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Allopregnanolone Treatment, Both As a Single Injection or Repetitively, Delays Demyelination and Enhances Survival of Niemann-Pick C Mice

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Abstract:

Niemann-Pick C Disease (NPC) is an irreversible neurodegenerative disorder without current treatment. It is a result of deficient intracellular cholesterol and/or ganglioside movement. We have investigated the effects of allopregnanolone treatments on survival, weight loss and motor function in the mouse model of Neimann-Pick C Disease (Npc1−/− mice). We confirmed previous results that a single injection of 250 micrograms of allopregnanolone on postnatal day 7 significantly extended the life span of Npc1−/− mice. This caused a marked difference in the weight curves of the treated mice but no statistical difference in the Rota-Rod performance. The effect of allopregnanolone treatment was also studied in mdr1a−/− mice, which have a deficient blood-brain barrier. The effect of the single, postnatal day 7 injection of 250 micrograms of allopregnanolone in these mice resulted in similar survival but poorer weight gain. T2-weighted magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) of treated mice showed values of signal intensity and fractional anisotropy closer to those of wild-type mice than those of untreated Npc1−/− mice. Neuropathology showed that day 7 treatment markedly suppressed astrocyte reaction and significantly reduced microglial activation. Furthermore, the steroid treatment also increased myelination in brains of Npc1−/− mice. A single injection on day 7 followed by injections every 2 weeks was also evaluated via survival and weight loss and had larger effects. We conclude that allopregnanolone treatment significantly ameliorates several symptoms of Neimann-Pick C Npc1−/− mice, presumably by effects on myelination or neuronal connectivity.
Key Words: neurodegeneration, neurosteroids, GABA receptors, mouse models, magnetic resonance imaging
**Introduction:**

Niemann-Pick type C (NPC) disease is a panethnic autosomal recessive disorder of unknown pathogenesis (Vincent et al. 2003). A major biochemical finding in this disorder is the intracellular accumulation of unesterified cholesterol within lysosomes and the Golgi apparatus. These findings have prompted the conclusion that NPC is a disorder of intracellular cholesterol trafficking (Patterson et al. 1995). When cloned, the predicted protein was found to contain a sterol-sensing domain consensus site and other motifs that suggest a direct causative role for a mutant \( NPC1 \) product in the altered cholesterol movement in NPC (Carstea et al. 1997). The finding that the gene for NPC2, a very similar disorder, encodes a soluble lysosomal protein with cholesterol binding properties also implicates cholesterol transport. The evidence that the combined lack of \( Npc1 \) and \( Npc2 \) did not significantly alter disease onset in these mouse models (Sleat et al. 2004) emphasizes the importance of cholesterol binding. However, the role of \( NPC1 \) in intestinal transport of cholesterol (Altman et al. 2004) is still moot (Kramer et al. 2005). NPC1 has also been implicated in the traffic of non-arachidonic fatty acids from late endosomes/lysosomes (Leventhal et al. 2004) by one group but this could not be confirmed by a second one (Passeggio and Liscun, 2005). In spite of the likely role in intracellular cholesterol trafficking, the pathophysiological basis for the symptoms present in NPC is unknown and the errant trafficking of gangliosides, which have been shown to accumulate within cells (Zervas et al. 2001), may play a significant role as well.

Previous studies with \( Npc1^{-/-} \) mice revealed a time-dependent accumulation of unesterified cholesterol in every organ except the brain (Patterson et al. 1995).
Subsequently, it was found that the brain’s apparent failure to accumulate cholesterol was due to a balance of neuronal accumulation and non-neuronal loss due to demyelination (Dietschy et al. 2001). Treatment of NPC patients with agents that lower somatic cholesterol has not had any significant effects on patient’s neurological symptoms (Patterson et al. 1993). Nifedipine and probucol, two agents that effectively reduce liver cholesterol, did not alter the progression of CNS disease in Npc1−/− mice (Erickson et al. 2000). A more recent study found that intra-peritoneal delivery of cholesterol-mobilizing cyclodextrins decreased liver cholesterol storage in Npc1−/− mice. However, both intra-peritoneal and intrathecal delivery of cyclodextrins, had only slight effects on the onset of neurological symptoms (Camargo et al. 2001).

