushroom powder CHORI found abnormal ergothionine in the girls and these replete it to boost glutathione (it was checked and low)

HOME EQUIPMENT:

MDI, spacers or compressors.

PAST MEDICAL HISTORY:

Birth History: Addie 4#6, Mom on bedrest and Terb about 32 wks. c/sperformed at St Mary's at 35 wks., Bottle fed, weak suck for BF. Similac used at first and changed to Horizon organic formula. Fed fine and gained weight.

Past Illnesses/Events: Normal development initially, tall for age and clumsy, not enough to raise suspicion, learning abc. With the infection at 2 the spleen already 12 cm and Mom thinks spleen already big earlier, but no one ever noticed.

Immunizations: UTD, no flu shot this year.

Hospitalizations: many usually Renown

Surgery: Port at CHRCO

Family History: neg for NPC, MGM carrier, no CF, DM, stroke, heart disease MGM needs valve replacement and AMI at 49 yo, asthma MGM, no allergies, cancer PGM breast, GGP stomach, PGGF heart issues

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Children's Hospital Oakland

HEMPEL, ADDISON

MR#: 893506

Social History: in school 3 d weekly, 6-7 kids, sp ed and ambulatory. MGP and Nanny for 6 years (Martha) (needs PPD) and Mom and Dad, no pets, ETS, Saddlehorn s Reno, they share bedroom carpeted, many stuffed animals always washed. Bedding washed every 3d, in diapers so changing all the time. House cleaner weekly too. Dust and vac daily.

Travel/Exposures: Mayo clinic, NIH, Children's Michigan for brain scans

Allergy History: never been evaluated

REVIEW OF SYSTEMS

Coughing or wheezing with exercise or activity? Only if sick

Coughing or wheezing with emotions or stress? None

Nocturnal coughing? None

Symptoms when sleeping? None

Recent acute attacks/flareups None

Recent use of rescue medication? Never

Recent use of systemic steroids? Never

Recent visits to ER or hospital? For infusions

Absences from activities (school, work, sports) NA

GERD symptoms? Easy gag and vomit

Endo mom not sure glucose, none known

Neuro loss of function and regaining balance and speech (some therapy at school)

Allergy symptoms? Always stuffy and drippy

Allergy exposures? None

Allergy Avoidance? Compliant

Tobacco Smoke Exposure None

Counseled re tobacco and/or allergy exposures Yes

PHYSICAL EXAM:

Age 5 HR 98 RR 36 Wt A 56.4#

Distress/Pain: congested nasal partial obstruction

Habitus: sitting obese child, in bed and IV running, watching TV avidly and vocalizing intermittently

Deformities: None

Skin: Normal

Conjunctiva/Lids: Normal

Eye Movement: Normal

PERRLA Normal

Tympanic Membranes: Normal

Nasal mucosa: externally normal, no discharge

Oral pharynx: healthy teeth, easy smile for mother

Tonsillar hypertrophy: None

Neck movement: Normal, no LAN and no gagging to touching her neck

Tracheal position: Normal

Jugular veins: Normal

Thyroid: Not visibly enlarged

Cough: twice during my visit, spontaneous and weak

Chest Shape: Normal

Retractions: None

Nasal Flaring: None

Air exchange: Normal with some transmitted upper airway noise

Stridor: None

Crackles: None

Wheezes or rhonchi: None

Percussion: Normal

Palpation: Normal

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Children's Hospital Oakland

HEMPEL, ADDISON

MR#: 893506

Heart sounds: Normal

Heart rate & Rhythm: Normal

Abdominal distension: None with enlarged liver and spleen.

Abdominal tenderness: None

GU normal female

Gait/station: some ataxia while sitting.

Clubbing, cyanosis, edema: None

Neuro/Development: development regression I will examine more thoroughly next visit since in midst of infusion now, hypotonic, but able to reach and grasp her stuffed dog, chew on it and wiggle around as she is sitting in bed.

LABS:

Sat 98%

STUDIES REVIEWED TODAY

8/9/07

per Dr Hastings work up at Stanford included:

EBV serology confirmed recent infection, EBV undetectable by PCR
quantitative immunoglobulins mild elevation

T- and B-cell studies, all normal

CD4, CD8, and alpha beta TCR; all normal

ectacytometry, normal

hemoglobin electrophoresis was normal

(not sure if she was evaluated for beta thalassemia or an unstable Hgb)

Abdominal ultrasound normal (unsure if with doppler)

CT with Doppler done at Renown Medical Center normal

storage diseases Gaucher and MPS negative

Niemann-Pick type C ultimately confirmed

8/14/08

platelet count of 140,000, hemoglobin 11.4, white count 5.4,
36 segs, 42 lymphs, 13 eosinophils, 8 monos. Glucose 86, SGOT 72, SCPT 13,
calcium 9.3, albumin 3.9, total bilirubin 0.4. Sodium 136, potassium 3.9,
chloride 105, bicarb 23, BUN 6, creatinine 0.4.

9/21/09

A CBC was done that revealed a white blood cell count of

5100, hemoglobin 11.2, hematocrit 32.8, and platelet count of 183,000. Her ANC
was 2500. A metabolic panel revealed a sodium of 137, potassium 4.2, chloride
105, CO2 23, BUN 13, and creatinine 0.2. Glucose was 95, and her calcium was
9.7. Her AST was 73, ALT 23, total bilirubin 0.4, and her direct bilirubin less
than 0.1. Her uric acid was 3.3, amylase 30, and LDH 272. Cholesterol 158,
triglycerides 165, and LDL 108. PT 13.7, INR 1.21, and PTT 38.4. Protein
creatinine ratio her urine from last Thursday was 0.167, which was acceptable.

Dr Hastings notes that lab consistently stable

ASSESSMENT:

Sleep disturbance: This is best evaluated with overnight polysomnogram. I explained the details of how sleep studies are performed and hope that we can get cooperation from Cassie for this. Due to the noted loss of muscle tone with laughing we will also check her MSLT multiple sleep latency testing.

Weak and poorly coordinated muscles: possible restrictive lung disease or poor chest muscle function and thus weak cough. Most children with NPC develop increased secretions and gradually diminishing cough and then pneumonias with recurrent infections leading to gradual respiratory failure. Sleep hypoventilation can

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Children's Hospital Oakland

HEMPEL,ADDISON

MR#: 893506

exacerbate this. For some GER and aspiration also add to lung damage.

Dysphagia: mild, able to maintain herself with oral feeding but has gagging and rarely aspiration. Parents are very careful with food offerings and work to feed her without causing gagging

NPC disease: genetics confirmed

PATIENT EDUCATION. As part of today's visit, we discussed the following:

- * Pathophysiology of disease
 - * Medications, including review of risks, benefits, and alternatives.
 - * Environmental & allergy issues
 - * Potential triggers to pulmonary symptoms.
 - * Signs and symptoms of respiratory distress.
 - * Use of equipment (such as spacers, nebulizers, peak flow meters, etc.)
 - * Prognosis and Treatment plan.
-

PLAN/RECOMMENDATIONS:

1. Continue same current medication plan
2. Obtain baseline PFT, PSG so that as new treatments are continued their affect on these parameters can be clearly monitored
3. Train the family in airway clearance techniques since these will be needed.

PENDING LABS: PSG, PFT with IOS, MIP and MEP

FOLLOW UP VISIT: monthly

Cc: PMD

Total visit time: 45 Educational time: 10

Thank you for referring your patient to us for pulmonary care. Please let us know if you have questions regarding our impression and plan. We look forward to working with you and your patient in the future.

KAREN A HARDY, MD

Electronically signed

10/13/09

1702

Report #: 1012-0043

Karen Hardy, M.D. (Director)

Edward Fong, M.D., Eric Zee, M.D., Manisha Newaskar, M.D.,

Danny Hsia, M.D., Haramandeep Singh, M.D.,

Sebnem Ozdogan, M.D., Pulmonary Fellow,

Renee Benson, M.D., Pulmonary Fellow, D. J. Kaley, RN

747 52nd Street, Oakland, CA 94609 Phone: (510) 428-3305 Fax: (510) 597-7154

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Children's Hospital Oakland
Sleep Disorders Laboratory
Karen Hardy, M.D. and Haramandeep Singh, M.D.
Co-Directors

RE: HEMPEL, ADDISON
DOB: 01/23/04
MR #: 893506

747 52nd Street

Oakland, CA 94609

Phone: 510-428-3311 Fax: 510-450-5857

Study Date: 12/09/09

Report Date: 12/17/09

Clinical Polysomnographic Report

MULTIPLE SLEEP LATENCY TEST

NAP	SLEEP ONSET	REM PERIODS
1	1 MIN 11 SECS	NO
2	5 MIN 22 SECS	NO
3	7 MIN 15 SECS	NO
4	20 MIN	NO
5	20 MIN	NO
	AVG: 10 MIN 46 SECS	0/5

IMPRESSIONS:

NORMAL STUDY. CRITERIA FOR NARCOLEPSY OR EXCESSIVE DAYTIME SLEEPINESS ARE NOT MET.

RECOMMENDATIONS:

CLINICAL CORRELATION IS RECOMMENDED. FOLLOW UP IN SLEEP CLINIC AS NEEDED.

HARAMANDEEP SINGH, MD Electronically signed 12/17/09 1235

Karen Hardy, M.D. (Director),
Edward Fong, M.D., Eric Zee, M.D., Manisha Newaskar, M.D.,
Jerrold Kram, M.D., Haramandeep Singh, M.D., Danny Hsia, M.D.,
Sebnem Ozdogan, M.D., Eli Sills, M.D., Renee Benson, M.D., Pulmonary Fellows
Bay Area Pediatric Pulmonary

Report #: 1217-0002

510-428-3305

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Children's Hospital Oakland
 Sleep Disorders Laboratory
 Karen Hardy, M.D. and Harmandeep Singh, M.D.
 Co-Directors

RE: HEMPEL, ADDISON
 DOB: 01/23/04
 MR #: 893506

747 52nd Street
 Oakland, CA 94609
 Phone: 510-428-3311 Fax: 510-450-5857

Study Date: 12/08/09
 Report Date: 12/11/09

Clinical Polysomnographic Report

CHIEF COMPLAINT: SLEEP DISTURBANCES, POSSIBLE HYPOVENTILATION OR OSA with known niemann pick type c disease on biweekly infusions of Cyclodextrin. Daytime sleepiness and staring spells noted as well.

The patient was recorded by means of a nocturnal polysomnogram. The following parameters were measured during the entire recording: Electroencephalogram (EEG), electromyogram (EMG) of the chin, electro-oculogram (EOG), electrocardiogram (ECG), air flow and carbon dioxide level from the nose, respiratory effort at the chest and abdomen, finger oximetry, and snoring microphone.

I. SUMMARY OF POLYSOMNOGRAPHIC VALUES:

VARIABLES	Patient's values	Reference values
Total Sleep Time (TST; min.)	402.4	>= 360
Sleep Efficiency (%)	81.4	>= 85
REM Sleep (% TST)	31.2	15-30
Apnea Index (AI; #/hr)	0.0	<= 1
Respiratory Disturbance Index (RDI; #/hr)	0.3	<= 10
Max ETCO2	42.0	<= 45
SaO2 nadir (%)	96.0	>= 92
% time SaO2 < 90%	0	0

Note: There are very few studies of normative values for polysomnography in children. Such values are based on very limited normative data and their predictive value should be used with caution. Most pediatric sleep laboratories define "acceptable" values for several of the above variables based on experience, and it is not possible to give numerical guidelines for these variables.

II. SLEEP ARCHITECTURE:

Total sleep time adequate.
 Sleep efficiency adequate.
 Proportion of REM sleep adequate.
 This is a technically adequate study.

III. DISTURBANCES OF SLEEP:

Addison did amazingly well for the hook up for the study. She slept well overnight without respiratory events or snoring. Gas exchange was excellent in room air.

IV. IMPRESSION:

Normal study

V. RECOMMENDATIONS:

Return for evaluation if clinically indicated

Clinical Polysomnographic Report**RE: ADDISON HEMPEL
893506**

Please contact us directly if you have any questions.

KAREN A HARDY, MD**Electronically signed****12/11/09****1243**

CC:

Dr Caroline Hastings, parents

HASTINGS, CAROLINE A MD

Sleep Lab Chart

Original: Medical Records Dept.

Karen Hardy, M.D. (Director),
Edward Fong, M.D., Eric Zee, M.D., Manisha Newaskar, M.D.,
Jerrold Kram, M.D., Haramandeep Singh, M.D., Danny Hsia, M.D.
Sebnem Ozdogan, M.D., Eli Sills, M.D., Renee Benson, M.D., Pulmonary Fellows
Bay Area Pediatric Pulmonary

Report #: 1211-0002**510-428-3305**

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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CHILDREN'S HOSPITAL OAKLAND
Pulmonary Function Laboratory
Karen Hardy, M.D., Director
747 52nd Street
Oakland, CA 94609
Phone: 510-428-3311 Fax: 510-450-5857

PATIENT: HEMPEL, ADDISON
BD: 01/23/04
MR#: 893506
Date of test: 12/08/2009

Primary Care Physician: HASTINGS, CAROLINE A MD

Ordering Physician: HARDY, KAREN A., M.D.

PULMONARY FUNCTION TEST

Interpreting Pulmonary Fellow: Renee Benson, M.D.
Interpreting Pulmonologist: Karen A. Hardy, M.D.

RESULTS

Standard pulmonary function testing assumes that patient's effort, cooperation, and ability to perform the tests do NOT limit measurements of the patient's lung function.

When parents arrived with the twin girls, it was stated that the girls are not cooperative, as neurologically they are only 2 years old and do not like anything whatsoever in their mouths. Therefore IOS testing was not accomplished today.

We then decided to try MIP/MEP testing, where Addison was laid down and held by Mom and Dad. It was very difficult for her, as she required to be held down, which she did not like at all. Therefore her testing was probably not optimal for results. A MEP was not able to be obtained with testing.

Effort, cooperation and ability to perform the MIP plus IOS tests WERE ADEQUATE. The test results are technically valid.

Effort, cooperation and/or ability to perform the MEP plus IOS tests were NOT ADEQUATE.

Respiratory muscle strength data

MIP (cmH2O): 70

CONCLUSIONS

- (X) PULMONARY FUNCTION TESTS WERE NORMAL.
() Normal inspiratory respiratory muscle strength.

COMMENTS AND SUGGESTIONS

If you wish a copy of the raw data, please call the PFT lab and the data can be sent to you.

There was no previous study for comparison.

_____, M.D.
RENEE BENSON, M.D.
Pediatric Pulmonary Fellow

_____, M.D.
KAREN A. HARDY, M.D.
Pediatric Pulmonary Attending

RENEE C BENSON, MD

CHILDREN'S HOSPITAL OAKLAND
Pulmonary Function and Sleep Laboratory

PATIENT: HEMPEL, ADDISON
BD: 01/23/04
MR#: 893506

KAREN A HARDY, MD

Karen Hardy, M.D. (Director),
Edward Fong, M.D., Eric Zee, M.D., Manisha Newaskar, M.D.,
Jerrold Kram, M.D., Haramandeep Singh, M.D., Danny Hsia, M.D.
Sebnem Ozdogan, M.D., Eli Sills, M.D., Renee Benson, M.D., Pulmonary Fellows
Bay Area Pediatric Pulmonary

Report #: 1208-0011

510-428-3305

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Run: 03/29/10-16:22 by BARCA, KAREN M.

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CHILDREN'S HOSPITAL OAKLAND
Pediatric Audiology
510-428-3344
510-450-5631 Fax

Name: HEMPEL, CASSIDY
B.D.: 01/23/04
M.R.: 893505
Evaluation Date: 12/10/09
Evaluation Time: 1050

AUDIOLOGICAL EVALUATION

REASON FOR VISIT: Cassidy, a 5-year 10-month-old twin girl, was referred to this clinic for an audiological evaluation. She and her twin have a history of Niemann-Pick disease Type C and continue on cyclodextrin infusions. Cassidy is followed in the Hematology/Oncology at CHRCO. Her history is also significant for global developmental delays, neurologic problems, and learning problems. Reportedly, her last hearing evaluation at NIH approximately 2 years ago recorded normal hearing sensitivity.

PAIN ASSESSMENT: No pain was reported.

TEST PROCEDURES: Visual Reinforcement audiometry with the aid of a test assistant. Reliability was good for speech but poor for tones as Cassidy was unable to condition to the auditory tasks.

TEST RESULTS: In soundfield, responses to speech were observed down to 20 dBHL, for at least one ear. No further behavioral audiologic results could be obtained as Cassidy was unable to condition to the auditory task. Acoustic immittance testing revealed normal eardrum mobility, bilaterally. Transient-Evoked Otoacoustic Emissions (TEOAEs) were present and robust for 1500-4000 Hz bilaterally, consistent with normal cochlear outer hair cell function in each ear.

SUMMARY & EDUCATION: Cassidy presents with normal hearing sensitivity to speech stimuli for at least one ear. TEOAEs were robust in both ears today, consistent with normal cochlear outer hair cell function. Cassidy PASSED the TEOAE hearing screen in each ear. Her current auditory status is adequate to support her speech-language development.

RECOMMENDATIONS: Cassidy may return to the audiology clinic as needed; sooner as recommended by her physicians for monitoring purposes.

If there are any questions regarding this report, please do not hesitate to call (510) 428-3344.

Rebecca Richards, M.S., CCC-A
Pediatric Audiologist

CC: CHRCO MEDICAL RECORD #893505
Caroline Hastings MD, CHRCO Hematology/Oncology Dept
Mr. & Mrs. Hempel, 14125 Shadow Bow Drive, Reno NV 89511

*The pediatric medical center for Northern California
747 Fifty Second Street Oakland, CA 94609-1809*

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Page 1 of 1

Bay Area Pediatric Pulmonary (BAPP)
Karen Ann Hardy, M.D.
747 - 52nd Street
Oakland, CA 94609-1809
Phone: 510-428-3305
Fax: 510-428-3123

NAME: HEMPEL, CASSIDY
M.R.: 893505
B.D.: 01/23/04
Date of Visit: 10/08/09
Time of Visit: 0927

PULMONARY CONSULTATION
INITIAL VISIT

Patient brought to visit by on 10/8/09 Office site: Reno
CC: trouble breathing and sleeping
Source: parents, record from PMD

HPI:

I met mother today and reviewed the history for Addie and her twin Cassie. Mother is very well informed regarding their illness and she took this opportunity to share much of the background information with me today.

Cassie was a healthy infant toddler and one year old. In retrospect mother thinks she was a little clumsy, but nothing dramatic enough to warrant attention. At about 2 y/o she had a bad virus, flu like symptoms with fatigue and cough. She continued with these symptoms and gradually developed clear signs of ataxia and dementia. She also has splenomegaly which Mom believes was missed before the diagnosis due to many photos showing abdominal enlargement. She was referred to Stand LPHC and underwent a number of tests. They also had consultation by Dr. Caroline Hastings. Mom estimated that it took about 16 months to clarify her diagnosis of Nieman Pick C disease in 10/08. Additionally very recently she has been determined to have XMRV a newly discovered retrovirus. Researchers believe that the initial illness at 2 y/o was likely due to this virus.

Cassie is now a 5 y/o with NPC and XMRV. Recently she and her sister were started onto a compassionate use treatment protocol with twice weekly infusions of cyclodextrin. The NIH first has them infused with very low dose. After a few months of stability they agreed to a gradually escalating dose of CD which is increased 100mg/k each infusion. Since the infusions were started she has improved with less ataxia, increased vocalization and shrinking of the spleen. Her lysosomes are decreasing in size. OHSU testing her urine and 70fold increase in cholesterol excretion is documented since starting cyclodextrin. Glutathione levels also assessed and decreased. She has elevated oxsterols, 7 keto chol and 6 triol chol (documented in alzheimers and heart disease). Carriers and patients have this finding. Amyloid beta levels in CSF are very high per NIH testing.

The parents have now met a number of parents with children who have or have died from NPC. Many have counseled them to get pulmonary assessments and assistance with the twins. Mom currently notes that Cassie has difficulty with sleeping, waking intermittently through the night but that cough is not clearly the cause of this. She walks but is ataxic, she lost ability to talk, but now is making a lot more noise as she seems better. Her cough is weak and is not triggered normally. She drools and chews on bibs and her shirt. She is fed orally, picky

RE: HEMPEL, CASSIDY
MR: 893505

Report #: 1012-0044

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCT: OE Database CHO)

eater, spits if bites are too big, better with softer foods. Her chewing and eating is much better than her sister. , Cassie has a vertical eye gaze palsy. She also has Echolalia. Occasional new words this week "I like orchids". She may have absence seizures. Flickering tree shadows and lights can trigger these spells. Wakes up nights, occ night cough if sick. Generally don't cough even who very congested. No parasomias, pattern wake 5am 1-3 nap down for night about 9pm. Gets weak if laughs, not overt cataplexy, sensitive to smells but has much less gagging than her sister.

Other information from mother:

Parents involved to raise money and do research on both NPC and Now XMRV.

The new retrovirus XMRV has been found in about 10 million folks have this. It was discovered in UNR and is related to prostate cancer and may relate to chronic fatigue syndrome. Articles about this virus are breaking on Science any day. Mom and Dad and MGM have latent virus. Mom getting involved in the virus research and how it relates to NPC. When AIDS comes into the body it attaches to cholesterol. Researches think the XMRV does something similar and uses cholesterol machinery to infect a host.

The kids are on Cyclodextrin study. It is the active ingredient helping cat and mice in studies. This molecule can surround cholesterol. No one knows why it works on cats and mice. It has been shown to double the life span and organs normalize organ by decreasing cholesterol deposition in NPC animals. Pulmonary penetration for the drug is poor. No one is sure if lung disease is now recognized because of the longer life, or if alters macrophage function. UT Southwestern trying to nebulize to the lung to see if it will affect lung function. Additionally CD does not cross the blood brain barrier easily and perhaps inhalation will facilitate crossing this barrier.

Cyclodextrin also kills the AIDS virus - possibly removing the cholesterol from HIV so that medications work better. Research studies on CD use on monkeys is promising. CD also kills anthrax.

MEDICATIONS:

NAC 3000mg day, Mom gradually worked her way up per info from researchers
Cyclodextrin IV started at 80mg/k for 3 months, then increased 100mg/k/infusion and FDA agreed. At 2800mg/k twice weekly plan over 8 hours to get to max of 5000mg/k. Monitoring renal function with urine 5am and blood for Cr and Ccr on each infusion day.
Zevessa 100mg bid (for the XMRV)
Fish oil
Sesame oil
Coconut oil
Circummin 1800mg once daily (makes mice live longer)
Ibuprofen 75mg bid
Vit D3 5000 IU (makes mice live longer) never got any baseline data
Vit B2 def and on B complex once daily
Brazil nut and mushroom powder CHORI found abnormal ergothioneine in the girls and these replete it to boost glutathione (it was checked and low)

HOME EQUIPMENT:

MDI, spacers or compressors.

PAST MEDICAL HISTORY:

Birth History: Cassie 4#8, Mom on bedrest and Terb about 32 wks. c/sperformed at St Mary's at 35 wks.. Bottle fed, weak suck for BF. Similac used at first and

RE: HEMPEL, CASSIDY

MR: 893505

Report #: 1012-0044

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

changed to Horizon organic formula. Fed fine and gained weight.

Past Illnesses/Events: Normal development initially, tall for age and clumsy, not enough to raise suspicion, learning abc. With the infection at 2 the spleen already 12 cm and Mom thinks spleen already big earlier, but no one ever noticed.

Immunizations: UTD, no flu shot this year.

Hospitalizations: many usually Renown

Surgery: Port at CHRCO

Family History: neg for NPC, MGM carrier, no CF, DM, stroke, heart disease
MGM needs valve replacement and AMI at 49 yo, asthma MGM, no allergies, cancer
PGM breast, GGP stomach, PGGF heart issues

Social History: in school 3 d weekly, 6-7 kids, sp ed and ambulatory. MGP and Nanny for 6 years (Martha) (needs PPD) and Mom and Dad, no pets, ETS, Saddlehorn s Reno, they share bedroom carpeted, many stuffed animals always washed. Bedding washed every 3d, in diapers so changing all the time. House cleaner weekly too. Dust and vac daily.

