



Published in final edited form as:

Brain Res Rev. 2008 March ; 57(2): 410–420. doi:10.1016/j.brainresrev.2007.05.012.

Endogenous and synthetic neurosteroids in treatment of Niemann Pick Type C Disease

Synthia H. Mellon, Wenhui Gong, and Marcus D. Schonemann

Department of Obstetrics, Gynecology and Reproductive Sciences, The Center for Reproductive Sciences University of California, San Francisco, CA 94143 USA

Abstract

The functions for neurosteroids during development and in response to nervous system injury are beginning to be identified. We focused on a mouse model in which we believed neurosteroid production would be altered, and which had a neurodegenerative phenotype. Niemann Pick Type-C (NP-C) is an autosomal recessive neurodegenerative disease caused by mutations in *NPC1* (95%) or *NPC2* (5%), resulting in lysosomal accumulation of unesterified cholesterol and glycolipids. The NIH mouse model of NP-C has a mutation in the *NPC1* gene, and exhibits several pathological features of the most severe NP-C patients. How lysosomal storage and trafficking defects lead to neurodegeneration is unknown. We found that these mice had normal neurosteroidogenic enzyme activity during development, but lost this activity in the early neonatal period, prior to onset of neurological symptoms. Neurons that expressed P450_{scc}, 3 β HSD, as well as those that expressed 3 α HSD and 5 α reductase were lost in adult NP-C brains, resulting in diminished concentrations of allopregnanolone. We treated NP-C mice with allopregnanolone and found that a single dose in the neonatal period resulted in a doubling of lifespan, substantial delay in onset of neurological symptoms, survival of cerebellar Purkinje and granule cell neurons, and reduction in cholesterol and ganglioside accumulation. The mechanism by which allopregnanolone elicited these effects is unknown. Our *in vitro* studies showed that Purkinje cell survival promoted by allopregnanolone was lost by treatment with bicuculline, suggesting GABA_A receptors may play a role. We treated NP-C mice with a synthetic GABA_A neurosteroid, ganaxolone (3 α -hydroxy-3 β -methyl-5 α -pregnan-20-one). Ganaxolone treatment of NP-C mice produced beneficial neurological effects, but these effects were not as robust as those obtained using allopregnanolone. Thus, allopregnanolone may elicit its effects through GABA_A receptors and through other mechanisms. Additional studies also suggest that allopregnanolone may elicit its effects through pregnane-X receptors (PXR). Our data suggest that mouse models of neurodegeneration may be beneficial in establishing both physiologic and pharmacologic actions of neurosteroids. These animal models further establish the wide range of functions of these compounds, which may ultimately be useful for treatment of human diseases.

Keywords

allopregnanolone; Niemann Pick Type C; GABA_A receptor; pregnane-X-receptor

Address correspondence to: Synthia H. Mellon, Ph.D. Professor Dept. of Ob, Gyn & Repro. Sci. UCSF San Francisco, CA 94143 Phone: 1-415-476-5329 Fax: 1-415-753-3271 Email: mellon@cgl.ucsf.edu.

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1. Introduction

Steroid hormones are essential for life. Glucocorticoids (cortisol, corticosterone) are necessary for carbohydrate metabolism and are synthesized and released in response to stress; mineralocorticoids (aldosterone) instruct the kidney to retain sodium; sex steroids (progesterone, testosterone, estradiol) are essential for reproduction. Neurosteroids are steroids synthesized *de novo* in the brain, or converted into neuroactive steroids in the brain from steroids derived from the circulation. In the 1980's, Etienne Baulieu coined the term "neurosteroid" to distinguish this class of steroids from glucocorticoids, mineralocorticoids, and sex steroids (Baulieu, et al., 1999, Compagnone and Mellon, 2000). This designation was ascribed to steroids that were synthesized *de novo* in the brain, as these steroids were identified in the rodent brain weeks after gonadectomy and adrenalectomy. At the same time, functions for neurosteroids, distinct from their function at nuclear receptors was being elucidated by several groups (Harrison and Simmonds, 1984, Majewska, et al., 1986). Over the past decade, the identification of the steroids found in the brains of many species has demonstrated a remarkable similarity. Functions associated with these neuroactive compounds has also been identified (reviewed in (Backstrom, et al., 2003, Barbaccia, 2004, Belelli, et al., 2006, Belelli and Lambert, 2005, Bernardi, et al., 2004, Brinton and Wang, 2006, Compagnone and Mellon, 2000, Finn, et al., 2004, Guarneri, et al., 2003, Lambert, et al., 2003, Mensah-Nyagan, et al., 2001, Morrow, et al., 2001, Reddy, 2002, Reddy, 2004, Rogawski and Reddy, 2002, Rupprecht and Holsboer, 1999, Schumacher, et al., 2004, Schumacher, et al., 2003, Stoffel-Wagner, 2003, Tsutsui and Mellon, 2006, Tsutsui, et al., 2004, Uzunova, et al., 2005, Vallee, et al., 2001). However, it is still unknown if these compounds are essential for life.