Recently, Griffen et al. (2004) reported that the Npc1−/− mouse had disrupted neurosteroidogenesis and showed a marked improvement with single postnatal day 7 injections of allopregnanolone and lesser improvement with single injections at older ages. These authors found that the activities of the allopregnanolone synthesizing enzymes, 5α-reductase, 3α-hydroxy steroid dehydrogenase (3α HSD) and 20α-hydroxy steroid dehydrogenase (20α HSD) were normal at embryonic day 16.5; however there was a greater than 50% decline in 3α HSD at birth and it was absent by 10 weeks, while 5α-reductase was decreased by more than 50% at 3 weeks and was very low at 10 weeks. There was no decrease in 20α HSD activity. These changes were found in the cortex, mid-brain and hind-brain of the Npc1−/− mice (Griffen et al. 2004). Purkinje cell survival in lobes 6 and 7 of the cerebellum were significantly increased by the day 7 injection, while storage of gangliosides GM1,2,3 were significantly decreased by the day 7 injection.
In the present study, we have confirmed and extended these findings by performing additional neuroimaging and neurohistological analysis to further characterize the effects of allopregnanolone. Allopregnanolone treatment was also carried out on \textit{mdr1a}\textsuperscript{−/−} mice, which have a deficient blood-brain barrier. Finally, the effect of a single injection on day 7 followed by injections every 2 weeks was studied in \textit{Npc1}\textsuperscript{−/−} mice to evaluate whether or not additional treatment of allopregnanolone would have a more significant positive effect.

\textbf{Materials and Methods:}

\textbf{Animals:}

\textit{Npc1\textsuperscript{NIH}} mutant (\textit{Npc1}\textsuperscript{−/−}) mice on the BALB/cJ background were maintained by brother-sister mating of heterozygous animals. Animals were kept at the University of Arizona Animal Care Facility (PHS Assurance No. A-3248-01) on mouse chow containing 6\% fat (or 10\% for breeding mothers) and water \textit{ad libitum}. Before or at weaning (postnatal day 14 or 21), tail tips were removed from mice and DNA was prepared. Polymerase chain reactions (PCRs) to identify genotypes at the \textit{Npc1} locus were performed using the primer pairs described in footnote 28 of Loftus et al. (1997). For PCR we used 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L Mg\textsubscript{2+}, 200 umol/L dNTPs, 1.25U \textit{Taq} polymerase, and 1 umol of each primer. DNA (20-40 ng) was added at room temperature, and cycles of 30s at 95°C, 30s at 61°C, 1 min at 72°C \times 35, and 10 min at 72°C were used. The products were separated on 1.5\% agarose gels.
Mdr1a<sup>−/−</sup> mice were the knockouts developed by Schinkel, et al (1994) and were obtained from the Jackson Laboratory (Bar Harbor, ME). This combination of mdr1a<sup>−/−</sup>/Npc1<sup>−/−</sup> has previously been shown to correct the sterility of Npc1<sup>−/−</sup> females (Erickson, et al. 2002).

Drug and Its Delivery:

Allopregnanolone (Sigma/Aldridge) was dissolved in a 20% solution of beta-cyclodextrin in water at 1.25 milligrams per ml. by brief sonication of the chilled solution and was injected at 25 milligrams per kilogram subcutaneously at day 7 and interperitoneally at later times.

The Npc1<sup>−/−</sup> mice were tail tipped and genotyped at 21 days of age. Npc1<sup>−/−</sup> pups and their litter mates had been injected on day 7 as described above. In the case of repeated injections, only the Npc<sup>−/−</sup> mice were again injected on day 21 and at 2 week-intervals thereafter until death.

Evaluation Criteria

All mice were evaluated using three criteria: weight loss, Rota-Rod performance and survival. Mouse body weights were recorded on a Monday, Wednesday, Friday schedule. Mice were allowed to survive their full life-span (but were euthanized when no longer eating) for Kaplan-Meyer analysis.
Each mouse was evaluated weekly on a Rota-Rod test instrument (Ugo-Basile, NY, USA). The Rota-Rod was driven at an accelerating rate, 11 - 25 rpm in 300 sec. The maximum testing time was 300 sec. The mouse was given 3 trials on the Rota-Rod, and the maximum trial time was recorded. Mice that could not remain on the Rota-Rod for at least 10 seconds were considered to have failed.