Travel/Exposures: Mayo clinic, NIH, Children's Michigan for brain scans

Allergy History: never been evaluated

REVIEW OF SYSTEMS

Coughing or wheezing with exercise or activity? Only if sick

Coughing or wheezing with emotions or stress? None

Nocturnal coughing? None

Symptoms when sleeping? None

Recent acute attacks/flareups? None

Recent use of rescue medication? Never

Recent use of systemic steroids? Never

Recent visits to ER or hospital? For infusions

Absences from activities (school, work, sports) NA

GERD symptoms? Easy gag and vomit

Endo mom not sure glucose, none known

Neuro loss of function and regaining balance and speech (some therapy at school)

Allergy symptoms? Always stuffy and drippy

Allergy exposures? None

Allergy Avoidance? Compliant

Tobacco Smoke Exposure? None

Counseled re tobacco and/or allergy exposures? Yes

PHYSICAL EXAM:

Age 5 HR 99 RR 33 Wt 44.6#

Distress/Pain: congested nasal partial obstruction

Habitus: sitting child, in bed and IV running, watching TV avidly and vocalizing intermittently

Deformities: None

Skin: Normal

Conjunctiva/Lids: Normal

Eye Movement: Normal

PERRLA Normal

Tympanic Membranes: Normal

Nasal mucosa: externally normal, no discharge

Oral pharynx: healthy teeth, easy smile for mother

Tonsillar hypertrophy: None

Neck movement: Normal, no LAN and no gagging to touching her neck

Tracheal position: Normal

Jugular veins: Normal

RE: HEMPEL, CASSIDY

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Thyroid: Not visibly enlarged

Cough: None
Chest Shape: Normal
Retractions: None
Nasal Flaring: None
Air exchange: Normal with some transmitted upper airway noise
Stridor: None
Crackles: None
Wheezes or rhonchi: None
Percussion: Normal
Palpation: Normal

Heart sounds: Normal
Heart rate & Rhythm: Normal
Abdominal distension: None with enlarged liver and spleen.
Abdominal tenderness: None
GU normal female
Gait/station: some ataxia while sitting.
Clubbing, cyanosis, edema: None
Neuro/Development: development regression I will examine more thoroughly next visit since in midst of infusion now

LABS:
Sat 98%

STUDIES REVIEWED TODAY

3/15/09 MEDIPORT PLACED AND CXR good position of tip in SVC
4/15/09

CBC shows a platelet count of 125,000, hemoglobin 10.4, white count 4.8, 40 segs, 37 lymphs, 7 monos, 15 eosinophils. Sodium 137, potassium 3.7, bicarb 24, chloride 104, BUN less than 5, creatinine 0.4, glucose 86, calcium 9.0, SGOT 75, SGPT 14, albumin 3.4, total bilirubin 0.4. On 04/08 her creatinine clearance was 118 mL/minute. This was a 24-hour collection with a volume of 1250 cc. Her CBC and chemistries are similar to the pre-infusion studies drawn on 04/07.

9/21/09

LABORATORY STUDIES: CBC revealed a white blood cell count of 4200, hemoglobin 10.3, hematocrit 30.3, and platelet count of 207,000. Her ANC was 2350. A metabolic panel revealed a sodium of 137, potassium 3.9, chloride 104, CO2 23, BUN 13, and creatinine of 0.2. Her glucose was 100, and her calcium was 9.9. Her AST was 83, ALT 17, total bilirubin 0.6, and her direct bilirubin was 0.1. Her sedimentation rate was 22. Her uric acid was 3.1, amylase 43, and LDH 291. Her cholesterol was 153, triglycerides 138, and LDL 14. PT 13.6, INR 1.2, and PTT 39.7. Her urine protein to creatine ratio was 0.18, which was acceptable.
** reviewed labs during infusions and all have been stable and acceptable

ASSESSMENT:

Sleep disturbance: This is best evaluated with overnight polysomnogram. I explained the details of how sleep studies are performed and hope that we can get cooperation from Cassie for this. Due to the noted loss of muscle tone with laughing we will also check her MSLT multiple sleep latency testing.

Weak and discoordinated muscles: possible restrictive lung disease or poor chest muscle function and thus weak cough. Most children with NPC develop increased

RE: HEMPEL, CASSIDY
MR: 893505

Report #: 1012-0044

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

secretions and gradually diminishing cough and then pneumonias with recurrent infections leading to gradual respiratory failure. Sleep hypoventilation can exacerbate this. For some, GER and aspiration also add to lung damage.

Dysphagia: mild, able to maintain herself with oral feeding without overt gagging or aspiration.

NPC disease: genetics confirmed

PATIENT EDUCATION. As part of today's visit, we discussed the following:

- * Pathophysiology of disease
 - * Medications, including review of risks, benefits, and alternatives.
 - * Environmental & allergy issues
 - * Potential triggers to pulmonary symptoms.
 - * Signs and symptoms of respiratory distress.
 - * Use of equipment (such as spacers, nebulizers, peak flow meters, etc.)
 - * Prognosis and Treatment plan.
-

PLAN/RECOMMENDATIONS:

1. Continue same current medication plan
2. Obtain baseline PFT, PSG so that as new treatments are continued their affect on these parameters can be clearly monitored
3. Train the family in airway clearance techniques since these will be needed.

PENDING LABS: PSG, PFT with IOS, MIP and MEP at CHRCC

FOLLOW UP VISIT: monthly

Cc: PMD

Total visit time: 45 Educational time: 10

Thank you for referring your patient to us for pulmonary care. Please let us know if you have questions regarding our impression and plan. We look forward to working with you and your patient in the future.

KAREN A HARDY, MD

Electronically signed

10/13/09

1652

Report #: 1012-0044

Karen Hardy, M.D. (Director)
Edward Fong, M.D., Eric Zee, M.D., Manisha Newaskar, M.D.,
Danny Hsia, M.D., Haramandeep Singh, M.D.,

RE: HEMPEL, CASSIDY

MR: 893505

Report #: 1012-0044

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Sabnem Ozdogan, M.D., Pulmonary Fellow,
Renee Benson, M.D., Pulmonary Fellow, D. J. Kaley, RN
747 52nd Street, Oakland, CA 94609 Phone: (510) 428-3305 Fax: (510) 597-7154

RE: HEMPFL,CASSIDY
MR: 893505

Report #: 1012-0044

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Children's Hospital Oakland
Sleep Disorders Laboratory
Karen Hardy, M.D. and Haramandeeep Singh, M.D.
Co-Directors

RE: HEMPEL, CASSIDY
DOB: 01/23/04
MR #: 893505

747 52nd Street
Oakland, CA 94609
Phone: 510-428-3311 Fax: 510-450-5857

Study Date: 12/09/09
Report Date: 12/17/09

Clinical Polysemnographic Report

MULTIPLE SLEEP LATENCY TEST

NAP	SLEEP ONSET	REM PERIODS
1	15 MIN 7 SECS	NO
2	16 MIN 3 SECS	NO
3	20 MIN	NO
4	6 MIN 14 SECS	NO
5	20 MIN	NO

AVG: 13 MIN AND 34 SECS 0/5

IMPRESSIONS:

NORMAL STUDY. CRITERIA FOR NARCOLEPSY OR EXCESSIVE DAYTIME SLEEPINESS ARE NOT MET

RECOMMENDATIONS:

CLINICAL CORRELATION IS RECOMMENDED. FOLLOW UP IN SLEEP CLINIC AS NEEDED.

HARAMANDEEP SINGH, MD Electronically signed 12/17/09 1242

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Report #: 1217-0003 510-428-3305

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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Page 1 of 1

Children's Hospital Oakland
Sleep Disorders Laboratory
Karen Hardy, M.D. and Haramandeeep Singh, M.D.
Co-Directors

RE: HEMPEL, CASSIDY
DOB: 01/23/04
MR #: 893505

747 52nd Street
Oakland, CA 94609
Phone: 510-428-3311 Fax: 510-450-5857

Study Date: 12/08/09
Report Date: 12/17/09

Clinical Polysomnographic Report

ADDENDUM TO OVENRNIGHT PSG FROM 12/08/09:

EPOCHS WITH POSSIBLE EPILEPTIFORM ACTIVITY WERE REVIEWED. THESE DID NOT APPEAR TO INDICATE GROSS EPILEPTIFORM ACTIVITY. CLINICAL SEIZURE ACTIVITY WAS NOT OBSERVED.

CLINICAL CORRELATION IS RECOMMENDED. IF THIS REMAINS A CONCERN, SUGGEST A REPEAT OVERNIGHT PSG USING FULL EEG MONTAGE AND/OR NEUROLOGY CONSULTATION.

HARAMANDEEP SINGH, MD Electronically signed 12/17/09 1252

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CHO - PATIENT CARE INQUIRY ** LIVE ** (PCT: OE Database CHO)

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Page 1 of 1

Children's Hospital Oakland
 Sleep Disorders Laboratory
 Karen Hardy, M.D. and Haramandeep Singh, M.D.
 Co-Directors

RE: HEMPEL, CASSIDY
 DOB: 01/23/04
 MR #: 893505

747 52nd Street
 Oakland, CA 94609
 Phone: 510-428-3311 Fax: 510-450-5857

Study Date: 12/08/09
 Report Date: 12/11/09

Clinical Polysomnographic Report

CHIEF COMPLAINT: sleep disturbances, daytime staring and sleepiness, possible hypoventilation or OSA. Known Niemann Pick type C disease on biweekly infusions of IV Cyclohextrin

The patient was recorded by means of a nocturnal polysomnogram. The following parameters were measured during the entire recording: Electroencephalogram (EEG), electromyogram (EMG) of the chin, electro-oculogram (EOG), electrocardiogram (ECG), air flow and carbon dioxide level from the nose, respiratory effort at the chest and abdomen, finger oximetry, and snoring microphone.

I. SUMMARY OF POLYSOMNOGRAPHIC VALUES:

VARIABLES	Patient's values	Reference values
Total Sleep Time (TST; min.)	390.7	≥ 360
Sleep Efficiency (%)	88.7	≥ 85
REM Sleep (% TST)	27.7	15-30
Apnea Index (AI; #/hr)	0.2	≤ 1
Respiratory Disturbance Index (RDI; #/hr)	1.8	≤ 10
Max ETCO2	44.0	≤ 45
SaO2 nadir (%)	97.0	≥ 92
% time SaO2 $< 90\%$	0	0

Note: There are very few studies of normative values for polysomnography in children. Such values are based on very limited normative data and their predictive value should be used with caution. Most pediatric sleep laboratories define "acceptable" values for several of the above variables based on experience, and it is not possible to give numerical guidelines for these variables.

II. SLEEP ARCHITECTURE:

Total sleep time adequate.
 Sleep efficiency adequate.
 Proportion of REM sleep adequate.
 This is a technically adequate study.

III. DISTURBANCES OF SLEEP:

Cassidy did well with set up and fell asleep with help from the parents. She had rare events with obstruction (hypopnea x7sec, apnea x5 sec) without desaturation, one mixed apnea 6 sec and nine central apneas all without desaturation. Soft snoring occurred rarely

Possible seizure activity (6 epochs) noted and this study will be reviewed by Dr Singh for this purpose.

IV. IMPRESSION:

This study has minor abnormalities which were not clinically significant

V. RECOMMENDATIONS:

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

Clinical Polysomnographic ReportRE: CASSIDY HEMPEL
893505

Dr Singh will be reading th MSLT and will also review this study for seizure activity
Repeat study as clinically indicated is suggested.

Please contact us directly if you have any questions.

KAREN A HARDY, MD

Electronically signed

12/11/09

1300

CC:

Dr Caroline Hastings, the parents

HASTINGS, CAROLINE A MD

Sleep Lab Chart

Original: Medical Records Dept.

Karen Hardy, M.D. (Director),
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Report #: 1211-0003

510-428-3305

CHO - PATIENT CARE INQUIRY ** LIVE ** (PCI: OE Database CHO)

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CHILDREN'S HOSPITAL OAKLAND
Pulmonary Function Laboratory
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747 52nd Street
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Phone: 510-428-3311 Fax: 510-450-5857

PATIENT: HEMPEL, CASSIDY
BD: 01/23/04
MR#: 893505
Date of test: 12/08/2009

Primary Care Physician: HASTINGS, CAROLINE A MD

Ordering Physician: HARDY, KAREN A., M.D.

PULMONARY FUNCTION TEST

Interpreting Pulmonary Fellow: Renee Benson, M.D.
Interpreting Pulmonologist: Karen A. Hardy, M.D.

RESULTS

Standard pulmonary function testing assumes that patient's effort, cooperation, and ability to perform the tests do NOT limit measurements of the patient's lung function.

When parents arrived with the twin girls, it was stated that the girls are not cooperative, as neurologically they are only 2 years old and do not like anything whatsoever in their mouths. Therefore IOS testing was not accomplished today.

We then decided to try MIP/MEP testing, where Cassidy had to lying down and held, which is not optimal with Cassidy, but measurements were taken with only a little fuss. However, since she did not understand how to perform the maneuver, nor was she sitting up, the measurements could be technically lower than predicted.

Effort, cooperation and ability to perform the tests WERE ADEQUATE. The test results are technically valid.

RESPIRATORY MUSCLE STRENGTH DATA

MIP (cmH2O) 100
MEP (cmH2O) 60

CONCLUSIONS

(X) PULMONARY FUNCTION TESTS WERE NORMAL.

() Normal combined respiratory muscle strength.

COMMENTS AND SUGGESTIONS

If you wish a copy of the raw data, please call the PFT lab and the data can be sent to you.

There was no previous study for comparison.

_____, M.D.
RENEE BENSON, M.D.
Pediatric Pulmonary Fellow

_____, M.D.
KAREN A. HARDY, M.D.
Pediatric Pulmonary Attending

RENEE C BENSON, MD

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Bay Area Pediatric Pulmonary
Report #: 1208-0010 510-428-3305

Use of 2-Hydroxypropyl- β -Cyclodextrin as an Intrathecal Drug Vehicle with Opioids^{1,2}

JONGDAE JANG, TONY L. YAKSH and HARLAN F. HILL³

Department of Anesthesiology, University of California, San Diego, La Jolla, California (J.J., T.L.Y.) and Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington (H.F.H.)

Accepted for publication January 31, 1992

ABSTRACT

2-Hydroxypropyl- β -cyclodextrin (CDEX), a seven-membered glucose pyranose structure, forms reversible inclusion complexes with the lipophilic portion of a drug molecule by noncovalent bonding. This can increase the water solubility of lipid-soluble drugs and reduce the rate of clearance of such agents from the spinal cord into the vasculature after i.t. administration. In this study, opioids (morphine, lofentanil, alfentanil and sufentanil) with and without CDEX (20, 2, 0.2 and 0.02% w/v in sterile water) were administered spinally in rats prepared with chronic i.t. catheters. CDEX prolonged the duration of analgesia (52.5°C hot plate) and reduced the incidence of catalepsy otherwise produced by a supermaximal i.t. dose of each of the opioids.

The magnitude of the potentiating effect of CDEX on opioids was dependent upon concentration of the CDEX and varied with drug lipid partition coefficients. The highest concentration of CDEX alone (20%) had no effect upon the volume-evoked micturition reflex, blood pressure, heart rate, or spinal reflexes. Our data indicate that CDEX may be a useful i.t. vehicle for modifying the redistribution characteristics of highly diffusible molecules after their i.t. administration, and that for each drug there is an optimal CDEX concentration. In the present case, CDEX prolongs the spinal analgesic action and reduces the supraspinal actions of i.t. drugs.

The β -cyclodextrins are nonionic cyclic polysaccharides composed of seven glucose moieties with the shape of a toroid or hollow truncated cone (Guttman *et al.*, 1988). To enhance β -cyclodextrin solubility and diminish its tendency to crystallize, substitutions onto the primary and secondary hydroxyl groups have been employed to create amorphous compounds such as CDEX (Pitha *et al.*, 1988). The cavity of these substituted cyclodextrins is relatively hydrophobic, whereas the external surfaces are hydrophilic, with the edge of the torus of the larger circumference containing chiral 2-hydroxypropyl groups.

An important property of cyclodextrins as drug carriers is that they form reversible inclusion complexes with drugs by noncovalent bonding (Uekama and Otagiri, 1984), mainly hydrogen bonds, within the hydrophobic cavity of the toroid. The reversibility of the complex formation is significantly influenced by the geometry of the CDEX molecule and the lipid solubility of the inserted portion of the guest molecule (Szejtli,

1982). Typically, drugs with high lipid solubility will form more stable CDEX complexes and be less likely to dissociate under any given set of conditions.

Of particular importance, inclusion complex formation can lead to the alteration of physical and chemical properties of the guest molecule. First, by virtue of the hydrophilic exterior of the CDEX molecule, complexation can increase the aqueous solubility of lipophilic drugs (Imai *et al.*, 1988). Second, the diffusional properties of the complexed drug can be dramatically altered by the large hydrophilic entity to which it is now complexed. Third, as the drug is reversibly complexed, it will typically display mass action kinetics, showing a time-dependent dissociation into the surrounding biophase (Szejtli, 1982). As a result, only a small fraction of the total drug may be free in the biophase and available for subsequent clearance or biological interaction. These properties thus provide a mechanism for markedly altering the kinetics of lipid-soluble agents.

Although considerable work has been done with systemic administration of CDEX drug complexes, one novel route is the use of CDEX molecules for delivery of drugs which are directed at sites in the central nervous system. One particular site of scientific and clinical attention is the spinal cord. A behaviorally relevant example of such a targeted drug effect is the analgesia produced by the local action of opioids on spinal

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² Portions of these data here were first reported in the abstract: Effect of β -cyclodextrin on analgesia produced by intrathecally administered opioids. *Neuroscience* 16: 211, 1990.

³ Deceased.

ABBREVIATIONS: CDEX, 2-hydroxypropyl- β -cyclodextrin; CSF, cerebrospinal fluid; AUC, area under the effect curve; %MPE, percent maximum possible effect; ANOVA, analysis of variance.

nociceptive processing observed after i.t. administration (Yaksh, 1987). Intrathecally administered drugs may undergo redistribution by 1) rostral movement in the CSF by bulk flow and/or 2) by diffusion into the spinal or epidural vasculature (Durant and Yaksh, 1986). In the first case, the rostral movement of significant quantities of agent depends upon the presence of large quantities of free drug in the CSF. In the second case, after i.t. administration, agents with high lipid solubility rapidly enter the spinal tissue, and free drug levels in the CSF are thus cleared rapidly from the i.t. space. Diffusion into tissue along the concentration gradient of the drug results in concurrent rapid movement from tissue into the parenchymal capillaries. There is thus an immediate clearance into plasma (Herz and Teschemacher, 1971). This vascular redistribution can result in short durations of action and unacceptable effects mediated by actions of the drug outside of the spinal cord. Thus, the primary side effect of spinal opioids is the depression of behavior (somnia, catalepsy) and respiration due to supraspinal movement and action of the drug (Yaksh, 1987; Cousins and Mather, 1984). In recent studies, we demonstrated that CDEX can be used as an i.t. delivery vehicle for insoluble agents such as capsaicin and peptides (Yaksh *et al.*, 1991). The vehicle itself does not display an effect on behavior or any evidence of neurotoxicity. Significantly, in these studies, CDEX appeared to augment the effect of the spinally administered agents, presumably in part by a modification of the redistribution or metabolism of the drug.

Given the above considerations, we sought in the present studies to characterize the action of CDEX in modifying the spinal antinociceptive action and the supraspinal side effects of a series of opioids, namely morphine and the 4-anilinopiperidines. These agents, although similar in molecular weight, vary widely in their lipid partition coefficients and ionization constants and in their propensity for redistribution to extraspinal sites (Yaksh *et al.*, 1986).

Methods

Intrathecal Model

Surgery. For placement of the chronic i.t. catheters, adult male Sprague-Dawley rats (250–300g) were anesthetized with halothane (2%) in air and placed in a stereotaxic headholder. A midline incision was made at the level of the superficial neck muscles beginning at the occipital crest and extending caudally about 2 cm. Subsequently, retraction of the underlying muscle layer exposed the atlanto-occipital membrane. The membrane was incised with a 22-gauge needle and then gently retracted with a small hook. The tip of the PE-10 i.t. catheter was inserted into the subarachnoid space and then carefully advanced in a caudal direction for 8 to 9 cm. The caudal end of the catheter was located at the level of the lumbar enlargement. The external tip emerged through the skin in the occipital region and was closed with a stainless steel pin. Neck muscles and skin were sutured in two planes. Testing was then carried out after 3 to 5 days.

Behavioral Assessment

Nociceptive tests. A cutaneous thermal (hot plate) test was used for assessing nociceptive responses. Briefly, the rat was placed on a metal surface maintained at 52.5°C and enclosed by a clear Plexiglas wall to form a space 20 × 30 cm. The test measure was the latency between the time of placing the animal on the surface and the behavioral endpoint. In the majority of animals, licking the hind paw was observed; in the remainder, jumping was the endpoint. Failure to respond by 60 sec on the hot plate (cutoff time) resulted in termination of the experiment and the assignment of that value as the response

latency. Response latencies were measured before and at 5, 15, 30, 60 and 120 min after injection.

General behavior. Catalepsy, rigidity and presence or absence of placing and righting response were assessed. Catalepsy was defined as the failure of the animal to move within 10 sec from a position in which the forepaw was placed on a horizontal bar 4 cm from the cage floor.

Cystometrography. Details of the *in vivo* chronic unanesthetized rat bladder model have been reported elsewhere (Durant and Yaksh, 1988). Briefly, under anesthesia, PE-90 tubing was permanently placed into the wall of the bladder through a laparotomy and externalized. The laparotomy was closed, and the animals were allowed 5 days to recover before being used for cystometrography. To carry out cystometrography, the rats were placed in a loose restrainer. A funnel leading to a strain gauge mounted cup was placed under the abdomen to measure urine outflow. The bladder was infused with saline at a rate of 200 μ l/min and the pressure in the bladder was continuously measured. With filling, the bladder displayed transient contractions, relaxation of the sphincter and an emission of bladder contents.

Cardiovascular responses. An arterial catheter was inserted into the tail artery of each rat with chronic i.t. catheter after induction of anesthesia with halothane. A tracheal tube was inserted and connected to a respirator. Heart rate and blood pressure were monitored throughout each experiment. Before and 5 min after i.t. injection of 20% hydroxypropyl- β -cyclodextrin (10 μ l), the tail was immersed in 53°C water for 15 sec. The magnitude of the tail flick response and the elevation in arterial blood pressure evoked by the immersion were measured.

Intrathecal Drugs and Injections

Drugs were delivered in either isotonic physiological saline (0.9% w/v) or in CDEX (Research Biochemicals, Natick, MA) dissolved in distilled water. This material was designated as being 40% substituted with 2-hydroxypropyl substituents. Dose-response curves were carried out with: morphine sulfate (MW = 285, log P = 0.14; Merck, Elmsford, NY), sufentanil citrate (MW = 579, log P = 3.2; Janssen), lofentanil oxalate (MW = 499, log P = 3.6; Janssen, Beerse, Belgium), alfentanil HCl (MW = 471, log P = 2.1; Janssen) (Meuldermans *et al.*, 1982). To examine the antagonistic action of naloxone on the high-dose actions of spinal opioids, rats received an i.p. injection of naloxone (1 mg/kg) followed by the i.t. injection of different doses of the opioids when they showed catalepsy.

All drugs were prepared for i.t. delivery in a volume of 10 μ l. Each injection was followed by injection of 10 μ l of saline to ensure that the drug was delivered.

Experimental Paradigms

In the present studies on the effects of CDEX, three separate sets of experiments were carried out. For each drug dose treatment group, five or eight animals were used.