All steroids and neurosteroids are synthesized from cholesterol through the participation and concerted action of a series of steroidogenic enzymes (Miller and Tyrell, 1994, Miller, 1988). The presence or absence of particular steroidogenic enzymes dictates that pathway of steroidogenesis that will be taken by a particular steroidogenic organ or cell type.

In conjunction with the proposed effect of the neurosteroid allopregnanolone on GABA_A receptors (Belelli and Lambert, 2005), there are several proposed roles for neurosteroids. Given exogenously, they are anxiolytic, anticonvulsant compounds. The neurosteroid pregnenolone has been shown to enhance memory when given intrathecally (Flood, et al., 1992, Flood, et al., 1995, Mathis, et al., 1994, Mayo, et al., 1993, Robel, et al., 1995). In rodent models of alcohol intoxication, one mechanism through which alcohol elicits its effects is through increased synthesis of allopregnanolone in the brain (Brot, et al., 1997, Caldeira, et al., 2004, Finn, et al., 2004, Finn, et al., 2003, Follesa, et al., 2004, Grobin, et al., 2005, Janis, et al., 1998, VanDoren, et al., 2000). Finally, the neurosteroid allopregnanolone has been implicated in a severe form of premenstrual disorder, called premenstrual dysphoric disorder (Backstrom, et al., 2003, Bernardi, et al., 2004, Bicikova, et al., 1998, Bixo, et al., 1997, Epperson, et al., 2002, Friedman, et al., 1993, Girdler, et al., 2001, Monteleone, et al., 2000, Rapkin, et al., 1997, Schmidt, et al., 1994, Smith, et al., 1998). Most recently, changes in GABA_A receptor subunit expression and sensitivity to the neurosteroid allopregnanolone has been implicated in changes in behavioral responses seen at puberty (Shen, et al., 2007).

In addition to behavioral effects of neurosteroids, neurosteroids have also been implicated in affecting neuronal function and differentiation (Brinton and Wang, 2006). These include neuroprotection against ischemia and stroke (Cutler, et al., 2005, Djebaili, et al., 2005, Hoffman, et al., 2003, Lapchak, 2004, Meffre, et al., 2007, Shear, et al., 2002, VanLandingham, et al., 2006), recovery of motor function after spinal cord injury (di Michele, et al., 2000, Fiore, et al., 2004, Labombarda, et al., 2006, Patte-Mensah, et al., 2004, Pomata, et al., 2000), regulation of myelination (Chavez-Delgado, et al., 2005, Gago, et al., 2001, Ghomari, et al., 2005, Ghomari, et al., 2003, Le Goascogne, et al., 2000, Schumacher, et al., 2000, Schumacher, et al.,

2004, Schumacher, et al., 2003), proliferation of neuronal stem cells (Suzuki, et al., 2004, Wang, et al., 2005), neurogenesis in the hippocampus (Keller, et al., 2004, Suzuki, et al., 2004, Wang, et al., 2005), and induction of analgesia (Pathirathna, et al., 2005, Todorovic, et al., 2004). Many of these actions of neurosteroids are discussed in detail in other papers in this issue.

We and others have taken several different approaches to understanding the role of neurosteroids *in vivo*. Mice in which several of the genes encoding several of the neurosteroidogenic enzymes have been ablated have been created. These include ablation of the P450scc (Hu, et al., 2002), P450c17 (Bair and Mellon, 2004), 5 α reductase type I and 5 α reductase type II (Mahendroo, et al., 2004, Mahendroo, et al., 2001, Mahendroo, et al., 1997). Among these knock out mice, only the P450c17 knock out mice are embryonic lethal at embryonic day 7. P450scc mice lack glucocorticoid production and need replacement at birth. Female mice lacking 5 α reductase type I and type II exhibited parturition and fecundity defects similar to those of animals without 5 α -reductase type I; male mice are phenotypically relatively normal, and the data from the knockout mice indicate T appears to be the only androgen required for differentiation of the male urogenital tract in mice and the synthesis of DHT serves largely as a signal amplification mechanism. Thus, global ablation of these genes does not provide insight into the roles of the neurosteroids they are involved in synthesizing, in the nervous system. Alternatively, these results may suggest that the neurosteroids do not play obligate and unique roles in the nervous system.