MRI Protocol
Magnetic resonance imaging (MRI) was carried out in four Npc1+/+ mice, four Npc1−/− without treatment, and four Npc1−/− treated with a single allopregnanolone injection at day 7. All mice were scanned at 67 ± 2 days old. For all MRI experiments, animals were anesthetized by isoflurane gas at a nominal ratio of isoflurane:oxygen of 1.5:1 and placed into a homemade mouse holder. The holder was constructed such that it would fit snugly into a 20 mm Litz coil (Doty Scientific Inc, Columbia, SC), which was used in a 4.7 T horizontal bore Bruker Biospec Avance® MRI instrument (Bruker, Karlsruhe, Germany) equipped with actively shielded gradients capable of 200 mT/m with rise times of 200 µs. Body temperature was monitored with a fiber optic rectal temperature probe (Luxtron, Santa Clara, CA) and maintained at 37 °C using a circulating heated water bath. Diffusion tensor imaging (DTI) was carried out in the transverse plane using a diffusion-weighted radial spin-echo pulse sequence (Trouard et al. 1999) with the following parameters: TR = 2 s, TE = 54 ms, slice thickness = 0.5 mm, Field of View (FOV) = 1.92 × 1.92 cm², acquisition matrix = 128 × 128 (data points along a radial line × number of radial lines), and the number of averages = 6. A total of seven image sets were collected from 12 contiguous sections: one without diffusion weighting and six with diffusion
weighting \( (b = 1010 \text{ s/mm}^2, \Delta = 25 \text{ ms}, \delta = 9 \text{ ms}) \) along six non-colinear directions (Hasan et al. 2001). The total scan time for each animal was approximately 3 hours. Images were reconstructed using a filtered back projection reconstruction (FBPR) onto a 128 x 128 image matrix. The three rank-ordered principle diffusivities of the diffusion tensor \( (\lambda_1, \lambda_2 \text{ and } \lambda_3) \) were calculated from the diffusion weighted images using standard algorithms (Pierpoli and Basser, 1996). From these principle diffusivities, the fractional anisotropy, FA, was calculated according to (LeBihan et al., 2001):

\[
FA = \sqrt\frac{\left(\lambda_1 - \langle \lambda \rangle\right)^2 + \left(\lambda_2 - \langle \lambda \rangle\right)^2 + \left(\lambda_3 - \langle \lambda \rangle\right)^2}{2\left(\lambda_1^2 + \lambda_2^2 + \lambda_3^2\right)}
\]

which has values between 0 (isotropic diffusion) and 1.0 (diffusion restricted to a single direction). Data analysis was carried out using programs written in Interactive Data Language (IDL, Research System, Boulder, CO).

**Immunohistochemistry**

Sagittal sections from cerebellum and coronal sections from the rest of the brains of \( \text{Npc1}^{+/+} \) and \( \text{Npc1}^{-/-} \) mice with or without allopregnanolone treatment were simultaneously processed for immunostaining. Immunohistochemistry was performed using the avidin-biotin horseradish peroxidase complex (ABC) method. Briefly, free-floating sections were first incubated in 10% normal horse serum diluted in PBS with 0.1% Triton X-100 for 1 hr at room temperature, followed by incubation with primary antibodies overnight at 4 °C. Antibodies used were: monoclonal rat anti-F4/80 antigen (Austyn and Gordon, 1981 [1:500; Serotec, Oxford, UK]), mouse anti-GFAP (1:7500; Sigma), and mouse anti-CNPase 2’, 3’-cyclic nucleotide 3’-phosphodiesterase (1:500,
Chemicon. After 3 washes in PBS, sections were incubated with corresponding
biotinylated secondary antibodies (1:400; Vector Laboratories, Burlingame, CA) in 5%
normal horse serum solution for 2-3 hrs, then in ABC diluted in PBS for 45 min.
Peroxidase reaction was carried out with 3,3’-diaminobenzidine tetrahydrochloride
(0.05% in 50 mM Tris-HCl buffer, pH 7.4) as chromogen and 0.03% H₂O₂ as oxidant.
Free-floating sections were mounted on pre-coated slides (SuperPlus, Fisher) and air-
dried. Sections were then dehydrated in graded ethanol and finally covered with
Permout.