Phase 1. The effects of saline and different concentrations of the CDEX vehicle on the dose-dependent antinociceptive effects of the four opioid agonists were determined. In each case, time-effect curves were obtained for different doses of the four agonists in vehicles consisting of saline and CDEX in concentrations of 0.02, 0.2, 2.0 or 20% w/v. For lofentanil and morphine, an additional group using 0.002% CDEX was used.

Phase 2. For each of the four opioid agonists, two opioid doses were selected which were observed to produce modest effects on the hot plate response when administered i.t. in a saline vehicle. The time-effect curves on the hot plate test for these doses were then determined when the drugs were administered in different concentrations of the CDEX vehicle (0.02, 0.2, 2.0 and 20%). For lofentanil and morphine, an additional group using 0.002% CDEX was used.

Phase 3. In separate groups of rats, the effects of the CDEX alone on blood pressure, the micturition reflex, were examined. In addition, in other animals, the incidence of catalepsy produced by the spinal injection of a maximal or a supermaximal dose of the several opioids in a saline vehicle was determined when that dose was administered in

an optimal (as defined in phase 1 and phase 2) or a maximal (20%) concentration of CDEX. In these animals, after the observation of catalepsy, 1 mg/kg naloxone was given i.p.

Statistics

Response latencies are presented as group means of the %MPE where $\%MPE = [(postdrug\ latency - predrug\ latency) / (cutoff\ time - predrug\ latency)] \times 100$. To compare the duration of action, AUC (%MPE *vs.* time in minutes after injection up to 120 min) were computed using the trapezoidal rule. For phase 1, dose-response curves for %MPE were calculated with $ED_{50} \pm 95\%$ confidence intervals. For the MPE curves, the data for each rat were the maximum obtained during the observation interval (2 hr). The AUC was calculated on the base of a 2-hr observation interval.

To permit comparison of doses and calculation of potency ratios, relative potency and duration ratios as compared to saline were computed with 95% confidence intervals. All analyses were carried out according to the statistical program of Tallarida and Murray (1986). Because the computer program to determine potency ratio requires equal animals and some drug groups were performed with five animals, the data from the saline vehicle ($N = 8$) were compared to the CDEX group ($N = 5$) by taking the data of the first five rats run with the saline group. Comparison of the ED_{50} calculated for this truncated control group with the ED_{50} of the complete control group revealed no difference. In the phase 2 study, to assess the effects of CDEX on the %MPE and AUC of different drug doses as a function of CDEX concentration, one-way ANOVA were performed. If the ANOVA values were statistically significant, a multiple means analysis (Duncan) was carried out. In phase 3, for the incidence of catalepsy, a χ^2 analysis was used.

Results

Phase 1: Intrathecal Opioid Dose-Response Curves in CDEX

Saline vehicle. The i.t. injection of the opioid agonists in saline vehicle resulted in a significant elevation of hot plate response latencies with no detectable effect on motor function. The magnitude of the effect produced by each opioid was monotonically dose dependent with the rank ordering of potency being lofentanil, sufentanil, morphine, alfentanil (see table 1 and fig. 1). At doses which just blocked the hot plate response, the ordering of the duration of action for i.t. doses yielding similar peak effect was morphine > lofentanil > sufentanil > alfentanil (figs. 2 and 3).

CDEX vehicle. CDEX alone had no effect on the hot plate response latency at the highest concentrations employed (10 μ l of 20% CDEX, see fig. 3). Complete dose-response curves were obtained in the presence of different concentrations of CDEX (0.02–20%) for each of the four opioids. As shown in figures 1 and 2, this resulted in a leftward shift in the %MPE and AUC dose-response curves. Comparison of slopes revealed no statistically significant changes as a elevation of CDEX concentrations ($P > .05$; data not shown). Over the range of CDEX concentrations (0.02% to 20% w/v), the degree of potentiation

TABLE 1

Summary of MPE (ED_{50}) of dose-response curves of drugs in saline with the lower (LI) and upper (UI) 95% confidence intervals for the ED_{50}

Values calculated from dose response curves in which each curves represents three to five doses with eight rats.

		Morphine	Sufentanil	Alfentanil	Lofentanil
ED_{50} (μ g)		3.5	0.3	8.5	0.04
95% CI	LI	0.3	0.04	1.8	0.01
	UI	14	1	13	0.2

tended to be related in a biphasic manner to CDEX concentration. To more clearly present this biphasic effect, a least squares linear regression was carried out on the dose-response data presented in figures 1 and 2 and respective %MPE and AUC dose ratios were calculated. Figures 4 and 5 present the ED_{50} dose ratios and AUC dose ratios with 95% confidence interval plotted *vs.* the respective CDEX concentration. These observations emphasize the fact each spinally administered opioid displayed an optimal concentration of CDEX. Thus, the optimal concentrations were 2% for lofentanil, 0.2% for sufentanil, 2% for alfentanil and 0.02% morphine. Moreover, it is clear that the degree of dose-effect augmentation differed between the different agents. Thus, for the peak effect and AUC, the ordering of the leftward shift as compared to saline vehicle was morphine > lofentanil > sufentanil > alfentanil (see figs. 4 and 5).

Phase 2: Intrathecal CDEX Dose-Response Curves with Opioids

In separate experiments, the above findings were replicated by preparing stock solutions of each agonist and adding these stock solutions to different CDEX concentrations. As indicated in figures 6 and 7, similar results were observed as described in phase 1 with certain CDEX concentrations found to be associated with a maximum response for each drug. In each case, a one-way ANOVA revealed a statistically significant main effect across CDEX concentration ($P < .05$). Using a multiple range test, as shown in figures 6 and 7, similar optimal CDEX concentrations were observed for each of the two opioid drug concentrations: morphine, 0.02%; sufentanil, 0.2%; alfentanil and lofentanil, 2.0%. It should be stressed that at the higher concentrations of each agonist (particularly lofentanil and morphine), the optimal CDEX concentrations tended to be obscured by the plateau effect on both the MPE and AUC measures resulting from a maximum cutoff latency.

Phase 3: Behavioral Effects of Intrathecal Opioids and CDEX

The administration of CDEX alone at the highest concentration used (10 μ l/20%) had no acute effect on gross behavior. No agitation or evidence of irritation was observed. There was no change in any index of motor function, including the placing or stepping reflex or ambulation after i.t. administration of 20% CDEX (10 μ l).

Cardiovascular system. Measurement of blood pressure and heart rate in the halothane (0.8%) -anesthetized rat revealed no change during a 30-min period after injection of CDEX solution ($N = 5$; maximum change from base line within 30 min = 5.2 ± 0.8 mm Hg; 7.0 ± 1.0 beats/min).

Cystometrography. In animals prepared with chronic bladder and i.t. catheters undergoing cystometrography, there was no change in the frequency (maximum change = 0.02 ± 0.001 contractions/min) or pressure contraction of the bladder (maximum change = 1.9 ± 0.1 cm H₂O) using the volume-evoked cystometrograph during a 60-min period after the i.t. administration of CDEX (10 μ l/20%).

Effects on supraspinal action of opioids. As shown in table 2, i.t. administration of high doses of the opioids in saline (lofentanil, 0.3 μ g; alfentanil, 100 μ g; morphine, 100 μ g; sufentanil, 3 μ g) resulted in a 100% incidence of catalepsy, indicating significant supraspinal redistribution of the drugs. Catalepsies with high doses of opioids were completely reversed by i.p.

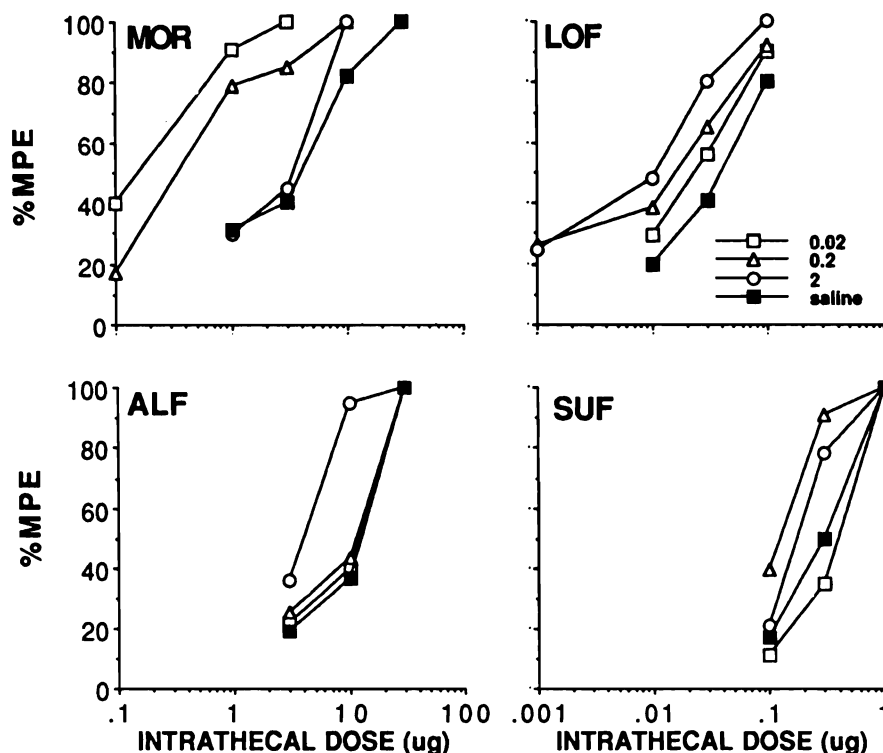


Fig. 1. Log dose-response (%MPE) curves for the hot plate response for morphine, lofentanil, alfentanil and sufentanil given i.t. in saline or CDEX (0.02–2%). Data for 0.002 and 20% CDEX solutions were deleted for clarity (see fig. 4). The %MPE is calculated on the basis of the maximum effect obtained within the 2-hr observation period. Doses above those which were supramaximal (i.e., produced greater than %MPE = 100%) are not shown and were not used to calculate dose-response regressions. Each point presents the mean of five to eight rats. S.E. bars were deleted for clarity. See figure 4 for dose ratios with 95% confidence intervals.

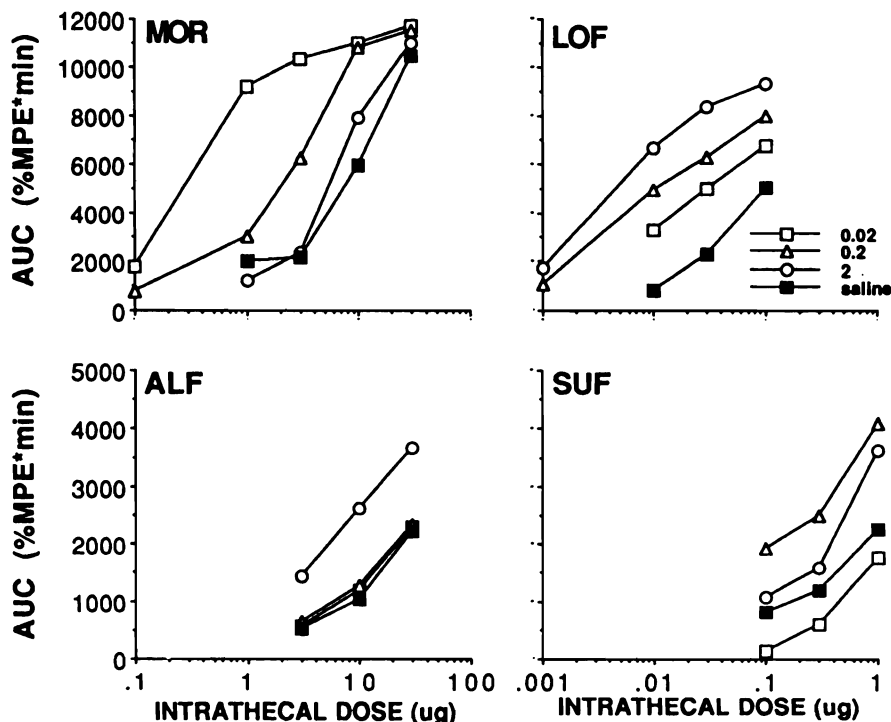


Fig. 2. Log dose-response (AUC) curves over the 2-hr observation interval for the hot plate response for morphine, lofentanil, alfentanil and sufentanil given i.t. in saline or CDEX (0.02–2%). Data for 0.002 and 20% CDEX solutions were deleted for clarity (see fig. 5). Each point presents the mean of five to eight rats. S.E. bars are deleted for clarity. See figure 5 for dose ratios with 95% confidence intervals.

administration of naloxone (1 mg/kg). To examine the decreased incidence of catalepsy with CDEX, rats received i.t. injections of the high dose of the agonist in the presence of the optimal concentration of CDEX (sufentanil, 0.2%; morphine, 0.02%; lofentanil alfentanil, 2%; see figs. 4 and 5) or in the maximum concentration of CDEX (20%). As indicated, catalepsy at the highest dose of opioid used was absent with the highest concentration of CDEX. These differences were significant according to a χ^2 test. Also indicated is the incidence of catalepsy produced by the optimal opioid dose and CDEX concentration which provided a maximum antinociceptive ef-

fect. At this concentration, producing maximum antinociceptive effects, catalepsy was also essentially absent. This separation is most clear for the lipid-soluble agents.

Discussion

Cyclodextrin derivatives have been used as drug carrier systems for the systemic administration of a variety of drugs. Aside from the ability to facilitate solubilization of lipophilic agents, the use of cyclodextrins for microencapsulation of a

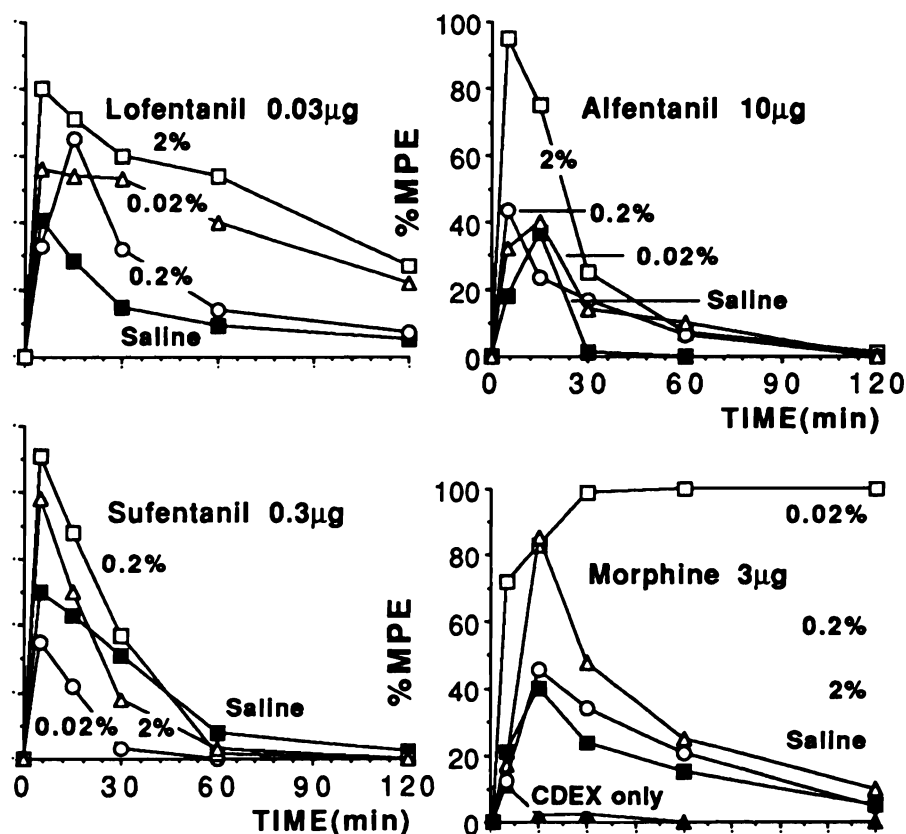


Fig. 3. Time course of hot plate response for i.t. administered morphine (3 μg), lofentanil (0.03 μg), alfentanil (10 μg) and sufentanil (0.3 μg) in saline or CDEX (0.02–2%) solution. Each line represents the mean data for five to eight rats. S.E. bars are deleted for clarity. Drug experiments were also carried out with 0.002 and 20% CDEX, but data were deleted for clarity. "CDEX only" indicates hot plate test with CDEX solution (20%/10 μl).

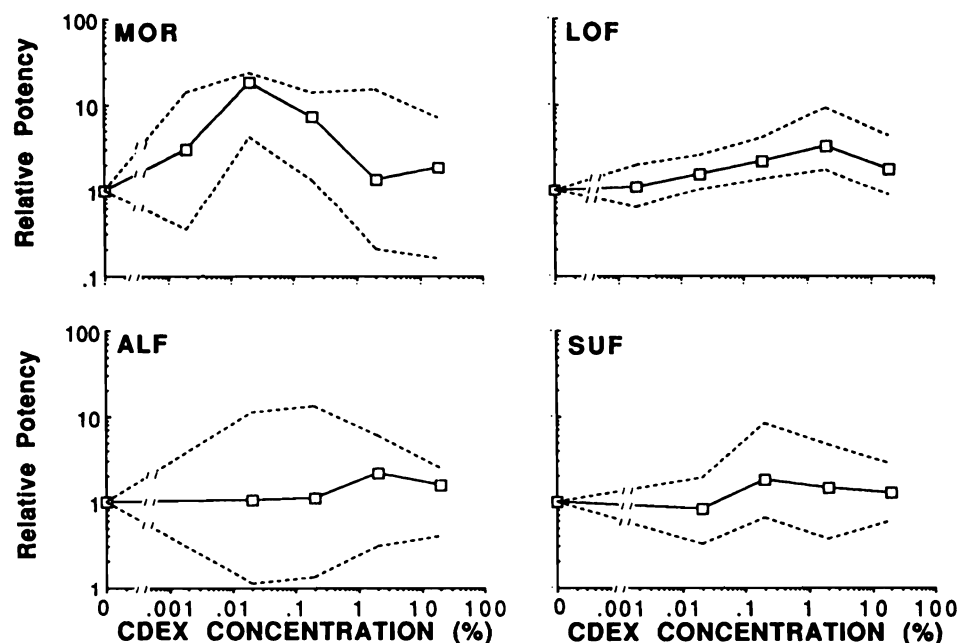


Fig. 4. Intrathecal potency ratios (ED_{50} of drug in CDEX/ ED_{50} of drug in saline) for i.t. morphine, lofentanil, alfentanil and sufentanil plotted as a function of CDEX concentration with 95% confidence interval (dotted line). Analyses are obtained from the data presented in figure 1. See text for details and analysis.

variety of agents has been shown to significantly alter the bioavailability of molecules (Szejtli, 1982).

In the present work, we specifically considered the qualities of CDEX as a vehicle for the delivery of drugs into the CSF. We demonstrated that even at the highest concentrations, spinally administered CDEX alone produced no observable effect. However, using water-based solutions of CDEX as a drug vehicle, we found that the time course and magnitude of the spinally mediated antinociceptive activities of the opioids examined were enhanced by combining the drugs with CDEX.

Similar results have been reported by others (Meert *et al.*, 1991). Thus, for morphine, alfentanil, sufentanil and lofentanil, CDEX served to 1) increase their duration of action, 2) enhance the apparent magnitude of antinociceptive effect and 3) diminish the extent to which high doses of the i.t. opioids, especially the lipid-soluble 4-anilino-piperidines, would evoke supraspinally mediated behavior. The present studies, using pharmacologically active agents which differ significantly in their lipid partition coefficients (Hug, 1984), revealed that these effects of CDEX appeared to be influenced by several properties,

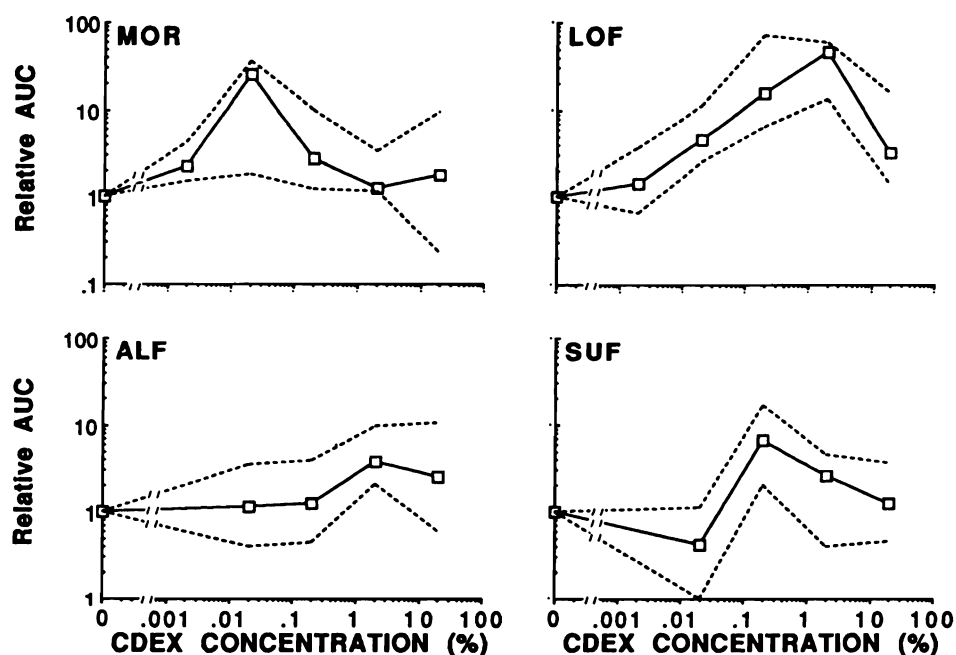


Fig. 5. Relative duration of action (AUC of drug in CDEX/AUC of drug in saline) for i.t. morphine, lofentanil, alfentanil and sufentanil plotted as a function of CDEX concentration with 95% confidence interval (dotted line). Analyses are obtained from the data presented in figure 2. See text for details and analysis.

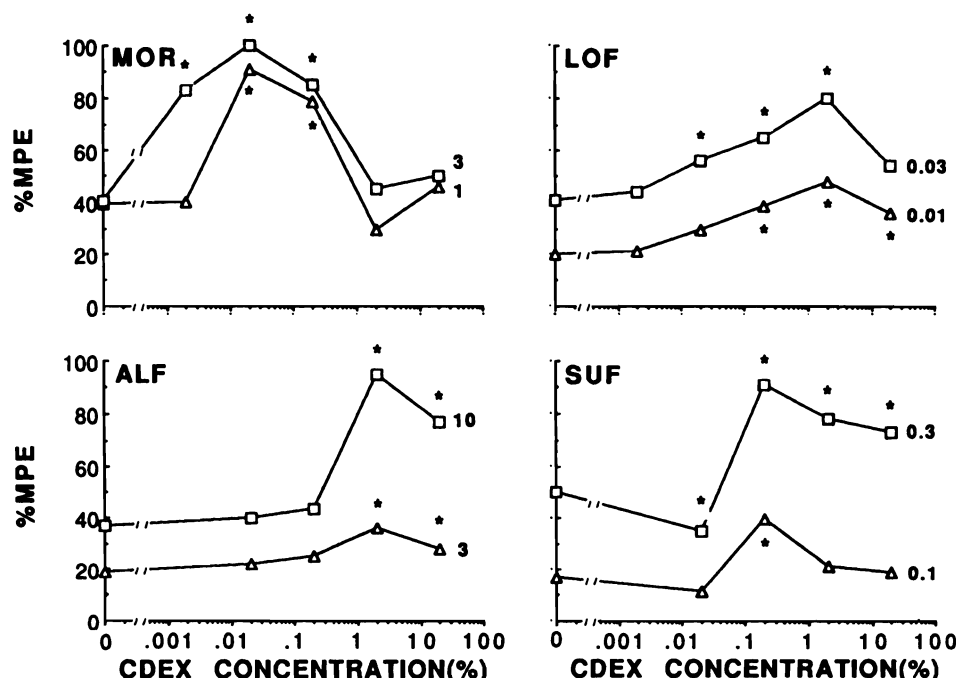


Fig. 6. Plot of %MPE, measured at the peak response observed in the 2-hr period, vs. the concentration of CDEX in vehicle for i.t. morphine (1 or 3 μ g), lofentanil (0.01 or 0.03 μ g), alfentanil (3 or 10 μ g) and sufentanil (0.1 or 0.3 μ g) in saline or CDEX (0.002–20%) solution. CDEX concentration 0 indicates saline groups (*i.e.*, drug in saline) ($N = 5-8$ rats/point). One-way ANOVA across CDEX concentrations were statistically significant for all drug doses ($P < .05$). * $P < .05$ vs. drug in saline as determined by Duncan multiple range test. See text for further details.

including the lipid solubility of the drug and the concentration of CDEX used with each drug.