To identify regions and cells of the nervous system that express the neurosteroidogenic enzymes, promoter-reporter constructs can be prepared, using the promoters for the genes encoding steroidogenic enzymes. Recent studies using a P450scc-cre reporter (Wu, et al., 2007) have shown that the P450scc promoter is expressed in the cortex, hippocampus, thalamus, hypothalamus (dorsomedial ventromedial hypothalamus and arcuate nucleus). We prepared P450c17-GFP transgenic mice, using a 1.5 kb promoter region of the rat P450c17 gene. In the brain, we found GFP expression in axonal tracts projecting rostrally from the midbrain and consolidating in the sub-cortical plate of the embryonic cortex and observed neuronal projections traversing along the dorsal ventral axis connecting the spinal cord and the brainstem extending basally towards the medulla and in condensed ganglia of the dorsal root. Hence these results are promising and may define neurosteroidogenic neurons and glia throughout development, and under different types of regulation.

Another strategy for understanding neurosteroid function *in vivo* is to identify existing mouse lines that may have altered neurosteroid production. We have used this approach, and have identified a mouse line for a childhood neurodegenerative disease, Niemann Pick Type C. We have used this mouse as a model for altered neurosteroidogenesis (Griffin, et al., 2004).

2. Niemann Pick Type C Disease

Niemann-Pick type C disease is a fatal autosomal recessive, childhood-onset, neurodegenerative disorder. This lysosomal lipid storage disorder is characterized by a defect in intracellular cholesterol trafficking, resulting in lysosomal accumulation of unesterified cholesterol (reviewed in Patterson, et al., 2001). The accumulation of cholesterol causes hepatomegaly with foamy macrophage infiltration, and chronic neurologic deterioration associated with accumulation of sphingomyelin and other glycolipids in neuronal tissues, leading to seizures, supranuclear ophthalmoplegia and progressive loss of motor and intellectual function in the second decade of life (Fink, et al., 1989, Norman, et al., 1967). NP-C has been linked to two genetic loci, *NPC1* (major locus) and *NPC2* (Millat, et al., 2001, Naureckiene, et al., 2000, Patterson, et al., 2001, Pentchev, et al., 1995, Vanier, et al., 1996). The human *NPC1* gene encodes a protein of 1,278 amino acids (Carstea, et al., 1997) that shares homology with other proteins that regulate cholesterol homeostasis, including 3-

hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase, sterol regulatory element binding protein cleavage-activating protein (SCAP), with Patched, the receptor for sonic hedgehog (Loftus, et al., 1997), and the RND family of prokaryotic permeases, suggesting NPC1 may function as a transmembrane efflux pump (Davies, et al., 2000). More than 95% cases of NP-C are caused by mutations in *NPC1* (Bauer, et al., 2002, Carstea, et al., 1997). NPC2, first identified as human epididymal protein 1 (HE1), is a widely expressed 151-amino acid lysosomal glycoprotein that binds cholesterol. About 5% of NP-C is caused by mutations in *NPC2*. NP-C patients from both complementation groups have similar clinical and biochemical phenotypes, suggesting that NPC1 and NPC2 may interact or function sequentially in a common metabolic pathway.

There are few data concerning the epidemiology of NP-C. The disease is pan-ethnic, and two genetic isolates have been described in French Arcadians in Nova Scotia, previously called NP-D (Crocker, 1961) and Spanish-Americans in southern Colorado (Wenger, et al., 1977). The prevalence of NP-C in the general population has been estimated at 1/150,000 live births (Patterson, et al., 2001). This estimate may be low, as about 50% of NP-C cases may present with neonatal liver disease (Kelly, et al., 1993, Vanier, et al., 1988). Thus, the true prevalence of NP-C is likely to be greater than 1/150,000.