Quantitative image analysis of F4/80 immunopositive cells in the primary somatosensory
cortex (SSp) and cerebellar cortex (CB) was carried out as previously described (Bi et al.
2004). Briefly, regions of interest were scanned and digitized with a Zeiss digital photo
camera (AxioCam Hrc) using Axivison v. 3.1. An automated computer program
generated by Dr. Fernando Brucher (University of California, Irvine) was used to
enhance image contrast, extract F4/80 immunopositive cells, and measure their total area.
Statistical significance was determined using two-tailed Student t-Test.

Results:

A) Mice treated with a single injection at day 7

1) Survival: Untreated Npc1+/− mice (whether on the regular or mdr1a+/− background,
where survivals are not statistically different) have a survival of 77.8 ± 1.2 days (mean ±
std error, n = 22). A single injection of allopregnanolone at day 7 increased the survival
to 118.6 ± 5.1 days (n = 7). This is statistically different at p <= 10⁻⁶. Treatment with
allopregnanolone at day 7 in blood/brain barrier deficient mdr1a<sup>−/−</sup> mice resulted in survival of 109.7 ± 4.7 days (n = 6). Kaplan-Meyer analyses of these data are shown in Figure 1.

Weight loss is a significant indicator of the progression of Npc-1<sup>−/−</sup> disease. As seen in Figure 2, weight loss was significantly delayed in mice treated with allopregnanolone on day 7. Treated mdr1a<sup>−/−</sup> mice were delayed in their attainment of the usual maximal weight, but then declined in parallel with the non-mdr1a<sup>−/−</sup> mice. This could suggest a negative effect of the early allopregnanolone injection on some neurological function in this genetic background.

2) **Rota-Rod:**

Rota-Rod performance was moderately enhanced until about 10 weeks in the day 7, treated mice but gradually decayed at older ages (Fig. 3). The mdr1a<sup>−/−</sup> single-injected mice were not significantly different than the controls (data not shown).

3) **Diffusion Tensor Imaging:**

Representative T2-weighted images and fractional anisotropy (FA) maps of mouse brain sections from a wild-type mouse, a Npc1<sup>−/−</sup> mouse without treatment, and a day 7 treated Npc1<sup>−/−</sup> mouse are shown in figure 4. Mice were scanned at approximately 67 days of age, which corresponds to near end-stage for untreated Npc1<sup>−/−</sup> mice. For similar age mice, the brain size of the Npc1<sup>−/−</sup> untreated mice is typically smaller than the wild-type mouse as can be seen in Panels A and B of Figure 4. Day 7 treated Npc1<sup>−/−</sup> mice have brain sizes in
between these two. An important difference between the mouse brain images is the hyperintensity in the T2-weighted images in the region of the corpus callosum in the untreated Npc1−/− mouse (see arrow in Fig. 4b). In wild-type mice, this same region shows a conspicuously low signal (Fig. 4a) due to the decreased T2 relaxation times in highly myelinated structures. In the treated Npc1−/− mouse, the corpus callosum is isointense with the surrounding structures. Fractional anisotropy maps reveal a generally reduced anisotropy in the white matter regions of the untreated Npc1−/− brain (Fig 4e) as compared to the wild-type (Fig 4d). Differences are particularly apparent in the corpus callosum, and within the internal capsule and external capsule. Day 7 treated Npc1−/− mice also show decreased anisotropy in white matter but it does not appear as severe as in the treated Npc1−/− mouse. These differences were observed in all the mice studied.