Factors Governing the Effects of CDEX as a Vehicle

CDEX encapsulates drugs by virtue of its hydrophobic interior which is thermodynamically favored by appropriately sized nonpolar portions of a drug molecule. The nominal size of the internal cavity in the β -cyclodextrin molecule (approximately 7–8 Å) represents a spatially limiting factor. Thus, a variety of chemical groups on the guest molecule, such as alkyl and phenolic groups, are amenable to insertion (Catena and Bright, 1989). The guest/host interaction is reversible and corresponds to a mass action relationship. Drugs with high lipid partition coefficients and a high propensity to form hydrogen bonds will result in a minimum energy conformation within the inclusion cavity, high complex stability corresponding to a higher affinity

binding and diminished likelihood of dissociation (Szejtli, 1982). It is conceivable that a drug complexed with CDEX may remain accessible and even bind to its specific receptor site, but the steric hindrance due to the bulky CDEX adjunct does not favor this possibility (Szejtli, 1982). The biological activity of a drug in a CDEX solution probably requires free drug. Thus, factors which govern dissociation from CDEX will influence drug activity (Kempfle *et al.*, 1987). Several factors likely influence guest/host dissociation.

First, a high lipid partition coefficient, as noted above, will result in a slow rate of drug dissociation (Szejtli, 1982). It should be emphasized that slow rates of dissociation do not *a priori* exclude the use of a CDEX complex. Drug dissociation is based on the law of mass action, and if the drug is diffusing into a relatively large volume, such as the CSF or the spinal cord, then the equilibrium conditions will permit the develop-

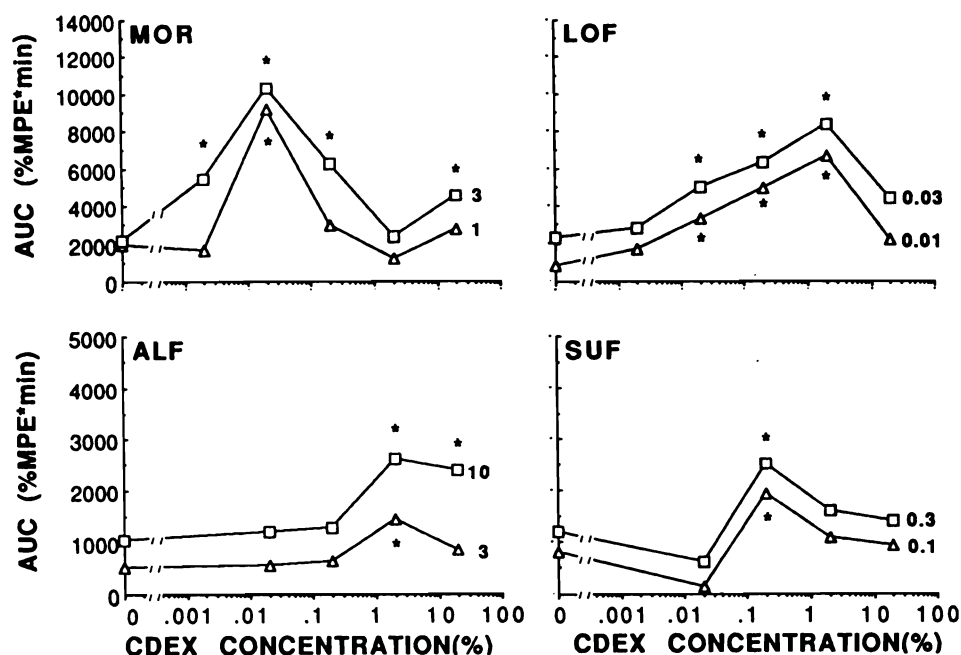


Fig. 7. Plot of AUC vs. the concentration of CDEX in vehicle for morphine (1 or 3 μ g), lofentanil (0.01 or 0.03 μ g), alfentanil (3 or 10 μ g) and sufentanil (0.1 or 0.3 μ g) in saline or CDEX (20%) solution. CDEX concentration 0 indicates saline groups (i.e., drug in saline) ($N = 5-8$ rats/point). One-way ANOVA across CDEX concentrations were statistically significant for all drug doses ($P < .05$). * $P < .05$ vs. drug in saline as determined by Duncan multiple range test. See text for further details.

TABLE 2

Summary of behavioral signs described after i.t. lofentanil, alfentanil, morphine and sufentanil at maximum and supermaximum doses with and without CDEX

Drug	Vehicle	Dose	N ^a	%Catalepsy ^b	Onset of catalepsy
μ g/10 μ l					
Lofentanil	Saline	0.1	0/10	0	
		0.3	5/5	100	<1 min
	CDEX (2%)	0.1	2/20	10	<1 min
		0.3	4/5	80	<1 min
	CDEX (20%)	0.1	0/6	0	
		0.3	0/5	0	
Alfentanil	Saline	30	0/6	0	
		100	6/6	100	<1 min
	CDEX (2%)	30	0/10	0	
		100	3/3	100	<1 min
	CDEX (20%)	30	0/7	0	
		100	0/6	0	
Morphine	Saline	30	0/6	0	
		100	5/5	100	<1 min
	CDEX (0.02%)	30	0/5	0	
		100	1/6	16.7	<30 min
	CDEX (2%)	30	0/20	0	
		100	0/11	0	<60 min
Sufentanil	Saline	1	0/4	0	
		3	5/5	100	<1 min
	CDEX (0.2%)	1	1/10	10	<1 min
		3	0/8	0	
	CDEX (20%)	1	0/8	0	
		3	0/5	0	

^a Number of rats which showed catalepsy/total number of rats.

^b Catalepsies observed in this experiment were reliably reversed with 0.3 to 1 mg/kg i.p. naloxone given after observation (data were not shown).

ment of sustained steady-state free drug concentrations in the space where CDEX concentrations are low. Indeed, in recent studies, we examined the neurotoxicity of the very lipophilic neurotoxin, capsaicin. Aside from providing a highly compatible drug delivery system, the agent in CDEX still clearly retained pharmacological activity, resulting in a significant depletion of substance P from the spinal dorsal horn (Yaksh *et al.*, 1991).

Based on results with morphine, sufentanil and lofentanil, the optimal CDEX concentration values appeared to be inversely related to the lipid partition coefficients. This observation that the optimal concentration of CDEX with alfentanil, in spite of its relatively low log P , would appear to correspond with the large fraction of alfentanil which exists in an unionized state at physiological pH.

Second, the availability of free drug in a drug-CDEX solution administered into a kinetically limited space is dependent upon the quantities of free CDEX present. If the CDEX/drug ratio is high, there will be an abundance of free CDEX, and the free CDEX (a pool of binding sites) will compete with the tissue for drug redistribution. Thus, if a relatively high CDEX/drug ratio is administered i.t. or epidurally, it appears that the effect of the drug will be diminished. If a relatively low CDEX/drug ratio is present, redistribution of the drug from the spinal space will not be significantly delayed. This theoretical position argues that in the CSF, there may be an optimal concentration of CDEX for any given drug at a specific dose. Other factors such as molecular shape or charge distribution may play important roles in CDEX-opioid complexation dynamics, but there are at present insufficient data to consider them.

Toxicology of Cyclodextrin

Studies on the toxic effects of cyclodextrin have been conducted for oral, i.v., s.c., i.p. and i.m. administration. Toxicity with nonsubstituted β -cyclodextrin was largely due to the crystallization after concentration in the kidney (Szejtli, 1982). No evidence of toxicity was observed in the present study after i.t. injection of 20% CDEX (10 μ l) as measured by motor function, blood pressure, heart rate or micturition. CDEX is known to be resistant to the usual hydrolyzing enzymes and may be metabolized by intestinal flora (Uekama and Otagiri, 1984). In view of its resistance to hydrolysis and relatively high molecular weight, clearance of CDEX from the central nervous system probably occurs by bulk flow of CSF. Studies on the metabolism and clearance of i.t. CDEX are few. In recent studies, we observed that the clearance of radiolabeled CDEX (β -cyclodextrin- 3 H) from spinal CSF after i.t. injection with serial CSF

sampling model in rats was identical to inulin [^{14}C], suggesting clearance by bulk CSF flow (J. Jang and T. L. Yaksh, unpublished observation).

Cyclodextrin on Spinal Drug Kinetics

In this study, CDEX augmented the antinociceptive effects of the i.t. administered opioids as compared to their effects when administered in the saline vehicle. As noted in the introduction, after i.t. administration, drugs are cleared from the CSF by movement into tissue, and thence by absorption into the spinal capillary net or by rostral transfer after the redistribution of CSF. It appears likely that the augmented effect and duration of action of opioids produced by CDEX reflects altered clearance of the drugs from the CSF/spinal axis, due to reduced diffusion of drug into the vasculature. The absence or decrease of the supraspinal effects otherwise typically observed after the spinal administration of high doses of these opioids (see table 2) supports this possibility. CDEX appears to provide a slow release reservoir, diminishing the amount of free drug in the CSF available to redistribute into blood. Kinetic distribution studies are currently in progress to confirm this consideration.

Clinical Application of Cyclodextrin

The spinal i.t. space represents an important route for the administration of agents, the actions of which are targeted at spinal sites or mechanisms. Spinal administration of receptor-selective agents such as opioids (morphine, sufentanil, alfentanil, lofentanil), α -agonists (dexmedetomidine, clonidine) (Sabbe and Yaksh, 1990; Sosnowski and Yaksh, 1990) as well as local anesthetics (bupivacaine) are used by the spinal route to achieve selective and therapeutically useful analgesia. Spinal administration of a variety of anticancer drugs (carmustine, doxorubicin, methotrexate), antibiotics (gentamicin, cephalosporin) and antifungal agents (amphotericin B, flucytosine) are also used to provide central nervous system concentrations not achievable by systemic administration due to poor diffusion or systemic toxicity (Blasberg and Groothuis, 1986; Devita *et al.*, 1967).

Though a useful therapeutic approach, several characteristics limit the applicability of the i.t. route. First, with regard to solubility, almost by definition, there are no currently useful agents delivered directly into the central nervous system which are not highly water soluble. Failure to have a suitable vehicle precludes water-insoluble agents from being tested. In recent studies, we characterized the spinal actions of opioid peptides which were insoluble in water, but displayed their potency after solubilization in CDEX and spinal administration (Yaksh *et al.*, 1991).

Second, spinally administered agents may undergo rapid redistribution into the vasculature of the epidural space or spinal tissue, proportional to drug lipid solubility. As a consequence, concentrations of lipophilic drugs in the central nervous system fall rapidly. To achieve a reasonable drug concentration-time profile would then require the use of a continuous infusion paradigm or the repeated injection of large quantities of drug. Bolus injection of high doses results in higher than necessary local tissue concentrations, which may be toxic. Either continuous infusion or high bolus doses results in elevated systemic drug concentrations which may also be deleterious, especially with antimetastatic agents (Green and Guillory, 1987). With the opioids (sufentanil or alfentanil), high lipid solubility accounts for rapid peripheral redistribution and

subsequent respiratory depression after i.t. or epidural doses (Hug, 1984; Gustafsson *et al.*, 1982; McQuay *et al.*, 1989). To achieve optimal pharmacokinetic properties with the minimal amount of i.t. administered drug and to maximize therapeutic effects and minimize side effects, there must be a minimization of drug clearance from the target site. To date, the use of hydrophilic agents has been the principle method of attempting to achieve these goals. For the opioids, this limits the selection to agents such as morphine, and this may impose some pharmacodynamic and pharmacokinetic (e.g., rostral redistribution) limitations on drug therapy (Payne, 1987; Yaksh and Noueihed, 1985; Yaksh *et al.*, 1988). The use of CDEX represents a theoretical alternative mechanism for spinal drug delivery by providing a central nervous system compatible vehicle which can increase the solubility of drug in CSF and retard the redistributional loss of spinally administered agent from the locus of desired effect to systemic circulation and extraspinal tissues. Recent studies by Meert *et al.* (1991) are in accord with these observations.

Results of the present studies indicate that the theoretical considerations outlined above regarding the role of CDEX/opioid concentrations and dosing are relevant to the *in vivo* animal model and suggest that the practical use of substituted β -cyclodextrins as modulators of i.t. or epidural drug redistribution and loss may have important clinical applications, but a systemic drug by drug analysis is essential.

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Use of 2-Hydroxypropyl- β -Cyclodextrin as an Intrathecal Drug Vehicle with Opioids^{1,2}

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ABSTRACT

2-Hydroxypropyl- β -cyclodextrin (CDEX), a seven-membered glucose pyranose structure, forms reversible inclusion complexes with the lipophilic portion of a drug molecule by noncovalent bonding. This can increase the water solubility of lipid-soluble drugs and reduce the rate of clearance of such agents from the spinal cord into the vasculature after i.t. administration. In this study, opioids (morphine, lofentanil, alfentanil and sufentanil) with and without CDEX (20, 2, 0.2 and 0.02% w/v in sterile water) were administered spinally in rats prepared with chronic i.t. catheters. CDEX prolonged the duration of analgesia (52.5°C hot plate) and reduced the incidence of catalepsy otherwise produced by a supermaximal i.t. dose of each of the opioids.

The magnitude of the potentiating effect of CDEX on opioids was dependent upon concentration of the CDEX and varied with drug lipid partition coefficients. The highest concentration of CDEX alone (20%) had no effect upon the volume-evoked micturition reflex, blood pressure, heart rate, or spinal reflexes. Our data indicate that CDEX may be a useful i.t. vehicle for modifying the redistribution characteristics of highly diffusible molecules after their i.t. administration, and that for each drug there is an optimal CDEX concentration. In the present case, CDEX prolongs the spinal analgesic action and reduces the supraspinal actions of i.t. drugs.

The β -cyclodextrins are nonionic cyclic polysaccharides composed of seven glucose moieties with the shape of a toroid or hollow truncated cone (Guttman *et al.*, 1988). To enhance β -cyclodextrin solubility and diminish its tendency to crystallize, substitutions onto the primary and secondary hydroxyl groups have been employed to create amorphous compounds such as CDEX (Pitha *et al.*, 1988). The cavity of these substituted cyclodextrins is relatively hydrophobic, whereas the external surfaces are hydrophilic, with the edge of the torus of the larger circumference containing chiral 2-hydroxypropyl groups.

An important property of cyclodextrins as drug carriers is that they form reversible inclusion complexes with drugs by noncovalent bonding (Uekama and Otagiri, 1984), mainly hydrogen bonds, within the hydrophobic cavity of the toroid. The reversibility of the complex formation is significantly influenced by the geometry of the CDEX molecule and the lipid solubility of the inserted portion of the guest molecule (Szejtli,

1982). Typically, drugs with high lipid solubility will form more stable CDEX complexes and be less likely to dissociate under any given set of conditions.

Of particular importance, inclusion complex formation can lead to the alteration of physical and chemical properties of the guest molecule. First, by virtue of the hydrophilic exterior of the CDEX molecule, complexation can increase the aqueous solubility of lipophilic drugs (Imai *et al.*, 1988). Second, the diffusional properties of the complexed drug can be dramatically altered by the large hydrophilic entity to which it is now complexed. Third, as the drug is reversibly complexed, it will typically display mass action kinetics, showing a time-dependent dissociation into the surrounding biophase (Szejtli, 1982). As a result, only a small fraction of the total drug may be free in the biophase and available for subsequent clearance or biological interaction. These properties thus provide a mechanism for markedly altering the kinetics of lipid-soluble agents.

Although considerable work has been done with systemic administration of CDEX drug complexes, one novel route is the use of CDEX molecules for delivery of drugs which are directed at sites in the central nervous system. One particular site of scientific and clinical attention is the spinal cord. A behaviorally relevant example of such a targeted drug effect is the analgesia produced by the local action of opioids on spinal

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ABBREVIATIONS: CDEX, 2-hydroxypropyl- β -cyclodextrin; CSF, cerebrospinal fluid; AUC, area under the effect curve; %MPE, percent maximum possible effect; ANOVA, analysis of variance.

nociceptive processing observed after i.t. administration (Yaksh, 1987). Intrathecally administered drugs may undergo redistribution by 1) rostral movement in the CSF by bulk flow and/or 2) by diffusion into the spinal or epidural vasculature (Durant and Yaksh, 1986). In the first case, the rostral movement of significant quantities of agent depends upon the presence of large quantities of free drug in the CSF. In the second case, after i.t. administration, agents with high lipid solubility rapidly enter the spinal tissue, and free drug levels in the CSF are thus cleared rapidly from the i.t. space. Diffusion into tissue along the concentration gradient of the drug results in concurrent rapid movement from tissue into the parenchymal capillaries. There is thus an immediate clearance into plasma (Herz and Teschemacher, 1971). This vascular redistribution can result in short durations of action and unacceptable effects mediated by actions of the drug outside of the spinal cord. Thus, the primary side effect of spinal opioids is the depression of behavior (somnia, catalepsy) and respiration due to supraspinal movement and action of the drug (Yaksh, 1987; Cousins and Mather, 1984). In recent studies, we demonstrated that CDEX can be used as an i.t. delivery vehicle for insoluble agents such as capsaicin and peptides (Yaksh *et al.*, 1991). The vehicle itself does not display an effect on behavior or any evidence of neurotoxicity. Significantly, in these studies, CDEX appeared to augment the effect of the spinally administered agents, presumably in part by a modification of the redistribution or metabolism of the drug.

Given the above considerations, we sought in the present studies to characterize the action of CDEX in modifying the spinal antinociceptive action and the supraspinal side effects of a series of opioids, namely morphine and the 4-anilinopiperidines. These agents, although similar in molecular weight, vary widely in their lipid partition coefficients and ionization constants and in their propensity for redistribution to extraspinal sites (Yaksh *et al.*, 1986).

Methods

Intrathecal Model

Surgery. For placement of the chronic i.t. catheters, adult male Sprague-Dawley rats (250–300g) were anesthetized with halothane (2%) in air and placed in a stereotaxic headholder. A midline incision was made at the level of the superficial neck muscles beginning at the occipital crest and extending caudally about 2 cm. Subsequently, retraction of the underlying muscle layer exposed the atlanto-occipital membrane. The membrane was incised with a 22-gauge needle and then gently retracted with a small hook. The tip of the PE-10 i.t. catheter was inserted into the subarachnoid space and then carefully advanced in a caudal direction for 8 to 9 cm. The caudal end of the catheter was located at the level of the lumbar enlargement. The external tip emerged through the skin in the occipital region and was closed with a stainless steel pin. Neck muscles and skin were sutured in two planes. Testing was then carried out after 3 to 5 days.

Behavioral Assessment

Nociceptive tests. A cutaneous thermal (hot plate) test was used for assessing nociceptive responses. Briefly, the rat was placed on a metal surface maintained at 52.5°C and enclosed by a clear Plexiglas wall to form a space 20 × 30 cm. The test measure was the latency between the time of placing the animal on the surface and the behavioral endpoint. In the majority of animals, licking the hind paw was observed; in the remainder, jumping was the endpoint. Failure to respond by 60 sec on the hot plate (cutoff time) resulted in termination of the experiment and the assignment of that value as the response

latency. Response latencies were measured before and at 5, 15, 30, 60 and 120 min after injection.

General behavior. Catalepsy, rigidity and presence or absence of placing and righting response were assessed. Catalepsy was defined as the failure of the animal to move within 10 sec from a position in which the forepaw was placed on a horizontal bar 4 cm from the cage floor.

Cystometrography. Details of the *in vivo* chronic unanesthetized rat bladder model have been reported elsewhere (Durant and Yaksh, 1988). Briefly, under anesthesia, PE-90 tubing was permanently placed into the wall of the bladder through a laparotomy and externalized. The laparotomy was closed, and the animals were allowed 5 days to recover before being used for cystometrography. To carry out cystometrography, the rats were placed in a loose restrainer. A funnel leading to a strain gauge mounted cup was placed under the abdomen to measure urine outflow. The bladder was infused with saline at a rate of 200 μ l/min and the pressure in the bladder was continuously measured. With filling, the bladder displayed transient contractions, relaxation of the sphincter and an emission of bladder contents.

Cardiovascular responses. An arterial catheter was inserted into the tail artery of each rat with chronic i.t. catheter after induction of anesthesia with halothane. A tracheal tube was inserted and connected to a respirator. Heart rate and blood pressure were monitored throughout each experiment. Before and 5 min after i.t. injection of 20% hydroxypropyl- β -cyclodextrin (10 μ l), the tail was immersed in 53°C water for 15 sec. The magnitude of the tail flick response and the elevation in arterial blood pressure evoked by the immersion were measured.

Intrathecal Drugs and Injections

Drugs were delivered in either isotonic physiological saline (0.9% w/v) or in CDEX (Research Biochemicals, Natick, MA) dissolved in distilled water. This material was designated as being 40% substituted with 2-hydroxypropyl substituents. Dose-response curves were carried out with: morphine sulfate (MW = 285, log P = 0.14; Merck, Elmsford, NY), sufentanil citrate (MW = 579, log P = 3.2; Janssen), lofentanil oxalate (MW = 499, log P = 3.6; Janssen, Beerse, Belgium), alfentanil HCl (MW = 471, log P = 2.1; Janssen) (Meuldermans *et al.*, 1982). To examine the antagonistic action of naloxone on the high-dose actions of spinal opioids, rats received an i.p. injection of naloxone (1 mg/kg) followed by the i.t. injection of different doses of the opioids when they showed catalepsy.

All drugs were prepared for i.t. delivery in a volume of 10 μ l. Each injection was followed by injection of 10 μ l of saline to ensure that the drug was delivered.

Experimental Paradigms

In the present studies on the effects of CDEX, three separate sets of experiments were carried out. For each drug dose treatment group, five or eight animals were used.

Phase 1. The effects of saline and different concentrations of the CDEX vehicle on the dose-dependent antinociceptive effects of the four opioid agonists were determined. In each case, time-effect curves were obtained for different doses of the four agonists in vehicles consisting of saline and CDEX in concentrations of 0.02, 0.2, 2.0 or 20% w/v. For lofentanil and morphine, an additional group using 0.002% CDEX was used.

Phase 2. For each of the four opioid agonists, two opioid doses were selected which were observed to produce modest effects on the hot plate response when administered i.t. in a saline vehicle. The time-effect curves on the hot plate test for these doses were then determined when the drugs were administered in different concentrations of the CDEX vehicle (0.02, 0.2, 2.0 and 20%). For lofentanil and morphine, an additional group using 0.002% CDEX was used.

Phase 3. In separate groups of rats, the effects of the CDEX alone on blood pressure, the micturition reflex, were examined. In addition, in other animals, the incidence of catalepsy produced by the spinal injection of a maximal or a supermaximal dose of the several opioids in a saline vehicle was determined when that dose was administered in

an optimal (as defined in phase 1 and phase 2) or a maximal (20%) concentration of CDEX. In these animals, after the observation of catalepsy, 1 mg/kg naloxone was given i.p.

Statistics

Response latencies are presented as group means of the %MPE where $\%MPE = [(postdrug\ latency - predrug\ latency) / (cutoff\ time - predrug\ latency)] \times 100$. To compare the duration of action, AUC (%MPE *vs.* time in minutes after injection up to 120 min) were computed using the trapezoidal rule. For phase 1, dose-response curves for %MPE were calculated with $ED_{50} \pm 95\%$ confidence intervals. For the MPE curves, the data for each rat were the maximum obtained during the observation interval (2 hr). The AUC was calculated on the base of a 2-hr observation interval.