Much of the work on NPC1 protein, neuronal histology, and cholesterol utilization has come from the mouse model of NP-C (Morris, et al., 1982), a strain of BALB/c mice with a retroposon insertion in *NPC1* (Loftus, et al., 1997, Morris, et al., 1982). These mice have defects in cholesterol metabolism morphologically and biochemically similar to human NP-C, and show most of the same neurological phenotypes as human beings with NP-C, although the neurological demise is much more rapid in the mouse than in human beings. Nevertheless, both the murine model and patients with NP-C show similar widespread histopathological abnormalities in the central and peripheral nervous systems, including cerebellar degeneration (Gilbert, et al., 1981, Morris, et al., 1982) Purkinje cell degeneration, irregular dendritic trees, decreased numbers of dendritic spines (Higashi, et al., 1993) and progressive dysmyelination of the CNS (Higashi, et al., 1995, Weintraub, et al., 1987, Weintraub, et al., 1985, Xie, et al., 2000), suggesting progressively defective utilization of cholesterol. The mechanisms of neuronal dysfunction and degeneration are not fully understood. Cholesterol content does not appear to be elevated in cortical neurons, even though these cells exhibit neuronal storage abnormalities (Spence and Callahan, 1989, Vanier, et al., 1991), and the rate of sterol synthesis and loss is lower in NP-C mice (Xie, et al., 2000). Human NP-C brains have cortical neurons with distended cytoplasm, ballooned neurons (Anzil, et al., 1973, Braak, et al., 1983, Norman, et al., 1967), and neurofibrillary tangles (Suzuki, et al., 1995). Cholesterol and sphingomyelin are decreased in white matter due to demyelination (Braak, et al., 1983, Xie, et al., 2000). In addition to accumulating cholesterol, cells from NP-C mice also accumulate gangliosides and glycosphingolipids (Zervas, et al., 2001).

3. Neurosteroids and NP-C

In addition to abnormal cholesterol trafficking in NP-C neurons, NP-C mice also show abnormalities in testicular steroidogenesis (Roff, et al., 1993) and ovarian steroidogenesis (Gevry, et al., 2004). Since we believe that neurosteroids are necessary for neuronal and glial function, we hypothesized that alterations in sequestration of intracellular cholesterol would result in altered neurosteroidogenesis, which we hypothesize would subsequently alter neuronal and glial function.

As a first step in determining if altered neurosteroidogenesis could contribute to the neuropathology seen in NP-C, we analyzed brains of adult NP-C mice for the endogenous concentrations of some neurosteroids. We determined that the concentration of pregnenolone,

DHEA and allopregnanolone were all significantly less in brains of NP-C mice than they were in brains of age-matched wild type mice (Griffin, et al., 2004). The concentrations of other neuroactive compounds such as tetrahydrodeoxycorticosterone or androstenediol were not assessed. Thus, NP-C mice have diminished neurosteroid concentrations, which could result from several different mechanisms.

We determined whether neurons and glia that express the steroidogenic enzymes required for allopregnanolone production are also diminished in brains of NP-C mice. Using immunohistochemistry, we found that adult NP-C brains had significantly diminished expression of P450_{scc}, 3 β HSD, 5 α reductase and 3 α HSD. This reduction in expression of these neurosteroidogenic enzymes was seen in the cortex and in the cerebellum. In the cerebellum, Purkinje neurons that express these enzymes are lost in the adult NP-C mouse. In the cortex, it is unknown which particular neurons or glia express these enzymes. Thus it is unknown if those cells are also lost in NP-C mouse brains, or if there is a reduction in expression of neurosteroidogenic enzymes.

Analysis of neurosteroidogenic enzyme activity throughout the life of the NP-C mouse showed that neurosteroidogenesis (allopregnanolone production) is normal at least at the end of gestation. However, at birth, we found that NP-C mouse brains had a significant reduction in 3 α HSD activity, and hence could not convert dihydroprogesterone to allopregnanolone. While not explicitly tested as substrates in these studies, it is also likely that NP-C mouse brains cannot convert corticosterone to tetrahydrodeoxycorticosterone, or testosterone to androstenediol, other neuroactive compounds. These conversion use the same enzymes as those used to convert progesterone to allopregnanolone (i.e. 5 α reductase and 3 α hydroxysteroid dehydrogenase).

The diminution in enzyme activity was seen in the cortex, midbrain and hindbrain, indicating that there was not region-specific reduction in enzymatic activity. Several weeks later, we also demonstrated a significant reduction in 5 α reductase activity (conversion of progesterone to dihydroprogesterone) in all brain regions. This reduction in 5 α reductase and 3 α HSD activities preceded, by several weeks, onset of behavioral symptoms of ataxia, tremor, and weight loss.