The differences in diffusion anisotropy between the three groups are shown quantitatively in Figure 5. The inverse cumulative histograms for FA values of pixels from the entire brain are given Figure 5a (n=4, each group). This histogram shows the percent of pixels in the brain having values greater than a given value of FA and the thickness of each line corresponds to the standard deviation in the measure within each group. The percentage of pixels above a particular FA value is always highest for wild-type mice. Untreated Npc1−/− mice have significantly fewer pixels at high FA values than the wild-type and this extends down to FA values of 0.2. Day 7 treated Npc1−/− mice also have fewer high FA pixels than wild-type mice, but have values much closer to wild-type mice than untreated mice. This same data is plotted in somewhat different manner in Figure 5b, where the percent pixels of the Npc1−/− mice (treated and untreated), normalized to the percent pixels
of wild-type mice are plotted versus FA values. The dashed line in the figure represents the case where two brains have identical histograms such that no differences are observed. Untreated Npc1<sup>−/−</sup> mice start showing differences from wild-type mice at FA ≈ 0.2 and these differences become more pronounced at higher FA values. For example, compared to wild-type mice, untreated Npc1<sup>−/−</sup> mice have only 20% of the pixels with FA above 0.8. Day 7 treated Npc1<sup>−/−</sup> mice also show fewer pixels at most FA values compared to wild-type mice, but start showing differences at higher an FA value (FA ≈ 3.75) compared to the untreated Npc1<sup>−/−</sup> mice. There is also a preservation of brain tissue with higher FA values compared to untreated Npc1<sup>−/−</sup> mice.

4) Neuropathology:

As reactive astrocytes and microglial activation are early onset signs of neuropathology in brains of Npc1<sup>−/−</sup> mice, potential effects of allopregnanolone treatment on these cell types were investigated by immunohistochemistry with anti-GFAP and anti-F4/80 antibodies. In wild-type mice, GFAP-immunopositive cells were mainly observed in the vicinity of blood vessels or adjacent to the ventricles and pia mater (Figure 6A and 6D). In contrast, GFAP labeled cells were prominent in all brain areas examined in Npc1<sup>−/−</sup> mice (Figure 6B and 6E), indicating the existence of massive astrocytic reaction. Allopregnanolone treatment markedly suppressed this reaction and brought the levels of GFAP staining closer to that in brains of wild-type mice (Figure 6C and 6F).

As previously reported, the number of reactive microglia labeled by anti-F4/80 antibodies were significantly increased in brains of Npc1<sup>−/−</sup> mice (Figures 7A and 7C) as compared
to that in wild-type mice (Figure 7E), and treatment with allopregnanolone markedly reduced microglial reaction (Figures 7B and 7D). Quantitative image analysis of F4/80 immunopositive cells in the primary somatosensory cortex (SSp) and cerebellar cortex (CB) indicated that the drug treatment resulted in an 80% decrease in the area of reactive microglia (Figure 7F; n=3, p<0.05, 2-tailed student t-Test).

Hypomyelination has been reported as a major neuropathology in human NPC and in animal models of the disease. Therefore, the status of oligodendrocytes was investigated by immunohistochemistry using anti-CNPase antibodies. As shown in Figure 8, myelination, as revealed by CNPase immunostaining, was also increased by allopregnanolone treatment, particularly in neocortex (compare Figures 8C to 8B) and cerebellum (compare Figures 8F to 8D). In neocortex of Npc1-/- mice, patches of CNPase labeled elements were only found in the deeper layers, mostly in layers V and VI. In treated mice, CNPase-labeled axons were more evenly distributed in layers IV, V, and VI. In cerebellum, more CNPase-decorated axons were observed in the granular layer. Unlike in control Npc1-/- mice, more myelinated axons reached the Purkinje cell layer in allopregnanolone-treated mice, with some even expanding into the molecular layer, a staining pattern closer to that found in wild-type mice.

B) Mice treated with single injections at day 7 and then q 14 days

1) Survival:
Treatment with allopregnanolone at day 7 and 14 day intervals increased survival to \(128.3 \pm 4.3\) days; see figure 1.

2) **Weight:**

Weight loss was more significantly delayed when the day 7 injection was followed by q 14 days injections; see figure 2.

3) **Rota-Rod Performance:**

Rota-Rod performance was markedly enhanced until week 11 in the day 7, then q.14 treated mice; see figure 3.