To permit comparison of doses and calculation of potency ratios, relative potency and duration ratios as compared to saline were computed with 95% confidence intervals. All analyses were carried out according to the statistical program of Tallarida and Murray (1986). Because the computer program to determine potency ratio requires equal animals and some drug groups were performed with five animals, the data from the saline vehicle ($N = 8$) were compared to the CDEX group ($N = 5$) by taking the data of the first five rats run with the saline group. Comparison of the ED_{50} calculated for this truncated control group with the ED_{50} of the complete control group revealed no difference. In the phase 2 study, to assess the effects of CDEX on the %MPE and AUC of different drug doses as a function of CDEX concentration, one-way ANOVA were performed. If the ANOVA values were statistically significant, a multiple means analysis (Duncan) was carried out. In phase 3, for the incidence of catalepsy, a χ^2 analysis was used.

Results

Phase 1: Intrathecal Opioid Dose-Response Curves in CDEX

Saline vehicle. The i.t. injection of the opioid agonists in saline vehicle resulted in a significant elevation of hot plate response latencies with no detectable effect on motor function. The magnitude of the effect produced by each opioid was monotonically dose dependent with the rank ordering of potency being lofentanil, sufentanil, morphine, alfentanil (see table 1 and fig. 1). At doses which just blocked the hot plate response, the ordering of the duration of action for i.t. doses yielding similar peak effect was morphine > lofentanil > sufentanil > alfentanil (figs. 2 and 3).

CDEX vehicle. CDEX alone had no effect on the hot plate response latency at the highest concentrations employed (10 μ l of 20% CDEX, see fig. 3). Complete dose-response curves were obtained in the presence of different concentrations of CDEX (0.02–20%) for each of the four opioids. As shown in figures 1 and 2, this resulted in a leftward shift in the %MPE and AUC dose-response curves. Comparison of slopes revealed no statistically significant changes as a elevation of CDEX concentrations ($P > .05$; data not shown). Over the range of CDEX concentrations (0.02% to 20% w/v), the degree of potentiation

TABLE 1

Summary of MPE (ED_{50}) of dose-response curves of drugs in saline with the lower (LI) and upper (UI) 95% confidence intervals for the ED_{50}

Values calculated from dose response curves in which each curves represents three to five doses with eight rats.

		Morphine	Sufentanil	Alfentanil	Lofentanil
ED_{50} (μ g)		3.5	0.3	8.5	0.04
95% CI	LI	0.3	0.04	1.8	0.01
	UI	14	1	13	0.2

tended to be related in a biphasic manner to CDEX concentration. To more clearly present this biphasic effect, a least squares linear regression was carried out on the dose-response data presented in figures 1 and 2 and respective %MPE and AUC dose ratios were calculated. Figures 4 and 5 present the ED_{50} dose ratios and AUC dose ratios with 95% confidence interval plotted *vs.* the respective CDEX concentration. These observations emphasize the fact each spinally administered opioid displayed an optimal concentration of CDEX. Thus, the optimal concentrations were 2% for lofentanil, 0.2% for sufentanil, 2% for alfentanil and 0.02% morphine. Moreover, it is clear that the degree of dose-effect augmentation differed between the different agents. Thus, for the peak effect and AUC, the ordering of the leftward shift as compared to saline vehicle was morphine > lofentanil > sufentanil > alfentanil (see figs. 4 and 5).

Phase 2: Intrathecal CDEX Dose-Response Curves with Opioids

In separate experiments, the above findings were replicated by preparing stock solutions of each agonist and adding these stock solutions to different CDEX concentrations. As indicated in figures 6 and 7, similar results were observed as described in phase 1 with certain CDEX concentrations found to be associated with a maximum response for each drug. In each case, a one-way ANOVA revealed a statistically significant main effect across CDEX concentration ($P < .05$). Using a multiple range test, as shown in figures 6 and 7, similar optimal CDEX concentrations were observed for each of the two opioid drug concentrations: morphine, 0.02%; sufentanil, 0.2%; alfentanil and lofentanil, 2.0%. It should be stressed that at the higher concentrations of each agonist (particularly lofentanil and morphine), the optimal CDEX concentrations tended to be obscured by the plateau effect on both the MPE and AUC measures resulting from a maximum cutoff latency.

Phase 3: Behavioral Effects of Intrathecal Opioids and CDEX

The administration of CDEX alone at the highest concentration used (10 μ l/20%) had no acute effect on gross behavior. No agitation or evidence of irritation was observed. There was no change in any index of motor function, including the placing or stepping reflex or ambulation after i.t. administration of 20% CDEX (10 μ l).

Cardiovascular system. Measurement of blood pressure and heart rate in the halothane (0.8%) -anesthetized rat revealed no change during a 30-min period after injection of CDEX solution ($N = 5$; maximum change from base line within 30 min = 5.2 ± 0.8 mm Hg; 7.0 ± 1.0 beats/min).

Cystometrography. In animals prepared with chronic bladder and i.t. catheters undergoing cystometrography, there was no change in the frequency (maximum change = 0.02 ± 0.001 contractions/min) or pressure contraction of the bladder (maximum change = 1.9 ± 0.1 cm H₂O) using the volume-evoked cystometrogram during a 60-min period after the i.t. administration of CDEX (10 μ l/20%).

Effects on supraspinal action of opioids. As shown in table 2, i.t. administration of high doses of the opioids in saline (lofentanil, 0.3 μ g; alfentanil, 100 μ g; morphine, 100 μ g; sufentanil, 3 μ g) resulted in a 100% incidence of catalepsy, indicating significant supraspinal redistribution of the drugs. Catalepsies with high doses of opioids were completely reversed by i.p.

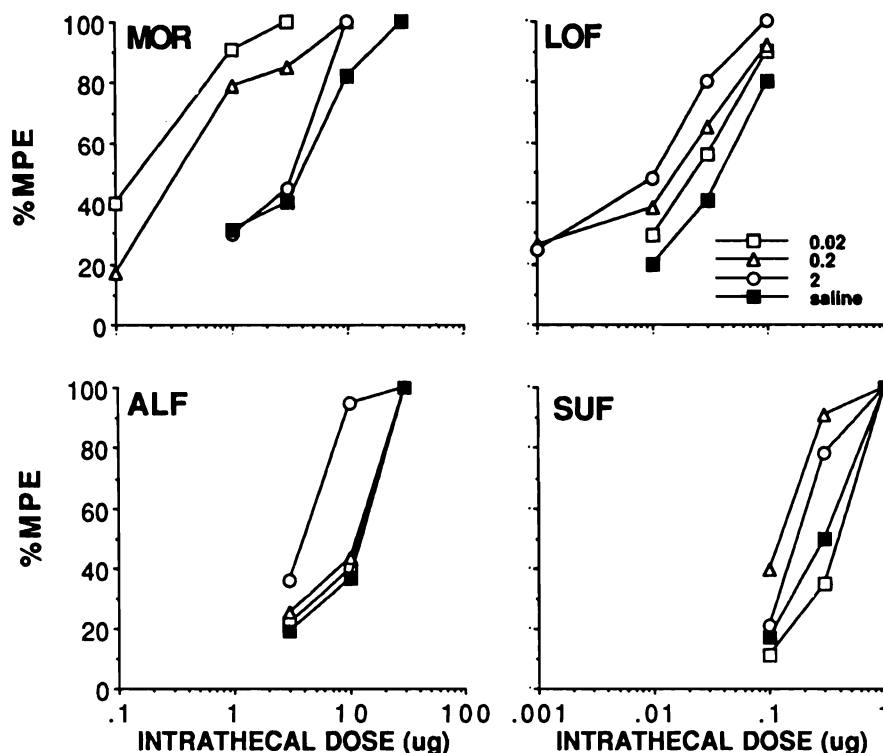


Fig. 1. Log dose-response (%MPE) curves for the hot plate response for morphine, lofentanil, alfentanil and sufentanil given i.t. in saline or CDEX (0.02–2%). Data for 0.002 and 20% CDEX solutions were deleted for clarity (see fig. 4). The %MPE is calculated on the basis of the maximum effect obtained within the 2-hr observation period. Doses above those which were supramaximal (i.e., produced greater than %MPE = 100%) are not shown and were not used to calculate dose-response regressions. Each point presents the mean of five to eight rats. S.E. bars were deleted for clarity. See figure 4 for dose ratios with 95% confidence intervals.

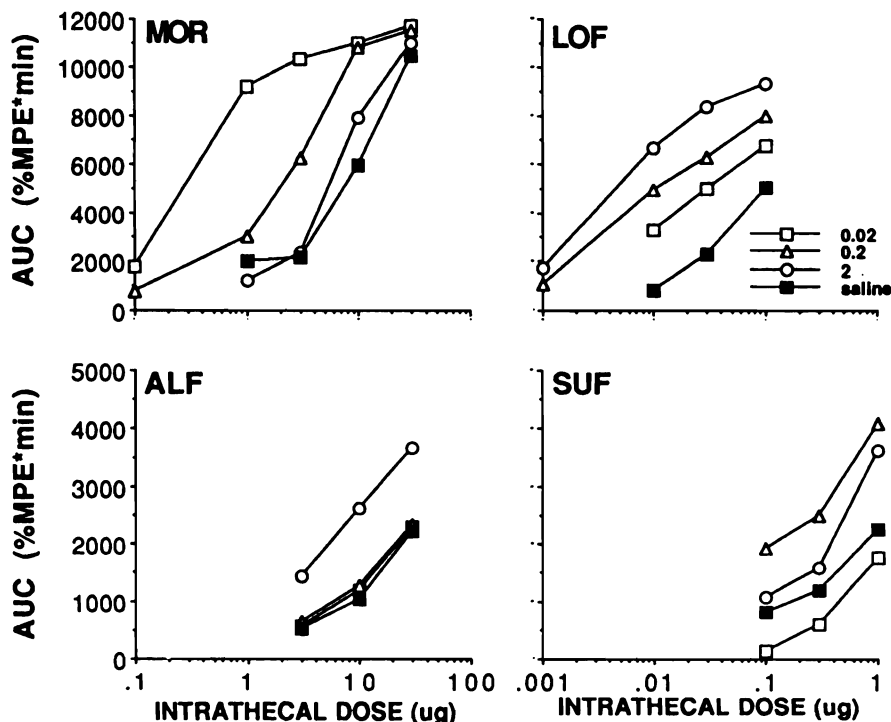


Fig. 2. Log dose-response (AUC) curves over the 2-hr observation interval for the hot plate response for morphine, lofentanil, alfentanil and sufentanil given i.t. in saline or CDEX (0.02–2%). Data for 0.002 and 20% CDEX solutions were deleted for clarity (see fig. 5). Each point presents the mean of five to eight rats. S.E. bars are deleted for clarity. See figure 5 for dose ratios with 95% confidence intervals.

administration of naloxone (1 mg/kg). To examine the decreased incidence of catalepsy with CDEX, rats received i.t. injections of the high dose of the agonist in the presence of the optimal concentration of CDEX (sufentanil, 0.2%; morphine, 0.02%; lofentanil alfentanil, 2%; see figs. 4 and 5) or in the maximum concentration of CDEX (20%). As indicated, catalepsy at the highest dose of opioid used was absent with the highest concentration of CDEX. These differences were significant according to a χ^2 test. Also indicated is the incidence of catalepsy produced by the optimal opioid dose and CDEX concentration which provided a maximum antinociceptive ef-

fect. At this concentration, producing maximum antinociceptive effects, catalepsy was also essentially absent. This separation is most clear for the lipid-soluble agents.

Discussion

Cyclodextrin derivatives have been used as drug carrier systems for the systemic administration of a variety of drugs. Aside from the ability to facilitate solubilization of lipophilic agents, the use of cyclodextrins for microencapsulation of a

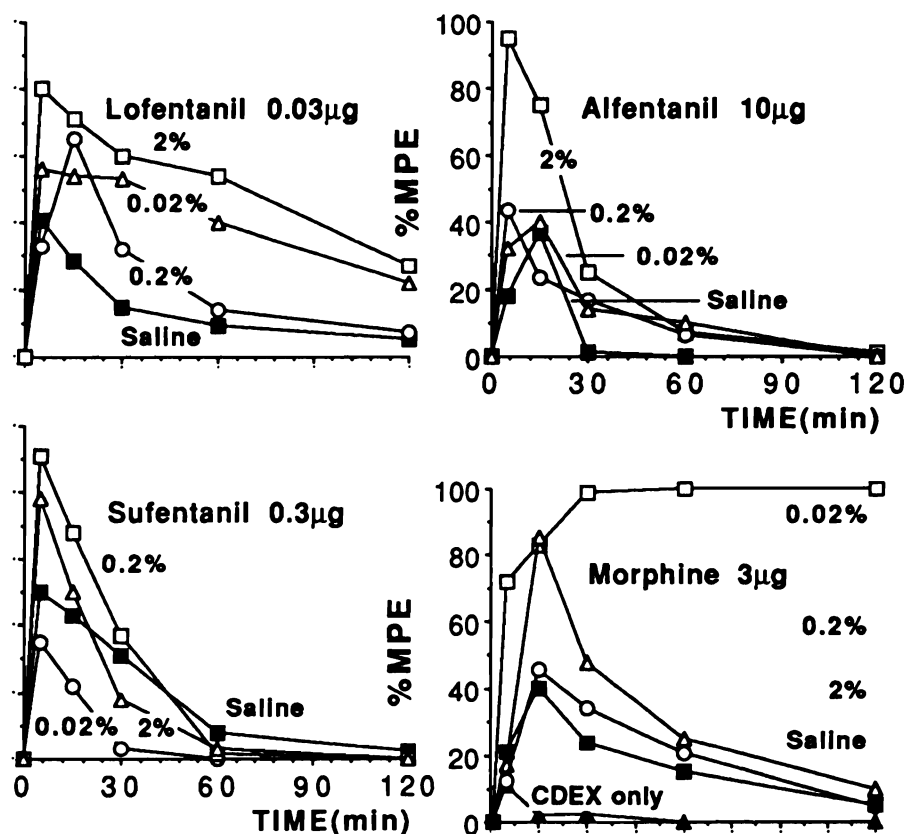


Fig. 3. Time course of hot plate response for i.t. administered morphine (3 μ g), lofentanil (0.03 μ g), alfentanil (10 μ g) and sufentanil (0.3 μ g) in saline or CDEX (0.02–2%) solution. Each line represents the mean data for five to eight rats. S.E. bars are deleted for clarity. Drug experiments were also carried out with 0.002 and 20% CDEX, but data were deleted for clarity. "CDEX only" indicates hot plate test with CDEX solution (20%/10 μ l).

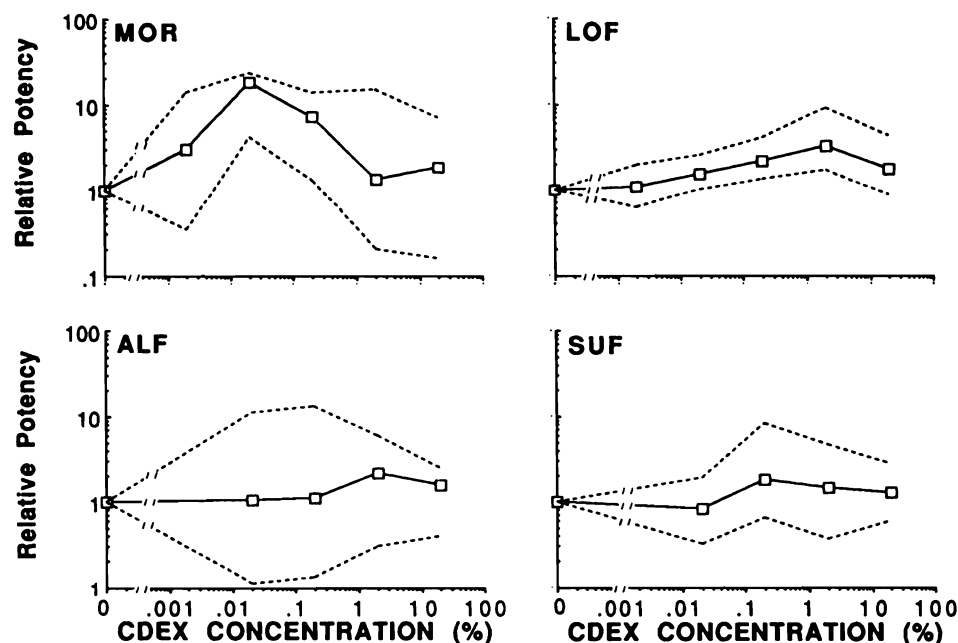


Fig. 4. Intrathecal potency ratios (ED_{50} of drug in CDEX/ ED_{50} of drug in saline) for i.t. morphine, lofentanil, alfentanil and sufentanil plotted as a function of CDEX concentration with 95% confidence interval (dotted line). Analyses are obtained from the data presented in figure 1. See text for details and analysis.

variety of agents has been shown to significantly alter the bioavailability of molecules (Szejtli, 1982).

In the present work, we specifically considered the qualities of CDEX as a vehicle for the delivery of drugs into the CSF. We demonstrated that even at the highest concentrations, spinally administered CDEX alone produced no observable effect. However, using water-based solutions of CDEX as a drug vehicle, we found that the time course and magnitude of the spinally mediated antinociceptive activities of the opioids examined were enhanced by combining the drugs with CDEX.

Similar results have been reported by others (Meert *et al.*, 1991). Thus, for morphine, alfentanil, sufentanil and lofentanil, CDEX served to 1) increase their duration of action, 2) enhance the apparent magnitude of antinociceptive effect and 3) diminish the extent to which high doses of the i.t. opioids, especially the lipid-soluble 4-anilino-piperidines, would evoke supraspinally mediated behavior. The present studies, using pharmacologically active agents which differ significantly in their lipid partition coefficients (Hug, 1984), revealed that these effects of CDEX appeared to be influenced by several properties,

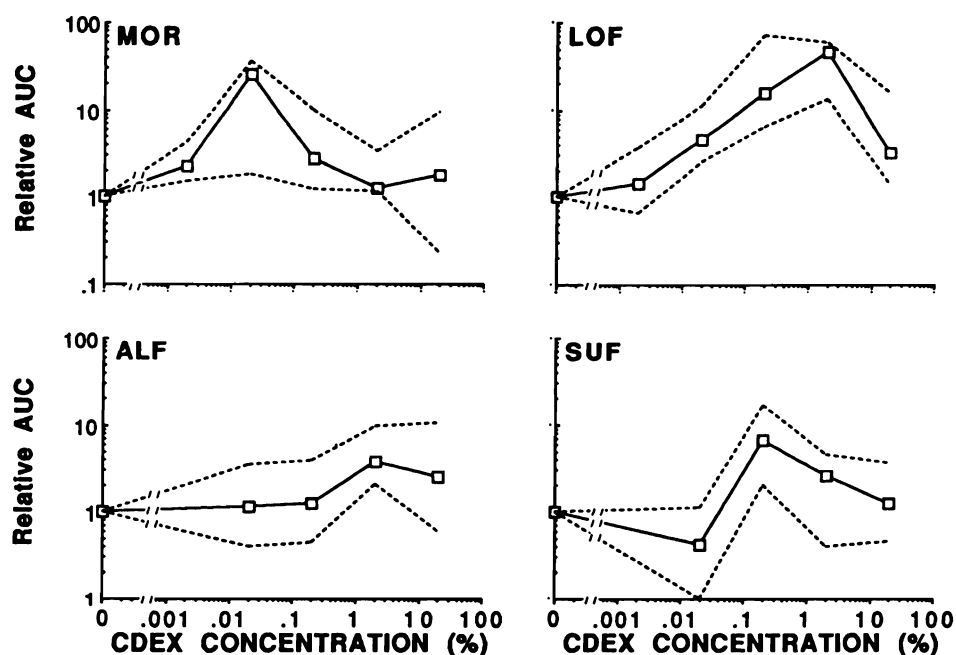


Fig. 5. Relative duration of action (AUC of drug in CDEX/AUC of drug in saline) for i.t. morphine, lofentanil, alfentanil and sufentanil plotted as a function of CDEX concentration with 95% confidence interval (dotted line). Analyses are obtained from the data presented in figure 2. See text for details and analysis.

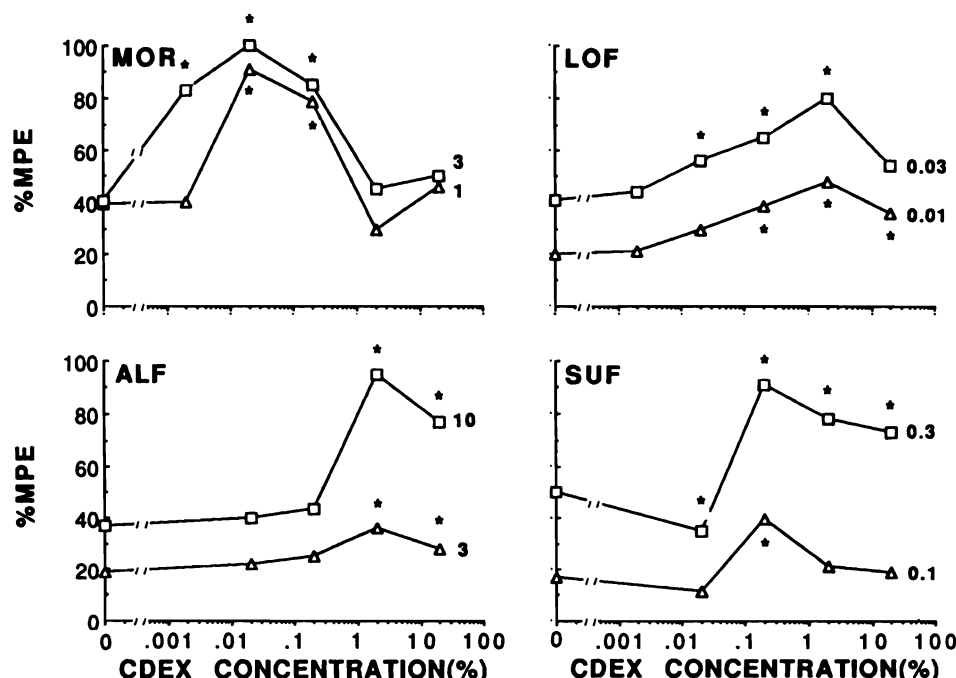


Fig. 6. Plot of %MPE, measured at the peak response observed in the 2-hr period, vs. the concentration of CDEX in vehicle for i.t. morphine (1 or 3 µg), lofentanil (0.01 or 0.03 µg), alfentanil (3 or 10 µg) and sufentanil (0.1 or 0.3 µg) in saline or CDEX (0.002–20%) solution. CDEX concentration 0 indicates saline groups (*i.e.*, drug in saline) ($N = 5-8$ rats/point). One-way ANOVA across CDEX concentrations were statistically significant for all drug doses ($P < .05$). * $P < .05$ vs. drug in saline as determined by Duncan multiple range test. See text for further details.

including the lipid solubility of the drug and the concentration of CDEX used with each drug.

Factors Governing the Effects of CDEX as a Vehicle

CDEX encapsulates drugs by virtue of its hydrophobic interior which is thermodynamically favored by appropriately sized nonpolar portions of a drug molecule. The nominal size of the internal cavity in the β -cyclodextrin molecule (approximately 7–8 Å) represents a spatially limiting factor. Thus, a variety of chemical groups on the guest molecule, such as alkyl and phenolic groups, are amenable to insertion (Catena and Bright, 1989). The guest/host interaction is reversible and corresponds to a mass action relationship. Drugs with high lipid partition coefficients and a high propensity to form hydrogen bonds will result in a minimum energy conformation within the inclusion cavity, high complex stability corresponding to a higher affinity

binding and diminished likelihood of dissociation (Szejtli, 1982). It is conceivable that a drug complexed with CDEX may remain accessible and even bind to its specific receptor site, but the steric hindrance due to the bulky CDEX adjunct does not favor this possibility (Szejtli, 1982). The biological activity of a drug in a CDEX solution probably requires free drug. Thus, factors which govern dissociation from CDEX will influence drug activity (Kempfle *et al.*, 1987). Several factors likely influence guest/host dissociation.