4, Treatment of NP-C mice with allopregnanolone

If the loss of allopregnanolone production contributed to the neuropathology of NP-C, we reasoned that appropriately timed treatment of NP-C mice with allopregnanolone should reduce the symptoms and pathology seen in untreated NP-C mice. We tried several approaches to treatment with allopregnanolone – providing the neurosteroid in drinking water, as a timed-release pellet, and as an injection, and each treatment had some efficacy (Griffin, et al., 2004). Efficacy was assessed by survival of mice, by time of onset of neurological symptoms, as well as by weekly assessment of locomotor function and coordination. All these markers of effective treatment changed in parallel with effective treatment, i.e. effective treatment delayed weight loss and onset of ataxia and tremor, prolonged locomotor function, and increased survival.

We reasoned that since there was a reduction in neurosteroidogenic enzyme activity in the early neonatal period, that lack of allopregnanolone at that time might be crucial for appropriate brain development. Hence, we treated mice during the first two weeks of life (Griffin, et al., 2004). Our results indicated that a single injection of allopregnanolone beginning at weaning (postnatal day 23) or earlier (to postnatal day 7) was effective, and that efficacy depended upon the day at which treatment was given. Hence, treatment at postnatal day 7 was the most effective time to treat NP-C mice, and resulted in a doubling of lifespan, a 5-week delay in loss of locomotor function, and a 3-4 week delay in onset of symptoms.

Additional studies treating mice at postnatal day 0 or day 3 indicated that treatment at these times was less effective than treatment at day 7 (unpublished results), again suggesting a time-dependency to treatment.

Our studies have now been repeated by other laboratories, which have demonstrated similar effects of allopregnanolone treatment on their colonies of NP-C mice (Ahmad, et al., 2005, Langmade, et al., 2006).

5. Assessment of biochemical markers of NP-C disease

Allopregnanolone treatment significantly increased survival and locomotor function in NP-C mice. We assessed whether there were changes in neuronal survival in the mice. As discussed previously, untreated NP-C mice have substantial loss of cerebellar Purkinje neurons at the end of life (~60 days). Analysis of brains of NP-C mice treated with allopregnanolone at postnatal day 7 indicated that those mice had substantial survival of cerebellar Purkinje neurons, which was seen in all lobes of the cerebellum (Griffin, et al., 2004, Langmade, et al., 2006). Concomitant with increased Purkinje neuronal survival, we also found that allopregnanolone-treated NP-C mice had significant reduction in both cortical and cerebellar ganglioside concentrations, and reduction in accumulation of cholesterol in the brain. Thus, many hallmarks of NP-C disease progression are ameliorated by a single injection of allopregnanolone at postnatal day 7.

6. Mechanism of allopregnanolone action: GABA_A receptor

Studies by other laboratories have demonstrated that one mechanism by which allopregnanolone functions is by augmentation of GABA_A receptor channel opening, through alteration of the kinetics of entry to and exit from desensitized states of the receptor (Zhu and Vicini, 1997, Zhu, et al., 1996). We used another GABA-ergic neurosteroid, ganaxolone, as a treatment in NP-C mice. Ganaxolone is a C3- β -methyl derivative of allopregnanolone, and was developed as an orally effective neurosteroid, presumably because of the inhibition of C3-hydroxyl oxidation due to the presence of the methyl group (Beekman, et al., 1998, Carter, et al., 1997, Gasior, et al., 2000, Gee, 1996). Pharmacokinetic and *in vivo* studies have shown that ganaxolone has greater efficacy than allopregnanolone at GABA_A receptors (Beekman, et al., 1998, Carter, et al., 1997, Gasior, et al., 2000, Gee, 1996, Kerrigan, et al., 2000, Laxer, et al., 2000, Monaghan, et al., 1997, Reddy and Rogawski, 2000, Reddy and Rogawski, 2000, Robichaud and Debonnel, 2005, Ungard, et al., 2000). We treated mice at postnatal day 7, using the same dose of ganaxolone as we used for allopregnanolone (25 mg/kg). We assessed onset of symptoms, locomotor function and coordination, and survival of mice. These data are shown in figure 1. Ganaxolone-treated mice resulted in a significant increase in longevity (average of 93 days vs. 68 days in untreated NP-C mice). However, this increased longevity was not as great as that seen with allopregnanolone treatment (allopregnanolone, 124 days vs. ganaxolone 93 days, figure 1). In addition to increasing longevity, ganaxolone treatment resulted in a significant delay in tremor, ataxia and weight loss in treated NP-C mice (figure 2 A). When we compared these beneficial effects of ganaxolone with the results obtained from allopregnanolone treatment, ganaxolone treatment was not as effective.