**Discussion:**

Our results confirmed those of Griffin, et al. (Griffin, 2004) that a single injection of allopregnanolone at day 7 considerably extends the life span of \(Npc1^{-/-}\) mice. We further discovered that repeated injections, starting at day 7 with 2-week intervals is moderately better than the single day 7 injection. In contrast, we observed that partially blood-brain barrier deficient \(mdr1a^{-/-}\) mice do not do better and may have some deficiency in weight gain compared to \(Npc1^{-/-}\) on the BALB/cJ background. Repeated injection of allopregnanolone also slightly improved the Rota-Rod performance in \(Npc1^{-/-}\) mice.

Magnetic resonance imaging indicated an improvement in myelination of long tracts in response to day 7 allopregnanolone treatement. Reductions of fractional anisotropy in \(Npc1^{-/-}\) is not surprising due to the known reductions in myelination in these mice.

Because high values of anisotropy are typically found in regions of highly myelinated
white matter tracts, decreases in the myelination of such tracts should result in decreases in anisotropy. Treatment of allopregnanolone on day 7 appears to correct some of the deficiencies of myelination as seen via MRI. The effect of treatment on myelination was also confirmed by neuropathological analyses. Furthermore, immunohistochemical studies showed that allopregnanolone treatment markedly decreased reactive astrocytes and significantly suppressed microglial reaction.

The etiology of the neuropathology has been studied in \( \text{Npc}1^{-/-} \) mice (a near perfect model of the human disease) with no definitive results. It has been established that the neurodegeneration seen in \( \text{Npc}1^{-/-} \) is an autonomous process in the central nervous system. The impact of visceral pathology on the neurodegeneration in \( \text{Npc}1^{-/-} \) was studied by Loftus et al. (2002) They reintroduced the wild-type \( \text{Npc}1 \) gene into \( \text{Npc}1^{-/-} \) mice by targeting its expression primarily to the CNS through the use of the prion protein promoter. Interestingly, neurodegeneration was prevented, life span was normalized, and the sterility of \( \text{Npc}1^{-/-} \) female mice was corrected. The rescue did not completely rectify the accumulation of GM2 or GM3 gangliosides in some neurons and glia (Loftus et al. 2002). This is relevant to the argument as to whether it is the storage of cholesterol or gangliosides that is most important in the neuropathology of \( \text{npc}1^{-/-} \) mice.

A genetic approach aimed at reducing the levels of gangliosides by mating \( \text{Npc}1^{-/-} \) mice with mice carrying a targeted mutation in the \( \beta-1-4\text{GalNAc} \) transferase gene, responsible for synthesis of GM2 and higher order gangliosides, successfully reduced CNS accumulation of GM2 and glycolipids GA1 and GA2, but did not improve the clinical
phenotype or neuronal pathology of the \( Npc1^{-/-} \) mice (Liu et al. 2000). A re-investigation of these mice showed that accumulation of unesterified cholesterol in the cerebral cortex, the hippocampus, and other subcortical regions was markedly decreased and that a few mice lived longer than controls (Gondre-Lewis et al. 2003). A pharmacological approach was used to target another key synthetic enzyme higher upstream in the glycosphingolipid synthetic pathway: glucosylceramide synthase. Oral administration of the inhibitor of this enzyme, N-butyldeoxynojirimein, to \( Npc1^{-/-} \) mice and cats resulted in reduced ganglioside accumulation in the brain, accompanied by a modest delay in onset of neurological dysfunction and death of the animals, and reduced Purkinje cell loss (Zervas et al. 2001).