First, a high lipid partition coefficient, as noted above, will result in a slow rate of drug dissociation (Szejtli, 1982). It should be emphasized that slow rates of dissociation do not *a priori* exclude the use of a CDEX complex. Drug dissociation is based on the law of mass action, and if the drug is diffusing into a relatively large volume, such as the CSF or the spinal cord, then the equilibrium conditions will permit the develop-

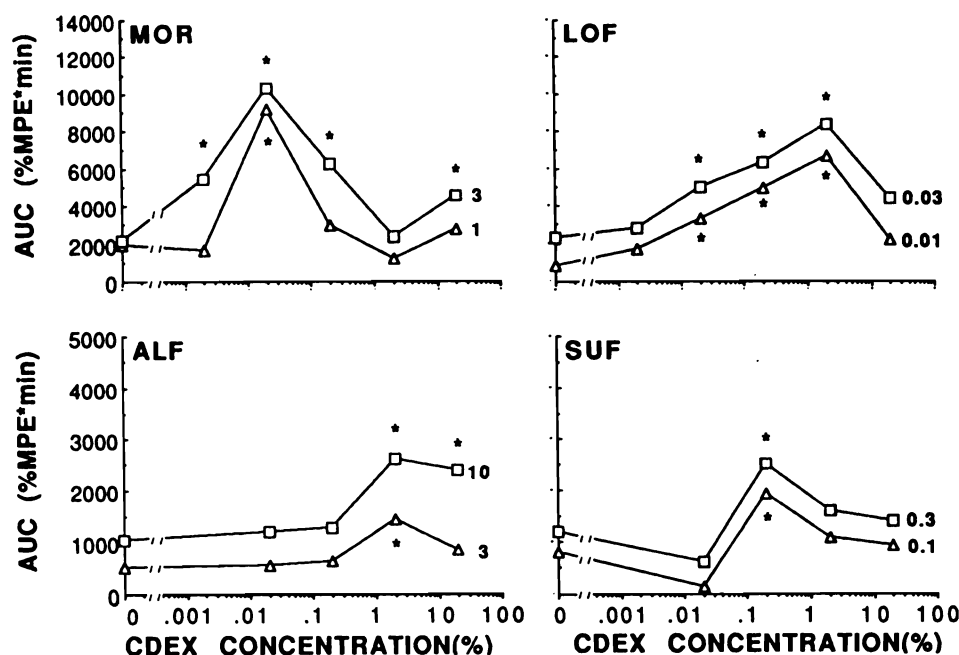


Fig. 7. Plot of AUC vs. the concentration of CDEX in vehicle for morphine (1 or 3 μ g), lofentanil (0.01 or 0.03 μ g), alfentanil (3 or 10 μ g) and sufentanil (0.1 or 0.3 μ g) in saline or CDEX (20%) solution. CDEX concentration 0 indicates saline groups (i.e., drug in saline) ($N = 5-8$ rats/point). One-way ANOVA across CDEX concentrations were statistically significant for all drug doses ($P < .05$). * $P < .05$ vs. drug in saline as determined by Duncan multiple range test. See text for further details.

TABLE 2

Summary of behavioral signs described after i.t. lofentanil, alfentanil, morphine and sufentanil at maximum and supermaximum doses with and without CDEX

Drug	Vehicle	Dose	N ^a	%Catalepsy ^b	Onset of catalepsy
μ g/10 μ l					
Lofentanil	Saline	0.1	0/10	0	
		0.3	5/5	100	<1 min
	CDEX (2%)	0.1	2/20	10	<1 min
		0.3	4/5	80	<1 min
	CDEX (20%)	0.1	0/6	0	
Alfentanil		0.3	0/5	0	
	Saline	30	0/6	0	
		100	6/6	100	<1 min
	CDEX (2%)	30	0/10	0	
		100	3/3	100	<1 min
Morphine	CDEX (20%)	30	0/7	0	
		100	0/6	0	
	Saline	30	0/6	0	
		100	5/5	100	<1 min
	CDEX (0.02%)	30	0/5	0	
Sufentanil		100	1/6	16.7	<30 min
	CDEX (2%)	30	0/20	0	
		100	0/11	0	
	CDEX (20%)	30	0/11	0	
		100	1/5	20	<60 min
Sufentanil	Saline	1	0/4	0	
		3	5/5	100	<1 min
	CDEX (0.2%)	1	1/10	10	<1 min
		1	0/8	0	
	CDEX (20%)	3	0/5	0	

^a Number of rats which showed catalepsy/total number of rats.

^b Catalepsies observed in this experiment were reliably reversed with 0.3 to 1 mg/kg i.p. naloxone given after observation (data were not shown).

ment of sustained steady-state free drug concentrations in the space where CDEX concentrations are low. Indeed, in recent studies, we examined the neurotoxicity of the very lipophilic neurotoxin, capsaicin. Aside from providing a highly compatible drug delivery system, the agent in CDEX still clearly retained pharmacological activity, resulting in a significant depletion of substance P from the spinal dorsal horn (Yaksh *et al.*, 1991).

Based on results with morphine, sufentanil and lofentanil, the optimal CDEX concentration values appeared to be inversely related to the lipid partition coefficients. This observation that the optimal concentration of CDEX with alfentanil, in spite of its relatively low log P , would appear to correspond with the large fraction of alfentanil which exists in an unionized state at physiological pH.

Second, the availability of free drug in a drug-CDEX solution administered into a kinetically limited space is dependent upon the quantities of free CDEX present. If the CDEX/drug ratio is high, there will be an abundance of free CDEX, and the free CDEX (a pool of binding sites) will compete with the tissue for drug redistribution. Thus, if a relatively high CDEX/drug ratio is administered i.t. or epidurally, it appears that the effect of the drug will be diminished. If a relatively low CDEX/drug ratio is present, redistribution of the drug from the spinal space will not be significantly delayed. This theoretical position argues that in the CSF, there may be an optimal concentration of CDEX for any given drug at a specific dose. Other factors such as molecular shape or charge distribution may play important roles in CDEX-opioid complexation dynamics, but there are at present insufficient data to consider them.

Toxicology of Cyclodextrin

Studies on the toxic effects of cyclodextrin have been conducted for oral, i.v., s.c., i.p. and i.m. administration. Toxicity with nonsubstituted β -cyclodextrin was largely due to the crystallization after concentration in the kidney (Szejtli, 1982). No evidence of toxicity was observed in the present study after i.t. injection of 20% CDEX (10 μ l) as measured by motor function, blood pressure, heart rate or micturition. CDEX is known to be resistant to the usual hydrolyzing enzymes and may be metabolized by intestinal flora (Uekama and Otagiri, 1984). In view of its resistance to hydrolysis and relatively high molecular weight, clearance of CDEX from the central nervous system probably occurs by bulk flow of CSF. Studies on the metabolism and clearance of i.t. CDEX are few. In recent studies, we observed that the clearance of radiolabeled CDEX (β -cyclodextrin- 3 H) from spinal CSF after i.t. injection with serial CSF

sampling model in rats was identical to inulin [^{14}C], suggesting clearance by bulk CSF flow (J. Jang and T. L. Yaksh, unpublished observation).

Cyclodextrin on Spinal Drug Kinetics

In this study, CDEX augmented the antinociceptive effects of the i.t. administered opioids as compared to their effects when administered in the saline vehicle. As noted in the introduction, after i.t. administration, drugs are cleared from the CSF by movement into tissue, and thence by absorption into the spinal capillary net or by rostral transfer after the redistribution of CSF. It appears likely that the augmented effect and duration of action of opioids produced by CDEX reflects altered clearance of the drugs from the CSF/spinal axis, due to reduced diffusion of drug into the vasculature. The absence or decrease of the supraspinal effects otherwise typically observed after the spinal administration of high doses of these opioids (see table 2) supports this possibility. CDEX appears to provide a slow release reservoir, diminishing the amount of free drug in the CSF available to redistribute into blood. Kinetic distribution studies are currently in progress to confirm this consideration.

Clinical Application of Cyclodextrin

The spinal i.t. space represents an important route for the administration of agents, the actions of which are targeted at spinal sites or mechanisms. Spinal administration of receptor-selective agents such as opioids (morphine, sufentanil, alfentanil, lofentanil), α -agonists (dexmedetomidine, clonidine) (Sabbe and Yaksh, 1990; Sosnowski and Yaksh, 1990) as well as local anesthetics (bupivacaine) are used by the spinal route to achieve selective and therapeutically useful analgesia. Spinal administration of a variety of anticancer drugs (carmustine, doxorubicin, methotrexate), antibiotics (gentamicin, cephalosporin) and antifungal agents (amphotericin B, flucytosine) are also used to provide central nervous system concentrations not achievable by systemic administration due to poor diffusion or systemic toxicity (Blasberg and Groothuis, 1986; Devita *et al.*, 1967).

Though a useful therapeutic approach, several characteristics limit the applicability of the i.t. route. First, with regard to solubility, almost by definition, there are no currently useful agents delivered directly into the central nervous system which are not highly water soluble. Failure to have a suitable vehicle precludes water-insoluble agents from being tested. In recent studies, we characterized the spinal actions of opioid peptides which were insoluble in water, but displayed their potency after solubilization in CDEX and spinal administration (Yaksh *et al.*, 1991).

Second, spinally administered agents may undergo rapid redistribution into the vasculature of the epidural space or spinal tissue, proportional to drug lipid solubility. As a consequence, concentrations of lipophilic drugs in the central nervous system fall rapidly. To achieve a reasonable drug concentration-time profile would then require the use of a continuous infusion paradigm or the repeated injection of large quantities of drug. Bolus injection of high doses results in higher than necessary local tissue concentrations, which may be toxic. Either continuous infusion or high bolus doses results in elevated systemic drug concentrations which may also be deleterious, especially with antimetastatic agents (Green and Guillory, 1987). With the opioids (sufentanil or alfentanil), high lipid solubility accounts for rapid peripheral redistribution and

subsequent respiratory depression after i.t. or epidural doses (Hug, 1984; Gustafsson *et al.*, 1982; McQuay *et al.*, 1989). To achieve optimal pharmacokinetic properties with the minimal amount of i.t. administered drug and to maximize therapeutic effects and minimize side effects, there must be a minimization of drug clearance from the target site. To date, the use of hydrophilic agents has been the principle method of attempting to achieve these goals. For the opioids, this limits the selection to agents such as morphine, and this may impose some pharmacodynamic and pharmacokinetic (*e.g.*, rostral redistribution) limitations on drug therapy (Payne, 1987; Yaksh and Noueihed, 1985; Yaksh *et al.*, 1988). The use of CDEX represents a theoretical alternative mechanism for spinal drug delivery by providing a central nervous system compatible vehicle which can increase the solubility of drug in CSF and retard the redistributional loss of spinally administered agent from the locus of desired effect to systemic circulation and extraspinal tissues. Recent studies by Meert *et al.* (1991) are in accord with these observations.

Results of the present studies indicate that the theoretical considerations outlined above regarding the role of CDEX/opioid concentrations and dosing are relevant to the *in vivo* animal model and suggest that the practical use of substituted β -cyclodextrins as modulators of i.t. or epidural drug redistribution and loss may have important clinical applications, but a systemic drug by drug analysis is essential.

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Hydroxypropyl- β -cyclodextrin can modulate the activity of spinally administered sufentanil

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Summary

Hydroxypropyl- β -cyclodextrin increased the effectiveness of sufentanil after epidural and intrathecal administration in rats, both in terms of a longer duration of analgesia after a fixed dose of sufentanil, and in a reduction of the lowest ED₅₀s to produce analgesia. There was also an increase in specificity, as indicated by the greater dissociation between the ED₅₀s for analgesia and for supra-spinal side-effects. Maximal activity was measured after inclusion complexation of sufentanil in 10% hydroxypropyl- β -cyclodextrin. At higher concentrations of hydroxypropyl- β -cyclodextrin, both the activity and the specificity were attenuated. The increased safety of sufentanil in 10% hydroxypropyl- β -cyclodextrin after spinal administration was also confirmed in terms of opioid-induced deviations in arterial PO₂, PCO₂ and oxygen saturation. At a dose of twice the ED₅₀ for deep surgical analgesia, the sufentanil/

hydroxypropyl- β -cyclodextrin complex produced no changes in these parameters. With sufentanil alone at comparable analgesic doses, significant shifts in all three parameters were present immediately after drug administration. At higher concentrations of sufentanil in hydroxypropyl- β -cyclodextrin changes in the three blood gases were present but the deviations were always smaller than those observed with comparable doses of plain sufentanil. These results support the notion that after complexation sufentanil is present longer at the spinal level after spinal administration. As a consequence, there is less free sufentanil available for redistribution into lipid tissue and into the circulatory system, producing less systemic side-effects.

Keywords: ANALGESIA, spinal, epidural; ANALGESICS, sufentanil, PHARMACOLOGY, cyclodextrin.

Introduction

Spinal injections of opioids are known to produce profound analgesia. The potency, duration and specificity of the opiates depend on different variables including the route of injection (epidural or intrathecal) and the water to octanol partition coefficient [1,2]. After epidural administration lipid soluble opioids, such

as sufentanil, produce analgesia with a rapid onset, which is more potent than morphine, with some specificity and a moderate duration of action. After big doses, early respiratory depression may occur but delayed depression has not been reported. After epidural application, significant plasma levels can be achieved rapidly [3]. As a result, early supra-spinal side-effects might occur. Given intrathecally, sufentanil is no more potent. The duration of activity is even shorter [1], probably due to rapid distribution and clearance of sufentanil from the cerebrospinal fluid (CSF) [3]. Water soluble

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opioids, such as morphine, have a different profile. Given epidurally, morphine has a slow onset and a prolonged residence time in the CSF. As a consequence, morphine can spread rostrally, resulting in late respiratory depression. Intrathecally, morphine has a faster onset, a higher potency and a longer duration of action [1].

To limit vascular uptake and to increase the availability of lipid soluble drugs when given spinally and, particularly, intrathecally, one has to limit the movement of the drug across the various membranes. Because this process depends on the concentration gradient of the free drug across a membrane, physical or chemical sequestration of an agent in a medium can reduce the diffusion gradient.

Molecular sequestration can be obtained by using cyclodextrins. Cyclodextrins are cyclic carbohydrates consisting of 6, 7 or 8 glucose units, respectively called α -, β - and δ -cyclodextrin. Cyclodextrins have a unique spatial configuration: the more polar hydroxyl groups are all orientated toward the outside of the circular structure. Cyclodextrins can be regarded as cylinders which are hydrophilic outside and hydrophobic inside. The hydrophobic cavity forms an ideal harbour in which poorly water-soluble molecules can shelter their most hydrophobic parts. Mixing such an insoluble compound with a cyclodextrin in an aqueous environment can therefore result in complexation. Because of the hydrophilic exterior of the cyclodextrin, such a complex is soluble. As a consequence, the solubility, dissolution rate and bioavailability of the lipophilic compound can change. The degree of complexation with a cyclodextrin depends on the dimensions and the lipophilicity of the guest molecule. For many drugs, β -cyclodextrin offers the most interesting cavity size. For the present investigation, the hydroxypropyl derivate of β -cyclodextrin was used, namely hydroxypropyl-beta-cyclodextrin (HP-B-CD). HP-B-CD has very low systemic toxicity [4,5] and is devoid of pharmacological activity *in vivo* [6]. Presented in such an inclusion complex, free sufentanil will be much less available, resulting in reduced diffusion of sufentanil into lipid rich systems such as epidural fat and vessels. Sufentanil might then be available longer in the CSF, increasing its

duration of action and causing less systemic side-effects.

In the present studies, the activity of sufentanil was evaluated in various concentrations of HP-B-CD after both epidural and intrathecal administration. Analgesia and other behavioural activities were taken into account. In order to control for the effects of such a sufentanil/HP-B-CD complexation on respiration, studies were performed to compare the effects of different doses of sufentanil alone and in 10% HP-B-CD, on PO_2 , PCO_2 and oxygen saturation after both epidural and intrathecal administration.

Materials and methods

Animals

Epidural/intrathecal catheterization. Male Wistar rats weighing 250 ± 20 g were used for spinal catheterization according to a technique described in detail elsewhere [2]. Briefly, under anaesthesia, 0.5 cm of a polyethylene catheter (PE 10) was introduced into the epidural space in a cephalad direction via a hole drilled in the fourth lumbar vertebra. After fixing the catheter to the vertebra, the loose end was tunnelled subcutaneously toward the neck region. For intrathecal catheterization, an analogous procedure with the catheter inside the dura was used.

The animals were allowed to recover for 5 days. During this time, they had free access to food and tap water. Animals showing any sign of neurological damage were discarded. After the experiments, in which the animals were used only once, the rats were killed and the position of the catheter tip was checked at autopsy. Only the results from those animals in whom the catheter tip appeared to be located at the proposed site of injection, were used for data analysis.

All experiments and housing after surgery took place in an air conditioned laboratory (temp.: $21 \pm 1^\circ\text{C}$; relative humidity: $65 \pm 10\%$).

Femoral artery catheterization. On the day of testing, the rats were given etomidate 2 ml (4 mg ml^{-1}) intraperitoneally. A polyethylene

and causing less systemic effects, the activity of sufentanil at various concentrations of epidural and intrathecal analgesia and other behaviour were taken into account. In the effects of such a sufentanil pre-anaesthesia on respiration, and to compare the effects of sufentanil alone and in 10% HP-B-CD, and oxygen saturation and oxygen saturation and intrathecal administration.

Methods

Catheterization. Male Wistar-Kyoto rats (200 g) were used for spinal catheterization according to a technique described elsewhere [2]. Briefly, under general anaesthesia a polyethylene catheter was inserted into the epidural space via a hole drilled in the vertebra. After fixing the catheter the loose end was tunneled under the skin in the neck region. For respiration, an analogous procedure was used for the catheter inside the dura was

allowed to recover for 5 days. They had free access to food and water. Animals showing any signs of distress were discarded. After surgery the animals were housed in cages in which the animals were kept. After 24 h the rats were killed and the catheter tip was checked at autopsy. Results from those animals in which the catheter appeared to be located at the intended site of injection, were used for

post-operative housing after surgery in a temperature conditioned laboratory (temperature: 22 ± 1°C, relative humidity: 65 ± 10%).

Pre-anaesthesia. On the day of surgery the rats were given etomidate 2 ml/kg intraperitoneally. A polyethylene

catheter (PE 50) was inserted for 3 mm into the right femoral artery, at the level of the groin, after it had been ligated. The catheter was rostrally inserted into the artery and kept in place by a surgical wire around the artery. The animals were then placed in Bolman cages for the entire duration of the experiment. A first arterial blood sample was taken 2 h after implantation as a check on recovery from etomidate. After each sampling (see below), the catheter was flushed with a heparin solution (Heparine Nove[®], 1 ml in 100 ml physiological solution).

Spinal injections. The method of injection has been described in detail elsewhere [7], and consisted of the administration through the catheter of 20 µl of sufentanil in consecutive steps of 1 µl. The injection procedure took approximately 60 s to complete. Sufentanil doses were given on a µg per rat rather than a µg per kg basis since this allowed both the drug concentration and the volume of the epidural injection to be held constant for the different animals.

Analgesia assay. The tail withdrawal procedure (TWR) used here has been described in detail elsewhere [8]. Briefly, the rat was placed in a cylindrical rat holder, with its tail hanging freely outside the cage. The distal 5 cm of the tail was immersed in a hot water (55 ± 1°C) bath and the time to tail withdrawal was measured to the nearest 0.1 s. In order to minimize tissue damage on repeated testing, a cut-off time of 10.0 s was used. A TWR latency > 6.0 s (and thus also ≥ 10.0 s) never occurred in control animals ($n > 1000$) and was used as the criterion of analgesia. Data were collected on five animals per condition except for the epidural studies with various concentrations of HP-B-CD where seven animals were used. Tail withdrawal latency, pinna and cornea reflexes and skeletal muscle tone (see other *in vivo* effects) were scored once before and at 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 min after injection by a single observer unaware of the pharmacological treatments.

Other *in vivo* effects. The blockade of the pinna and the corneal reflexes and muscle rigidity were monitored as indices of the central pharmacological

activity of the opiates. Blockade of these reflexes is a characteristic effect of opiates on the tenth and fifth cranial nerves, whereas rigidity of skeletal muscles probably originates in the striatum and substantia nigra [9,10,11]. The pinna reflex consisted of a characteristic head twitch and was assigned scores ranging from 0 (normal reflex) to 3 (absence of any apparent motor response), depending on the response of the animal to gentle mechanical stimulation by means of a blunt metal rod (diam.: 0.5 mm); scores 1 and 2 indicate that the reflex was slightly or markedly retarded, respectively. The corneal reflex was tested by stimulating the eye in a similar manner and was also scored from 0 (normal) to 3 (absence of any apparent motor response). The scores given for overall skeletal muscle tone ranged from 0 (normal tone) to 3 (lead pipe rigidity); scores 1 and 2 represent weakly and moderately increased tone. In control animals ($n > 1000$) a score ≥ 2 never occurred for the pinna and corneal reflexes or for muscle rigidity.

Arterial blood sampling. Arterial blood samples of 0.2 ml were taken at 5, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min after injection. Blood gas parameters (P_{O_2} , P_{CO_2} and oxygen saturation) were calculated using an ABL3 Acid Base Laboratory[®] (Radiometer Copenhagen) pH/Blood gas analyzer. Data were collected on five animals per treatment condition.

Experimental designs

Analgesia experiment 1. In order to assess the effects of various concentrations of HP-B-CD, 1.00 µg per rat sufentanil was injected epidurally in 0, 2.5, 5, 10, 15, 20 and 25% HP-B-CD. Intrathecally, concentrations between 0 and 20% were tested.

Analgesia experiment 2. Because of the results obtained with sufentanil in 10% HP-B-CD, a dose response function for epidural and intrathecal sufentanil in 10% HP-B-CD was determined. The tested doses included 0.016, 0.063, 0.25 and 1.00 µg of sufentanil per rat.

Blood gases experiments. In a first series of

experiments, the effects of fixed epidural doses of 1.00, 3.00 and 6.00 μg sufentanil alone and 0.16, 0.40 and 1.00 μg sufentanil in 10% HP-B-CD were tested. For intrathecal administration doses of 1.00, 2.50 and 9.00 μg sufentanil alone and 0.063, 0.25 and 1.00 μg sufentanil in 10% HP-B-CD were used. The doses were selected on the basis of the data obtained on the analgesic properties of sufentanil alone and sufentanil in 10% HP-B-CD. For the epidural route, the selected doses represent 2, 5, 10–12 times the lowest ED_{50} for a prolongation of the TWR latency ≥ 10.0 s; for the intrathecal route of administration the doses correspond to 2–3, 8 and 31 times the lowest ED_{50} .

Drugs

Sufentanil citrate, in inclusion complexation with various concentrations of hydroxypropyl-beta-cyclodextrin (HP-B-CD), was prepared as an aqueous solution in water with NaCl. The pH of the solution was kept constant at 7.4 using NaOH 0.1 N.

Statistics

At each experimental condition, the test results of five or seven rats were collected. For the analgesia testing, criterion values were defined for each of the four variables that were examined. The TWR latency was evaluated using the >6.0 and ≥ 10.0 s criterion. Score 3 was used to evaluate the blockade of the pinna and corneal reflexes and muscle rigidity. Because the blockade of the pinna reflex always appeared first, only these results are presented. ED_{50} values and 95% confidence limit were calculated according to Finney's iterative method [12]. Differences between two experimental conditions were evaluated using a two-tailed Mann-Whitney *U*-test [13]. For the blood gases analyses, changes from baseline were evaluated by Wilcoxon two-tailed matched-pairs signed-ranks test [13].

Results

Analgesia testing

None of the tested rats had a pre-injection TWR latency ≥ 6.0 s, or a blockade of the pinna

and corneal reflexes or any muscle rigidity. Epidural and intrathecal administration of 1 μg per rat sufentanil produced a TWR latency ≥ 10.0 s and a blockade of the pinna reflex in all rats. The mean (\pm SEM) duration of a TWR latency >6.0 and ≥ 10.0 s after epidural and intrathecal administration are given in the top lines of each panel in Table 1, which also gives the duration of antagonism of the pinna reflex. Complexation of 1 μg per rat sufentanil in HP-B-CD increased the duration of analgesia and of blockade of the pinna reflex. A maximal potentiation of the duration of analgesia was observed at 10% HP-B-CD (Table 1). The longest blockade of the pinna reflex was measured with sufentanil in 15% HP-B-CD.

Because 10% HP-B-CD was demonstrated to produce an almost maximal increase in the duration of analgesia, a dose-response function was established at this concentration. The lowest ED_{50} s of sufentanil in 10% HP-B-CD for a TWR latency >6.0 and ≥ 10.0 s were 0.025 and 0.079 μg per rat after epidural treatment and 0.032 and 0.032 μg per rat after intrathecal administration (Table 2; right panel). A blockade of the pinna reflex was measured at respectively 0.85 and 0.31 μg per rat. The specificity ratio (ED_{50} blockade pinna reflex/ ED_{50} TWR >6.0 or ≥ 10.0 s) ranged epidurally between 34.0 (TWR >6.0 s) and 10.76 (TWR ≥ 10.0 s) whereas for intrathecal administration it was 9.69.

Blood gas analysis

Plain sufentanil. A dose of 1.00 μg plain sufentanil, after both epidural and intrathecal administration, increased the arterial PCO_2 and decreased the PO_2 and oxygen saturation levels (Figs 1 and 2, left panels). After epidural administration (Fig. 1), a maximal increase in PCO_2 levels was observed with 5 min and significant differences from baseline remained present up to 90 min after treatment. Concomitant with the increase in PCO_2 there was also a maximal decrease in PO_2 and oxygen saturation within 5 min. Both parameters returned to baseline within 1½ h. After intrathecal administration of 1.00 μg sufentanil similar results were obtained (Fig. 2).

or any muscle rigidity. Intrathecal administration of 1 µg sufentanil produced a TWR latency of 10.0 s after epidural and intrathecal administration are given in the top panel of Table 1, which also gives the duration of the pinna reflex. A maximal potentiation of analgesia and of the pinna reflex was observed (Table 1). The longest TWR was measured with 10% HP-B-CD. HP-B-CD was demonstrated to produce a maximal increase in the TWR in a dose-response function with a concentration of 10%. The lowest ED₅₀ in 10% HP-B-CD for a TWR ≥ 10.0 s were 0.025 and 0.032 µg per rat for epidural treatment and intrathecal treatment (Table 2; right panel). A blockade of the pinna reflex was measured at respectively 0.025 and 0.032 µg per rat. The specificity of the pinna reflex/ED₅₀ TWR was 10.76 (TWR ≥ 10.0 s) after epidural administration it was

Table 1. Effects of various concentrations of hydroxypropyl-beta-cyclodextrin on the activity of 1 µg per rat sufentanil after epidural (upper panel) and intrathecal (lower panel) administration. The average durations for analgesia (TWR latency > 6 or ≥ 10.0 s) and for the blockade of the pinna reflex (*n* = 7 epidural, *n* = 5, intrathecal) are given

Epidural administration			
Cyclodextrin (%)	TWR > 6.0 s mean ± SEM (min)	TWR ≥ 10.0 s mean ± SEM (min)	Blockade pinna reflex mean ± SEM (min)
0	49.3 ± 5.4	21.4 ± 3.0	5.7 ± 1.7
2.5	36.4 ± 5.5	28.6 ± 5.5	10.0 ± 2.4
5.0	43.6 ± 8.4	26.4 ± 6.7	6.4 ± 2.4
10.0	62.1 ± 5.1	40.7 ± 5.4†	9.3 ± 2.8
15.0	55.7 ± 2.8	45.0 ± 3.3†	22.1 ± 3.9†
20.0	53.6 ± 3.0	36.4 ± 3.0†	17.1 ± 2.1†
25.0	47.5 ± 4.2	37.5 ± 3.4*	15.8 ± 3.3†
Intrathecal administration			
Cyclodextrin (%)	TWR > 6.0 s mean ± SEM (min)	TWR ≥ 10.0 s mean ± SEM (min)	Blockade pinna reflex mean ± SEM (min)
0	36.0 ± 6.0	16.7 ± 7.3	6.0 ± 2.5
2.5	42.0 ± 3.0	36.0 ± 6.0	10.0 ± 3.2
5.0	33.0 ± 5.6	27.0 ± 5.6	10.0 ± 3.2
10.0	72.0 ± 8.8†	48.0 ± 3.0†	22.0 ± 5.2*
15.0	66.0 ± 10.2*	54.0 ± 9.0†	27.0 ± 3.0†
20.0	51.0 ± 7.6	39.0 ± 6.0*	19.0 ± 4.8*

* *P* < 0.05; † *P* < 0.01 compared to control (0% cyclodextrin)

Table 2. *In vivo* comparison between sufentanil dissolved in water and in 10% hydroxypropyl-beta-cyclodextrin. Given are the lowest ED₅₀s to produce analgesia (TWR latency > 6.0 or ≥ 10.0 s) and to produce a blockade of the pinna reflex. Ratio c/a is the specific ratio for the ED₅₀ for the blockade pinna reflex; ratio c/b is the specific ratio for the lowest ED₅₀s for analgesia. Doses are given in µg per rat

Index	Sufentanil		Sufentanil/cyclodextrin	
	Epidural	Intrathecal	Epidural	Intrathecal
TWR > 6.0 s				
LED ₅₀ (a)	0.26	0.26	0.025	0.032
TWR ≥ 10.0 s				
LED ₅₀ (b)	0.58	0.29	0.079	0.032
Pinna reflex				
ED ₅₀ (c)	1.02	0.77	0.85	0.31
Ratio c/a	3.92	2.96	34.0	9.69
Ratio c/b	1.75	2.66	10.76	9.69

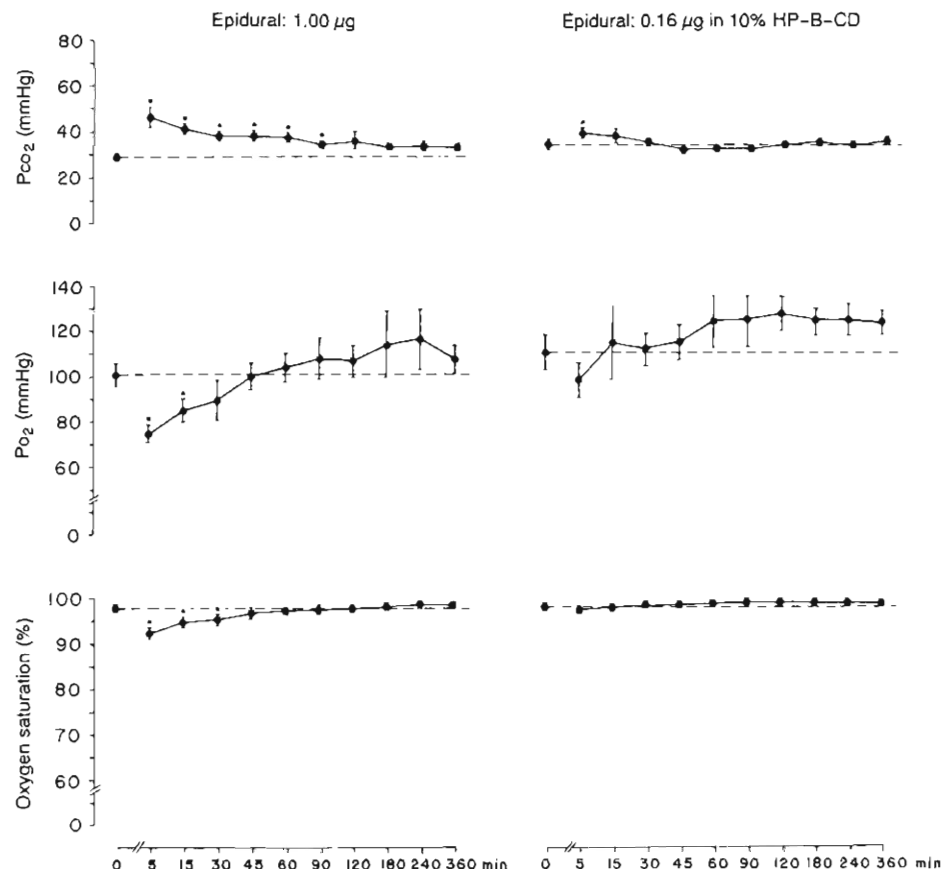


Fig. 1.. Effects of 1.00 µg epidurally administered sufentanil and 0.16 µg sufentanil in 10% HP-B-CD on PCO₂ (in mm Hg), PO₂ (in mm Hg) and oxygen saturation (in %) over time. Given are the mean (\pm 1 SEM) values of five rats before and at different periods after epidural treatment. The doses of sufentanil in 0 and 10% HP-B-CD were selected on the basis of equipotency in terms of analgesia. The horizontal dotted lines indicate the preinjection control base-lines. Differences from base-line were evaluated by means of the Wilcoxon matched-pairs signed-ranks test (two-tailed; * P < 0.05).

Higher doses of sufentanil caused more pronounced and longer lasting deviations from control levels for all three blood gas parameters after both epidural and intrathecal administration (Figs 3–6, left panels). Again, maximal effects were observed within 5 min after treatment and, depending on the parameter used, values returned to baseline within 90–180 min.

Sufentanil in 10% HP-B-CD. Sufentanil in 10% HP-B-CD produced somewhat different results. With a similar dose of twice the ED₅₀ for a TWR prolongation \geq 10.00 s, almost no

effects on blood gas parameters were detected (Figs 1 and 2, right panels). After epidural administration, 0.16 µg sufentanil in 10% HP-B-CD increased the PCO₂ only slightly over 5 min; there was no effect on PO₂ and oxygen saturation. Intrathecally given, 0.063 µg sufentanil in 10% HP-B-CD had no effects on PCO₂, PO₂ and oxygen saturation.

Increasing the amount of sufentanil in 10% HP-B-CD to 1.00 µg resulted in an increase in PCO₂ levels and a decrease in PO₂ and oxygen saturation (Figs 3–6, right panels). Given epidurally, 1.00 µg sufentanil in 10%

10% HP-B-CD

90 120 180 240 360 min

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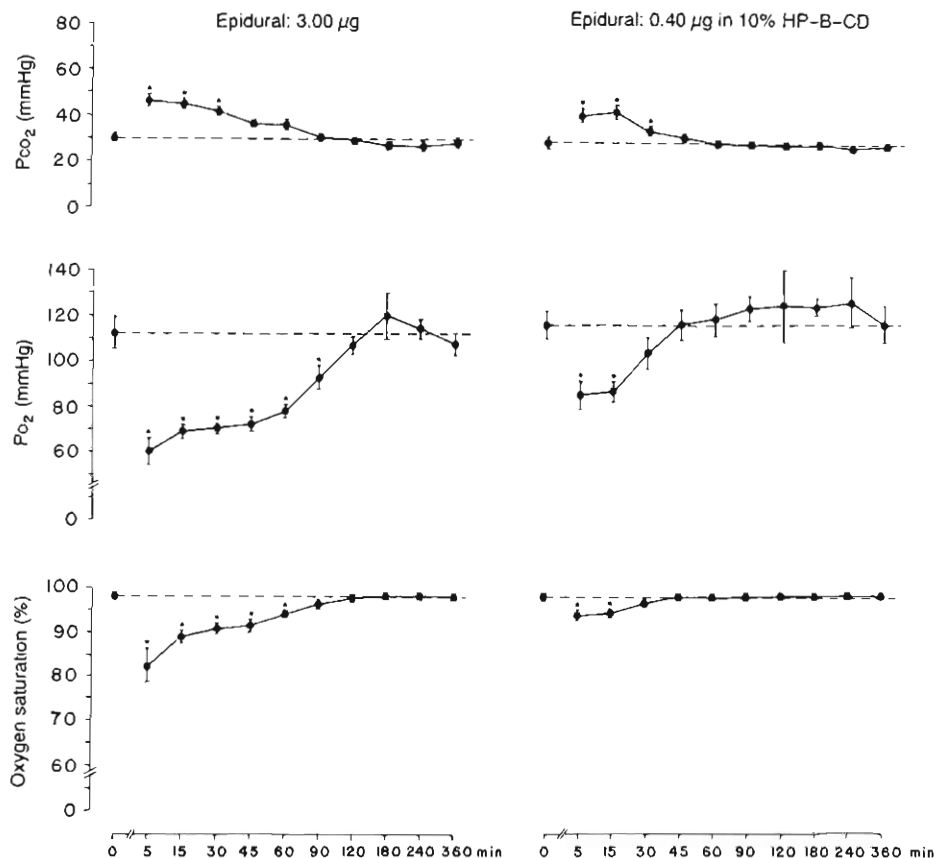


Fig. 2. Effects of 3.00 μ g sufentanil and 0.40 μ g sufentanil in 10% HP-B-CD after epidural administration on arterial blood gas parameters over time. See also legend to Fig. 1.

HP-B-CD resulted in a maximal raise in PCO₂ levels at 15 min after treatment. Concomitantly, the PO₂ and oxygen saturation dropped. Differences from baseline were observed up to 45 min (PCO₂ and PO₂) and 60 min (oxygen saturation) after treatment. Given intrathecally, 1.00 μ g sufentanil in 10% HP-B-CD produced a maximal increase in PCO₂ at 15 min after treatment. The lowest values of PO₂ and oxygen saturation were found within 5 min after starting treatment. PCO₂ returned to baseline within 30 min, and PO₂ and oxygen saturation within 60 min, of starting treatment.

Discussion

These results indicate that complexation of sufentanil with hydroxypropyl- β -cyclodextrin

(HP-B-CD) potentiates the duration, potency and specificity of epidurally and intrathecally administered sufentanil. The maximum increase in duration of analgesia for both spinal routes for light (TWR > 6 s) and deep surgical analgesia (TWR ≥ 10.0 s) was associated with 10% HP-B-CD. Higher concentrations of HP-B-CD did not increase the duration of analgesia further. The potentiation of sufentanil was also shown in the longer duration of the blockade of the pinna reflex, an *in vivo* index for opiate activity within the brain [9,10]. However the increase in duration of analgesia was the more pronounced effect, implying a longer-lasting analgesia without central side-effects.

The potentiation of activity and the increased specificity are also reflected in the dose response function of sufentanil in 10% HP-B-CD (Table

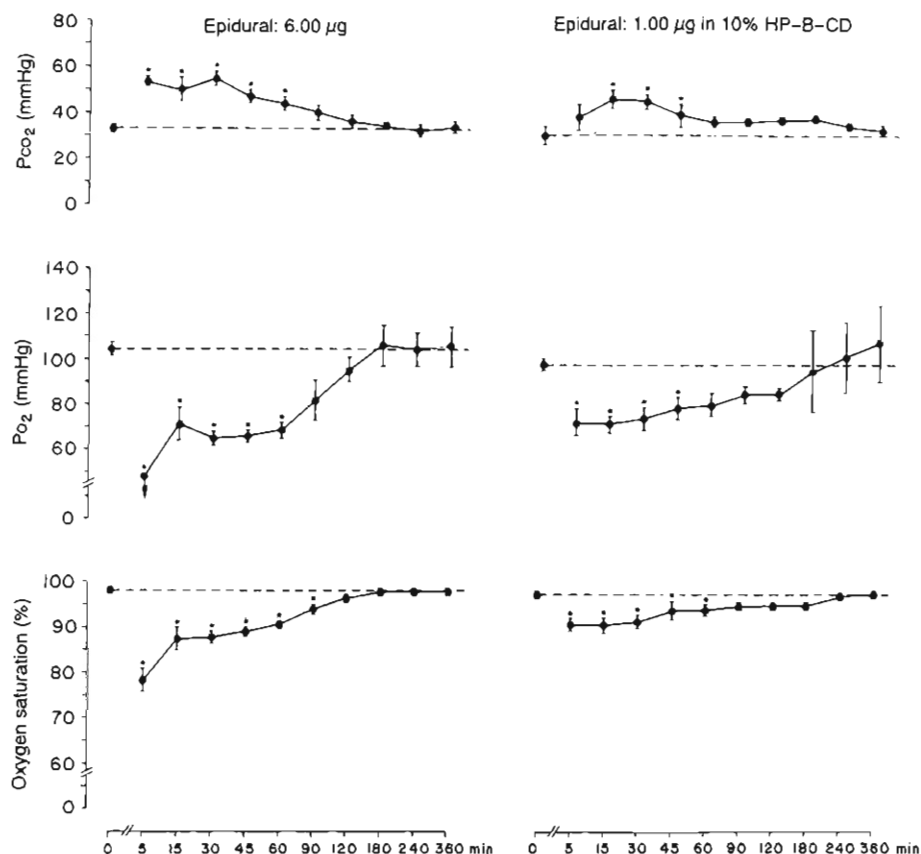


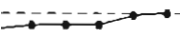
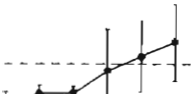
Fig. 3. Effects of 6.00 µg sufentanil and 1.00 µg sufentanil in 10% HP-B-CD after epidural administration on arterial blood gas parameters over time. See also legend to Fig. 1.

2). As compared to sufentanil alone [1], sufentanil complexation decreased the lowest ED_{50} s of both epidural and intrathecal sufentanil. The complexation also diminished the ED_{50} for the blockade of the pinna reflex by factors of 1.2 (epidural) and 2.5 (intrathecal). Since, again, the potentiation of analgesia was more pronounced than that of the blockade of the pinna reflex, higher specificity ratios could be obtained. In fact, the specificity ratios obtained here with sufentanil in 10% HP-B-CD were higher than those reported for morphine in comparable test situations [1]. On the basis of these increased specificity ratios, it can be expected that after administration of sufentanil in HP-B-CD, lower concentrations of sufentanil will be found in the plasma, reducing the

possibility of systemic side-effects, including respiratory depression.

At twice the ED_{50} for deep surgical analgesia, sufentanil in 10% HP-B-CD did not produce any changes in arterial PO_2 , PCO_2 and oxygen saturation levels after both epidural and intrathecal administration. Higher concentrations of sufentanil in 10% HP-B-CD caused early shifts in all three blood gas parameters. However, the deviations were smaller and shorter lasting than those observed with comparable doses of plain sufentanil. Thus, for both spinal routes, complexation of sufentanil in 10% HP-B-CD increases the safety ratio between the doses needed to produce analgesia and those producing respiratory depression. Furthermore, it appeared that overdose led to a lower risk of

n 10% HP-B-CD



90 120 180 240 360 min

er epidural administration

side-effects, including deep surgical analgesia, HP-B-CD did not produce P_{O_2} , P_{CO_2} and oxygen both epidural and intra-. Higher concentrations of HP-B-CD caused early shifts parameters. However, the and shorter lasting than comparable doses of plain both spinal routes, com- in 10% HP-B-CD in- io between the doses gesia and those produc- sion. Furthermore, it e led to a lower risk of

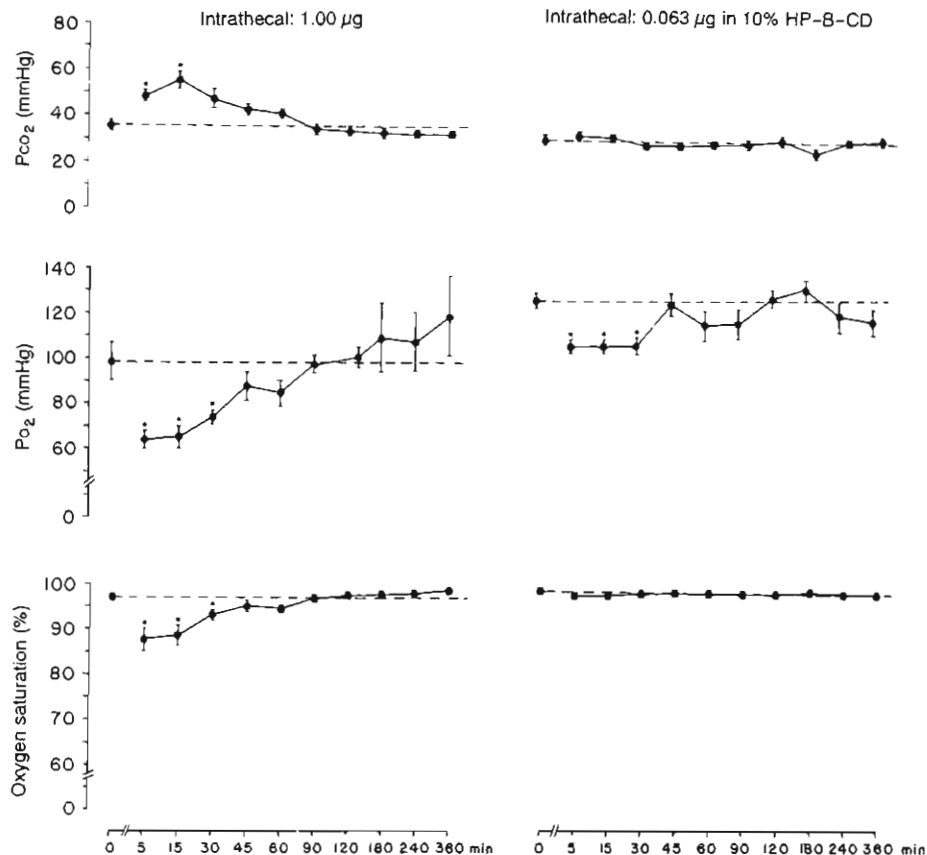


Fig. 4. Effects of 1.00 µg sufentanil and 0.063 µg sufentanil in 10% HP-B-CD after intrathecal administration on arterial blood gas parameters over time. See also legend to Fig. 1.

respiratory depression when sufentanil was complexed in 10% HP-B-CD when compared with sufentanil alone, thus confirming that complexation of sufentanil in 10% HP-B-CD reduces supraspinal side-effects. It is also of interest that sufentanil, alone or in 10% HP-B-CD, did not produce late respiratory depression in this preparation. These results imply that either the sufentanil/HP-B-CD complex will not spread rostrally or that all the sufentanil had left the complex before the HP-B-CD molecule reached the upper brain stem. Further studies, including distribution and elimination experiments, may be needed to clarify the dissociation and redistribution processes of both sufentanil and HP-B-CD after spinal administration in complexation.

In conclusion, the results presented here indicate that hydroxypropyl-β cyclodextrin can serve as a carrier molecule for sufentanil. Such a sufentanil/HP-B-CD complexation increases the duration, potency and specificity of the analgesia produced by sufentanil after both epidural and intrathecal administration. The best results were obtained after complexation of sufentanil in 10% HP-B-CD. The better and safer analgesic activity of sufentanil after complexation can probably be explained in terms of a longer availability of the drug at the site of injection, resulting in a diminished vascular redistribution with less systemic side-effects.