The deficiency in neurosteroidogenesis described by Griffin et al. (2004) provides a pathogenetic mechanism related to cholesterol availability. Cholesterol is, of course, the precursor of steroids and \( Npc1^{-/-} \) has been associated with decreased testosterone production in the testes (Roff et al. 1993). The normal levels of 3\( \alpha \) HSD and 5\( \alpha \) reductase at embryonic day 16.5 with a gradual decrease after birth might suggest that the cholesterol deficiency led to down regulation of these enzymes. Since these two enzymes were already significantly decreased on the day of birth, it is not surprising that an injection of allopregnanolone at day 7 could make up for a deficiency of allopregnanolone (Griffin et al. 2004); what is much more surprising is the long term effects of a single injection at day 7. Effects of allopregnanolone on cultured embryonic rat hippocampal neurons in astrocyte conditioned media from 3 day old rat neonates have been studied (Liu et al. 2002). The activation of GABA\(_A\) receptor/Cl\(^-\) channels found in
the cultured cells occurred in a triphasic manner and could indicate changes in conduction induced by allopregnanolone at day 7 in mouse brains. Alternatively, stimulation of myelin basic protein expression and, subsequently, presumably myelin accumulation, occurred in organotypic slice cultures of 7-day-old mouse cerebellum (Ghoumari et al. 2003a). Since demyelination is a feature in the brains of older Npc1⁻/⁻ mice, such stimulation in myelin basic protein syntheses in the younger mice might be protective at later ages. This stimulatory effect of allopregnanolone on myelin basic protein synthesis was also found in Schwann cell cultures (Melcangi et al. 1999), which are the cells which myelinate axons. Such neurosteroids can also activate the pregnane X receptor (PXR) which induces cytochrome CYP3A4 (Lamba et al. 2004). However, PXR is only expressed in human thalamus and spinal cord rather than NPC1 critical tissues (Lamba et al. 2004).

In adult rats, allopregnanolone has been shown to decrease neuronal loss after traumatic brain injury and to enhance the rats’ performance in the Morris water maze (Djebaili et al. 2004). This might be related to the inhibitory effect of allopregnanolone on IL-1β and TMF-α cytokine expression after traumatic brain injury (He et al. 2004). Of interest, of course, is the activation of inflammatory cells seen very early in Npc1⁻/⁻ brains (Baudry et al. 2003). This effect is thought to be directly on anti-apoptotic Bcl-2 proteins rather than through the GABA receptor (Charalampopoulos et al. 2004). However, over expression of Bcl-2 in transgenic mice (Erickson and Bernard 2002) did not prevent neuronal cell death. Thus, an effect of allopregnanolone on this pathway in Npc1⁻/⁻ seems unlikely.
Effects of allopregnanolone on neurotransmission may be magnified in Npc1<sup>−/−</sup> mice, since it has been shown that cholesterol enrichment potentiated GABA<sub>Α</sub> receptors by the steroids but did not affect the non-steroidal potentiators (Sooksawate and Simmonds 2001). Given the alterations of cholesterol metabolism in Npc1<sup>−/−</sup>, with excess storage in the late lysosomal fraction, it could be argued that there is membrane depletion of cholesterol and that the effects of allopregnanolone would be potentiated in Npc1<sup>−/−</sup> mice.

It is important to emphasize that while allopregnanolone injections delay the onset of neurological symptoms in Npc1<sup>−/−</sup> mice, the weight loss and early demise are mostly reflective of the cerebellar degeneration. It is quite possible that altered neuronal circuits and/or distribution of myelination could have harmful effects in other parts of the brain. It has been shown that neonatal allopregnanolone administration disrupts the normal development of the prefrontal cortex and medial dorsal thalamus, increasing the number of parvalbumin-expressing neurons while the total neuronal number is decreased in the medial dorsal nucleus (Gizerian et al. 2004). Similarly, alterations of GABAergic interneuron localization by neurosteroids have been shown in pre-frontal cortex (Grobin et al. 2003). Treatment of cultured rat cerebellar granular cells with Ganaxolone, a synthetic analog of allopregnanolone had no immediate effects, but its withdrawal changed amounts of several GABA receptors, thus, withdrawal of allopregnanolone might also result in potentially harmful effects (Mascia et al. 2002). It is of interest that the progesterone agonist mifepristone, but not progesterone, prevents Purkinje cell death in organotypic slice cultures, an effect not ascribed to GABA receptor responses (Ghoumari et al. 2003b).
In summary allopregnanolone treatment of young mice has dramatic effects on the cerebellar degeneration normally found in Npc1/− mice, however, memory (dementia) and other aspects of Niemann-Pick C have not been studied in the animal model. Given the diverse effects of allopregnanolone reviewed above, it seems essential that fuller evaluation of such treatments on a variety of neurobehavioral parameters should be performed.
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References:


**Figure Legends:**

**Figure 1:**
Kaplan Meyer Survival Curves for *Npc1<sup>−/−</sup>* mice (BALB/cJ and *mdr1a* backgrounds, combined for untreated): untreated, (———); BALB/cJ day 7, (—.—.—.-); *mdr1a* day 7, (—.—.—.-); BALB/cJ day 7 and then q 14, (—.—.—.-).