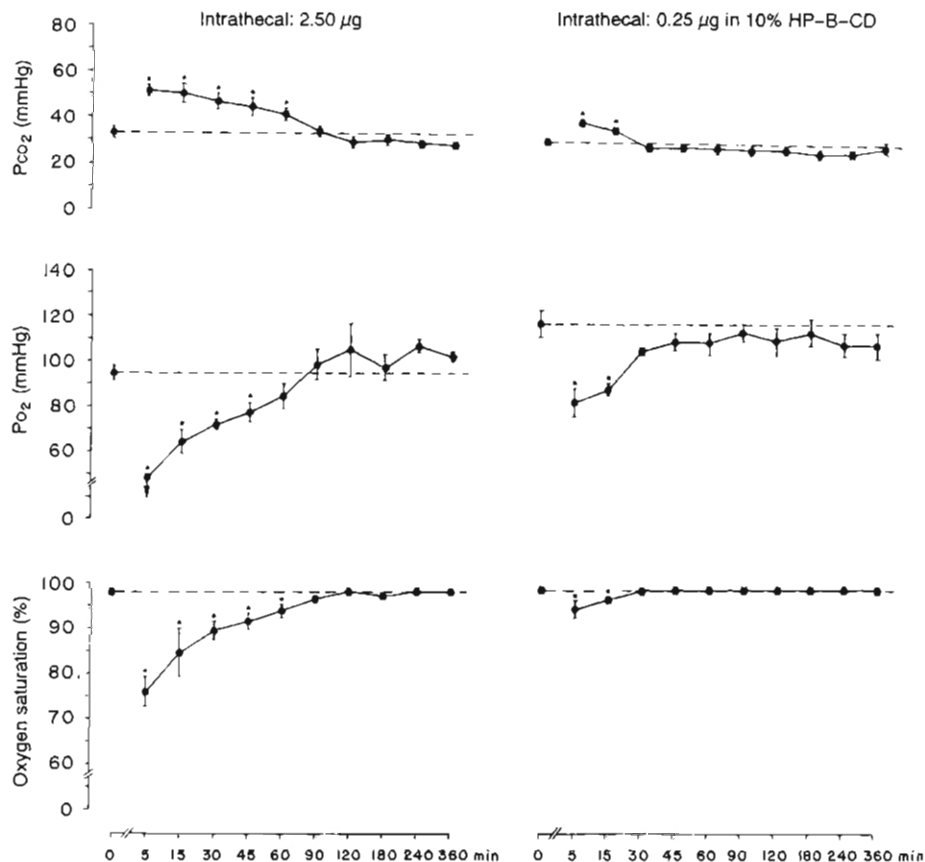


Fig. 5. Effects of 2.50 µg sufentanil and 0.25 µg sufentanil in 10% HP-B-CD after intrathecal administration on arterial blood gas parameters over time. See also legend to Fig. 1.

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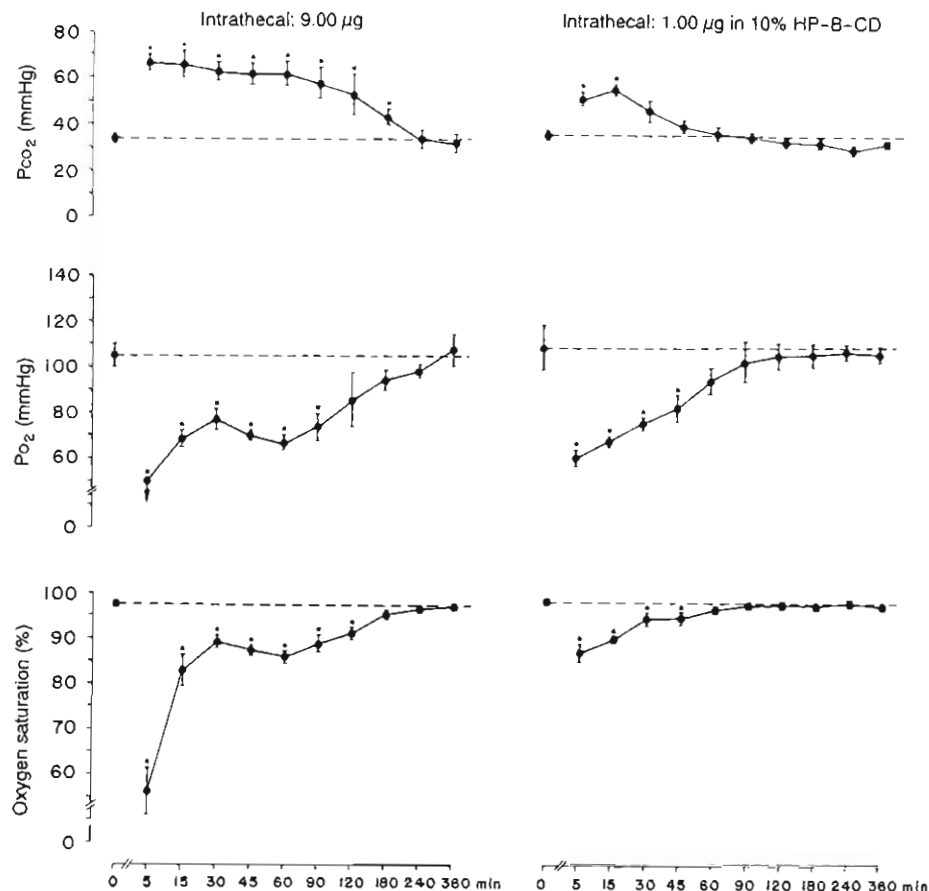


Fig. 6. Effects of 9.00 µg sufentanil and 1.00 µg sufentanil in 10% HP-B-CD after intrathecal administration on arterial blood gas parameters over time. See also legend to Fig. 1.

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Effect of (Hydroxypropyl)- β -cyclodextrin on Flux of Morphine, Fentanyl, Sufentanil, and Alfentanil through the Spinal Meninges of Monkey

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Received January 25, 1993, from the University of Washington School of Medicine, Department of Anesthesiology, RN-10, Seattle, WA 98195. Accepted for publication December 2, 1993*.

Abstract □ Previous studies have demonstrated that (hydroxypropyl)- β -cyclodextrin behaves as a slow-release reservoir when used as a vehicle for intrathecal administration of opioids. The goal of the current investigation was to determine if (hydroxypropyl)- β -cyclodextrin might serve as a slow-release vehicle for epidural opioid administration as well. An *in vitro* diffusion cell model was used to determine the flux of morphine, fentanyl, alfentanil, and sufentanil through the spinal meninges of *Macaque nemestrina* monkeys in the absence or presence of varying concentrations of (hydroxypropyl)- β -cyclodextrin. No concentration of cyclodextrin slowed the flux of any of the opioids through the meninges, indicating that (hydroxypropyl)- β -cyclodextrin will not behave as a slow-release reservoir for these opioids in the epidural space. This finding suggests that the rate-limiting step in opioid transfer was diffusion through the meninges not dissociation of the opioid cyclodextrin complex. However, (hydroxypropyl)- β -cyclodextrin significantly increased the flux of sufentanil through the meninges. Since sufentanil's hydrophobicity has previously been shown to impede its meningeal flux, this finding suggests that cyclodextrin effectively decreases sufentanil's hydrophobicity by formation of inclusion complexes in the aqueous environments of the spinal meninges.

Use of spinal opioids to treat acute pain, cancer pain, and non-malignant chronic pain is a common technique throughout the world. While this technique affords excellent analgesia without motor impairment, it is not without its limitations. The principal limitations encountered in the clinical use of neuraxial opioids are related to their relatively short duration of action and to their redistribution to supraspinal opioid receptors with associated sedation and respiratory depression. To overcome the limited duration of analgesia, catheters are commonly placed in the epidural or intrathecal space to allow repeated dosing. However, this approach requires either trained personnel to reinject the catheter at frequent intervals or continuous infusion pumps, and it does not eliminate the problem of opioid redistribution to supraspinal sites.

In an effort to solve both the duration and redistribution problems, a variety of delivery vehicles have been investigated for intrathecal opioid administration. Bernards et al.¹ demonstrated both prolonged duration of analgesia and decreased supraspinal redistribution in the rat by intrathecal administration of alfentanil encapsulated in liposomes. Similarly, Langerman et al.² demonstrated prolonged duration of meperidine analgesia in the rabbit when administered intrathecally in a lipid solution. More recently, Jang et al.³ demonstrated that use of (hydroxypropyl)- β -cyclodextrin as a delivery vehicle for intrathecal opioid administration in the rat resulted in prolongation of the opioid spinal analgesic effect and a reduction in systemic side effects related to supraspinal redistribution.

(Hydroxypropyl)- β -cyclodextrin is a cyclic polymer made up of seven glucopyranose subunits with hydroxypropyl substituents.⁴ This substituted cyclodextrin forms a torus with a hydrophobic interior and a hydrophilic exterior. The hydrophilic exterior renders the cyclodextrin molecule water soluble. The hydrophobic interior allows the cyclodextrin molecule to form

reversible complexes with drug molecules by forming noncovalent bonds with hydrophobic regions of the "guest" molecule.⁵ Jang et al.³ suggested that it was the ability of opioid molecules to reversibly form inclusion complexes with (hydroxypropyl)- β -cyclodextrin that explained their finding of prolonged duration of action when cyclodextrin was used as an intrathecal drug delivery vehicle. In short, the authors speculated that (hydroxypropyl)- β -cyclodextrin formed inclusion complexes with the opioids and served as a slow-release opioid reservoir within the intrathecal space.

The work by Jang et al.³ suggests that (hydroxypropyl)- β -cyclodextrin may be a useful agent to favorably modify the pharmacokinetics of intrathecally administered opioids. However, clinically, the majority of spinal opioids are administered by the epidural route, not the intrathecal route. Epidural opioid administration requires that the opioids diffuse across the spinal meninges—dura, arachnoid, and pia mater—in order to reach their sites of action in the spinal cord dorsal horn. Use of (hydroxypropyl)- β -cyclodextrin as a delivery vehicle for epidural opioid administration might be clinically useful if it either served as a slow-release reservoir or improved the meningeal permeability of epidurally administered opioids.

The goal of the current investigation was to determine the effect of (hydroxypropyl)- β -cyclodextrin on transmeningeal flux rates of clinically useful opioids. To answer this question, a previously established *in vitro* diffusion cell model⁶⁻⁸ was used to measure the flux of morphine, fentanyl, alfentanil, and sufentanil through the spinal meninges of the *Macaque nemestrina* monkey in the absence and presence of (hydroxypropyl)- β -cyclodextrin.

Experimental Section

Materials and Animals—Pharmaceutical-grade morphine sulfate (Elkins-Sinn, Cherry Hill, NJ), fentanyl citrate (Janssen Pharmaceutica, Inc., Piscataway, NJ), alfentanil citrate (Janssen Pharmaceutica, Inc., Piscataway, NJ), and sufentanil citrate (Janssen Pharmaceutica, Inc., Piscataway, NJ) were dissolved in mock cerebrospinal fluid (CSF) to yield 1.2 mM base stock solutions. Mock CSF consisted of 140 mequiv NaCl, 25 mequiv NaHCO₃, 2.9 mequiv KCl, 0.4 mequiv MgCl₂, 3.5 mequiv urea, 4.0 mequiv glucose, and 2.0 mequiv CaCl₂. Pharmaceutical-grade (hydroxypropyl)- β -cyclodextrin was the gift of Janssen Pharmaceutica, Inc. [³H]Morphine (specific activity = 50 Ci/mmol; New England Nuclear, Boston, MA), [³H]fentanyl (specific activity = 11.7 Ci/mmol; Research Diagnostics Inc., Flanders, NJ), [³H]sufentanil (specific activity = 54.4 Ci/mmol; Research Diagnostics Inc., Flanders, NJ), [³H]alfentanil (specific activity = 12 Ci/mmol; Research Diagnostics Inc., Flanders, NJ), and [¹⁴C](hydroxypropyl)- β -cyclodextrin (gift of J. Pitha) were used as radiotracers to determine drug concentrations in flux experiments.

Monkey (*M. nemestrina*) tissues were obtained from anesthetized animals of both sexes weighing 4.5–10.5 kg.

Tissue Dissection—Spinal cords of 25 *M. nemestrina* monkeys were removed en block and an incision was made along the ventral surface of the spinal cord through all three meningeal tissues (dura, arachnoid, and pia mater). By grasping the cut edge of the pia mater, the dura, arachnoid, and pia mater were carefully reflected from the spinal cord, preserving their normal anatomic relationships. Three tissue specimens were obtained from each animal and all three specimens were tested simultaneously.

The meningeal specimens were placed between two halves of a temperature controlled (37 °C) diffusion cell. Ten milliliters of mock

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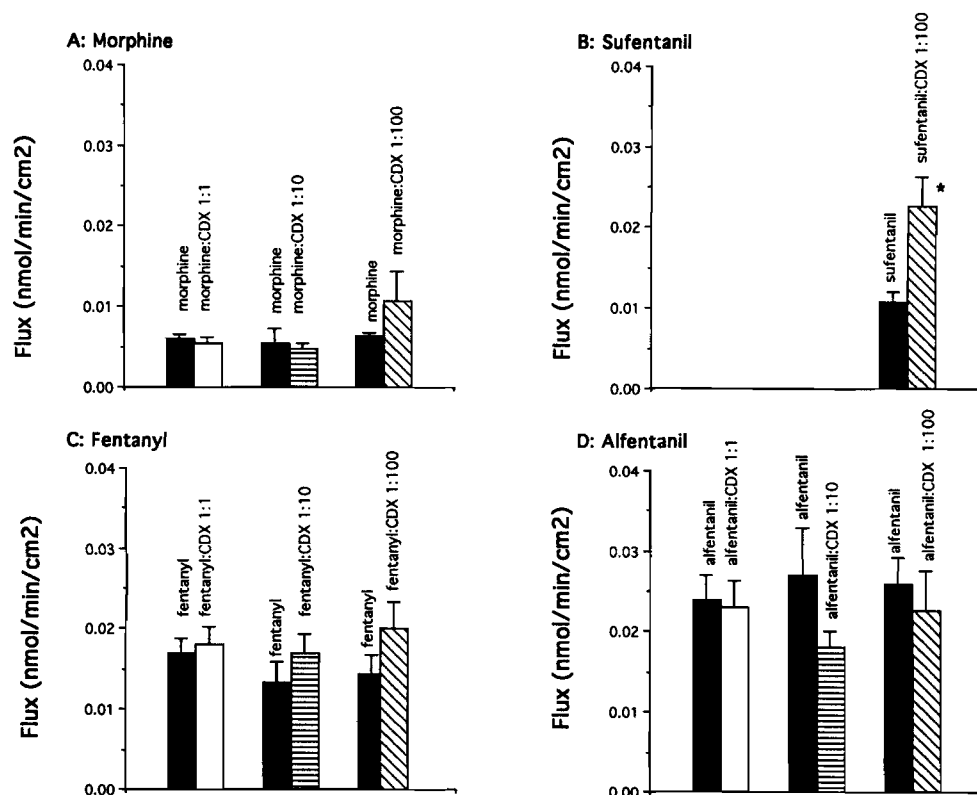


Figure 1—Histograms of drug flux in the presence and absence of (hydroxypropyl)- β -cyclodextrin. Black bars indicate flux of the study drug in the absence of cyclodextrin and adjacent light bars indicate flux of the same drug through the same tissues in the presence of the indicated concentration of cyclodextrin. Data are mean \pm SEM. The asterisk indicates $p < 0.05$. $n = 5$ –8 for all data points.

CSF was placed in the fluid reservoirs on either side of the meningeal tissue. Each fluid reservoir was vigorously stirred by a magnetic stirrer to minimize unstirred layer effects.⁹ Oxygen (95%) and carbon dioxide (5%) were bubbled through each fluid reservoir to maintain normal pH and to provide oxygen for meningeal cells.

Flux Measurements—At time 0, 100 μ L of mock CSF containing the study drug (1.2 mM) and 3 H-labeled radiotracer were added to the donor reservoir on the dura mater side of the diffusion cell. Thereafter, at 5-min intervals for 45 min, 200- μ L samples were removed simultaneously from the donor reservoir on the dura mater side of the diffusion cell and from the recipient reservoir on the pia mater side of the diffusion cell. The samples were placed into borosilicate glass scintillation vials for later scintillation counting to determine drug concentrations.

At the completion of the experiment, the fluid reservoirs were emptied and rinsed five times with mock CSF to remove any remaining drug/radiotracer. The experiment was then repeated identically except that (hydroxypropyl)- β -cyclodextrin was added to the solution containing the study drug/radiotracer to produce a final cyclodextrin concentration of 12, 120, or 1200 μ M. Again, samples were taken from the fluid reservoirs on both sides of the meningeal tissue at 5-min intervals for 45 min. Previous studies using this model have shown that repeated flux measurements through the same tissue are reproducible over a 4-h period, indicating that the tissue remains viable throughout the study period.⁶

The flux of (hydroxypropyl)- β -cyclodextrin through the spinal meninges in the absence of any study drugs was determined in the same manner.

Flux Calculation—Flux (Q) was determined from drug concentration data by plotting the amount of drug (nanomoles) in the recipient reservoir at each time point. The slope of the line relating drug content versus time data was determined using least-squares linear regression and is equal to the test drug's flux through the meninges in units of nanomoles per minute. The flux was then divided by the area of the port connecting the two fluid reservoirs of the diffusion cell to yield flux per square centimeter.

All experiments were conducted over a time period during which the concentration gradient of the drug under study decreased by less than 2% from its value at time zero. This condition assures that flux is effectively unidirectional and that the concentration gradient across the tissues is nearly constant throughout the experiment.

Drug Concentration Analysis—Samples were placed into borosilicate glass vials containing 5–10 mL Hydroflour scintillation cocktail (National Diagnostics, Manville, NJ). Samples were counted in a Packard liquid scintillation counter (Tri-Carb 2000, Packard Instrument, Downers Grove, Illinois) for 20 min or until the standard deviation of depurations per minute (dpm) was $\leq 2\%$. Background counts from blank mock CSF were subtracted from the sample dpm before calculating drug concentration.

Statistical Analyses—Differences in each drug's flux rate in the absence of (hydroxypropyl)- β -cyclodextrin vs flux rate in presence of (hydroxypropyl)- β -cyclodextrin was assessed by the paired t -test. Differences were considered significant at the $p \leq 0.05$ level. All experimental results are reported as mean \pm standard error.

Results and Discussion

Determination coefficients (r^2) for regression lines used to determine opioid flux averaged 0.941 ± 0.003 , indicating excellent fit of the data to a linear model.

Figure 1 demonstrates that no concentration of (hydroxypropyl)- β -cyclodextrin significantly decreased the meningeal flux of any of the opioids studied. Thus, cyclodextrin does not appear to behave as a slow-release reservoir for movement of these opioids through the spinal meninges. In order for cyclodextrin to act as a slow-release reservoir for epidural opioids, it must be less permeable through the meninges than the drugs one seeks to retain in the epidural space. The flux of (hydroxypropyl)- β -cyclodextrin (12 μ M) was less than that of all of the opioids studied (0.0046 ± 0.0018 nmol/min/cm²; $n = 5$), indicating that it is, in fact, less permeable. The fact that (hydroxypropyl)- β -cyclodextrin's flux was less than that of the opioids suggests that it is the free opioid which crosses the meninges, not the opioid-cyclodextrin complex.

The fact that (hydroxypropyl)- β -cyclodextrin did not slow the meningeal flux of any of the opioids does not mean that these drugs do not form complexes with cyclodextrin. In fact,

morphine has been shown to form inclusion complexes with β -cyclodextrin.¹⁰ It is possible that the opioids form inclusion complexes but that dissociation from (hydroxypropyl)- β -cyclodextrin is not the rate-limiting step in meningeal diffusion. In order for a drug to cross the meninges, it must dissociate from the cyclodextrin cavity and then diffuse through the meninges. Which of these steps is rate limiting will determine the effect of cyclodextrin on meningeal flux. The dissociation of guest molecules from cyclodextrins follows the laws of mass action and the rate of dissociation will depend upon the relative affinity of the guest molecule for the hydrophobic center of the cyclodextrin. If an opioid's rate of dissociation from cyclodextrin is significantly faster than its meningeal diffusion rate, then meningeal diffusion will be rate limiting and cyclodextrin will not have an effect on the drug's meningeal permeability.

The difference between our findings and those of Jang et al.³ may reflect a difference in rate-limiting step. Jang et al. administered cyclodextrin-complexed opioids into the intrathecal space and found that analgesia was prolonged by cyclodextrin. To reach the spinal cord from the intrathecal space, opioids must cross only the pia mater. In contrast, to reach the spinal cord from the epidural space, opioids must cross all three spinal meninges, dura mater, arachnoid mater and pia mater. The arachnoid mater is the principal meningeal permeability barrier and resistance to diffusion through it is 1 order of magnitude greater than resistance to diffusion through the pia mater.⁶ Therefore, it is possible that in the intrathecal space dissociation from (hydroxypropyl)- β -cyclodextrin is rate limiting, while in the epidural space diffusion through the arachnoid mater is rate limiting. If dissociation from cyclodextrin is in fact rate limiting in the intrathecal space, then it could serve as a slow-release reservoir as Jang et al.³ observed.

An interesting finding of potential clinical use is that (hydroxypropyl)- β -cyclodextrin more than doubled the flux of sufentanil (Figure 1). An understanding of the nature of the arachnoid mater as a permeability barrier is important for interpreting this finding. The arachnoid mater consists of overlapping tiers of flattened cells connected to one another by frequent tight junctions and occluding junctions.¹¹ Diffusion through the arachnoid mater requires that a drug molecule repeatedly negotiate hydrophobic lipid bilayers and hydrophilic intra- and intercellular spaces. The necessity for drug molecules to partition repeatedly into both hydrophilic and hydrophobic environments likely accounts for the fact that hydrophobicity is the principal determinant of meningeal permeability.⁸ While hydrophobicity is the major determinant of meningeal permeability, the relationship is not linear. Drugs of intermediate hydrophobicity (octanol-buffer distribution coefficient = 100–200) are significantly more permeable than are drugs of greater or lesser hydrophobicity.⁸ The presumed reason for this relationship between hydrophobicity and permeability is that hydrophilic drugs do not readily partition into the lipid bilayers of the arachnoid cells and this slows their diffusion. On the other hand, hydrophobic drugs readily partition into and thus diffuse through the hydrophobic environments of the arachnoid mater. However, when hydrophobic drugs encounter the lipid/aqueous interface on the opposite side of the lipid bilayer they re-enter the aqueous phase with difficulty and this becomes rate limiting.

This relationship between hydrophobicity and meningeal permeability may explain the effect of (hydroxypropyl)- β -cyclodextrin on the meningeal flux of sufentanil. Sufentanil is a very hydrophobic drug (octanol-buffer distribution coefficient = 1737) that should readily partition into the lipid environments of the arachnoid mater. However, the same forces which favor partitioning of sufentanil into hydrophobic environments will impede its movement back into the aqueous environments of the arachnoid mater, or the subarachnoid space. The ability of (hydroxypropyl)- β -cyclodextrin to solubilize hydrophobic drugs

in aqueous environments may favorably alter the thermodynamics of sufentanil's partitioning from lipid bilayers back into aqueous environments and thereby increase its flux.

The reason that cyclodextrin did not increase the flux of any of the other opioids may reflect the fact that they are significantly less hydrophobic than sufentanil (octanol-buffer distribution coefficients: morphine = 1; alfentanil = 129; fentanyl = 955). The flux of morphine and alfentanil is not limited by their hydrophobicity; thus, increasing their aqueous solubility by formation of inclusion complexes with cyclodextrin would not be expected to increase their flux. Though fentanyl's flux is somewhat limited by its hydrophobicity, the effect is less than that for sufentanil. Thus, the ability of cyclodextrin to alter the kinetics of partitioning between hydrophobic and hydrophilic environments would be expected to have less effect on fentanyl flux than on sufentanil flux.

Another possible explanation for the increase in sufentanil flux is that (hydroxypropyl)- β -cyclodextrin alters the arachnoid mater in such a way as to decrease its performance as a diffusion barrier. In fact, cyclodextrin solutions have been shown to extract membrane-bound proteins which could conceivably disrupt arachnoid cell membranes and thereby increase drug flux. However, since the flux of other opioids was not increased at any concentration of cyclodextrin, it seems unlikely that a generalized change in arachnoid mater barrier characteristics explains the specific increase in sufentanil flux. (Hydroxypropyl)- β -cyclodextrin has also been shown to improve the permeability of drugs by solubilizing molecules which are otherwise poorly soluble in aqueous solutions. Since all opioids used in this study were at concentrations well below their solubility product constants and were already in aqueous solution when added to the diffusion cell, dissolution of undissolved drug does not explain sufentanil's increased flux in the presence of cyclodextrin.

Conclusions

(Hydroxypropyl)- β -cyclodextrin does not behave as a slow-release reservoir for opioids diffusing through the spinal meninges of the *M. nemestrina* monkey. This finding is in contrast to work indicating that (hydroxypropyl)- β -cyclodextrin does behave as a slow-release reservoir in the subarachnoid space. However, (hydroxypropyl)- β -cyclodextrin does significantly improve the meningeal permeability of the hydrophobic opioid sufentanil.

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