**Figure 2:**
Weight curves for *Npc1<sup>−/−</sup>* mice (BALB/cJ and *mdr1a* backgrounds combined for untreated): untreated, (■■); BALB/cJ day 7, (■■); *mdr1a* day 7, (▼▼); BALB/cJ day 7 and then q 14, (▲▲).

**Figure 3:**
Rota-Rod scores of BALB/cJ *Npc1<sup>−/−</sup>* mice (BALB/cJ and *mdr1a* backgrounds combined for untreated): untreated, (■■); BALB/cJ day 7 (■■), BALB/cJ day 7 and then q 14 (▲▲).

**Figure 4:**
Representative T2-weighted images and fractional anisotropy (FA) maps of wild-type, untreated *Npc1<sup>−/−</sup>* mice and day 7 treated *Npc1<sup>−/−</sup>* mice.
Figure 5:
Whole brain histogram analysis of fractional anisotropy in wild-type, untreated $Npc1^{-/-}$ mice and day 7 treated $Npc1^{-/-}$ mice. Panel a) shows inverse cumulative histogram of FA in the three groups. Panel b) shows inverse cumulative histograms normalized by the wild-type data. The dashed line is included for reference and represents the results of such analysis if the NPC brains were identical to wild-type brains. Reductions indicate a loss of brain volume as a function of FA. The most significant percentage loss in both NPC groups was at the highest FA values. However, treated mice show less reduction than their untreated counterparts.

Figure 6:
Effect of allopregnanolone-treatment on astrocytes in primary somatosensory cortex and ventral lateral thalamus. Brain sections from 65 day-old wild-type (A,D), $Npc1^{-/-}$ (B,E), and allopregnanolone-treated $Npc1^{-/-}$ mice (C,F) were processed for immunohistochemistry with anti-GFAP antibodies as described in Materials and Methods. (A-C) Primary somatosensory cortex (10x objective); (D-F) ventral lateral thalamus (20x).

Figure 7:
Effect of allopregnanolone-treatment on reactive microglia in primary somatosensory cortex and cerebellar cortex. Brain sections from 65 day-old wild-type (E), $Npc1^{-/-}$ (A,C), allopregnanolone-treated $Npc1^{-/-}$ mice (B,D) and wild-type mice (E), were
processed for immunohistochemistry with anti-F4/80 antibodies as described in Materials and Methods. (A,B,E) Primary somatosensory cortex (10x objective); (C,D) cerebellar cortex (20x). (F) Quantitative results of F4/80 immunopositive cells in primary somatosensory cortex and cerebellum; allopregnanolone treatment (allo) significantly reduced the total area occupied by reactive microglia (p<0.05; n=3).

Figure 8:
Effect of allopregnanolone-treatment on oligodendrocytes in primary somatosensory cortex and cerebellar cortex. Brain sections from 65 day-old wild-type (A,D), Npc1-/- (B,E), and allopregnanolone-treated Npc1-/- mice (C,F) were processed for immunohistochemistry with anti-CNPase antibodies as described in Materials and Methods. (A-C) Primary somatosensory cortex (10x objective); (D-F) cerebellar cortex, lobe 10 (20x).
Figure 1

184x138mm (150 x 150 DPI)
Effect of ALLO on Weights of BALB/c and mdrla-/- Mice

Figure 2
187x128mm (150 x 150 DPI)
**Figure 3**

Effect of ALLO on Rota-Rod Scores of BALB/c $Hpc1^{+/-}$ Mice

![Graph showing the effect of ALLO on Rota-Rod Scores of BALB/c $Hpc1^{+/-}$ Mice.](image-url)

186x125mm (150 x 150 DPI)
Figure 4

152x95mm (150 x 150 DPI)
Figure 5

120x159mm (150 x 150 DPI)
Figure 6
228x121mm (150 x 150 DPI)
Figure 7
228x121mm (150 x 150 DPI)
Figure 8
228x120mm (96 x 96 DPI)