We also assessed locomotor function and coordination in ganaxolone-treated NP-C mice. Ganaxolone treatment resulted in a significant delay in loss of locomotor function; furthermore, the loss of locomotor function was much more gradual in ganaxolone-treated mice than it was in untreated mice (Figure 2 B and C). When compared with allopregnanolone-treated mice, ganaxolone-treated mice had an earlier loss of locomotor function. Delays in loss of coordination were similar between allopregnanolone- and ganaxolone-treated mice. Both groups of treated mice had normal coordination for more than 4 weeks after untreated NP-C

mice showed loss of coordination. The loss of coordination was also more gradual in both ganaxolone- and allopregnanolone-treated mice than it was in untreated NP-C mice. Taken together, these results indicate that ganaxolone, a synthetic neurosteroid that is being developed clinically for treatment of pediatric seizure disorders, is an effective treatment in NP-C mice. However, the differences we found in the effects of ganaxolone and allopregnanolone treatment suggest that the mechanism(s) by which allopregnanolone elicits its effects is not completely identical to that of ganaxolone. Since ganaxolone is thought to elicit its effects solely through GABA_A-mediated mechanisms, our data suggest that in addition to affecting GABA_A receptors, allopregnanolone likely has other mechanisms of action *in vivo* that are not mimicked in entirety by ganaxolone. Treatment of NP-C mice with other ligands of the GABA_A receptor, such as benzodiazepines, has not been tested.

Another reason that ganaxolone may not be as effective as allopregnanolone maybe due to ganaxolone's inability to be metabolized to other neuroactive compounds, as allopregnanolone can. Allopregnanolone can be converted to dihydroprogesterone (5 α -pregnan-3, 20-dione) by the enzyme 3 alpha hydroxysteroid dehydrogenase. This is a reversible enzymatic reaction, although the reduction (production of allopregnanolone) is favored enzymatically. Dihydroprogesterone, unlike allopregnanolone, is active at nuclear progesterone receptors. Because of the 3 β -methyl group on ganaxolone, it cannot be converted to a similar compound. Hence, some of the additional benefits of allopregnanolone may be due to its metabolism to other neuroactive compounds.

The data also suggest that the neurodegeneration that occurs in NP-C mice is a result of multiple different pathologies that result from lysosomal accumulation of cholesterol and other compounds, like gangliosides, that traffic via the same intracellular pathway. Hence, targeting several of these pathways would likely be more beneficial than targeting a single pathway. For example, NP-C mice fail to use lipoprotein-derived cholesterol for synthesis of 25- and 27-hydroxycholesterol (Frolov, et al., 2003, Zhang, et al., 2004). These compounds are ligands for the liver X receptor (LXR) that promote cellular cholesterol efflux and catabolism. Treatment of NP-C mice with both allopregnanolone and a synthetic LXR ligand resulted in even better neurological outcome than treatment with either compound alone (Langmade, et al., 2006). These data indicate that directing treatments toward multiple different mechanisms, pathways, and targets provides synergistic benefits. The data also indicate that these different ligands work through different mechanisms, since the result of their combined treatments are synergistic. These data may suggest that treatment of NP-C mice with allopregnanolone plus ganaxolone may result in a different, perhaps more beneficial outcome, than treatment with either compound alone.

7. Allopregnanolone is a ligand for the nuclear pregnane-X receptor (PXR)

How can a single injection of a neurosteroid in the early postnatal period, result in long-term effects on neuronal survival and intracellular cholesterol and ganglioside accumulation more than 10 weeks later? Furthermore, how can a GABA_A receptor-mediated action elicit these results? The concentration of allopregnanolone used in our studies (25 mg/ml) (Griffin, et al., 2004) was a maximally effective dose (Figure 3, onset of symptoms). At this dose of treatment, we determined that the maximum concentration of allopregnanolone achieved in the brain is ~25 μ M (~8 μ g/g tissue). Allopregnanolone has a very short half life in the plasma of wild type and NP-C mice ($t_{1/2}$ ~ 30 minutes) as well as in human beings ($t_{1/2}$ ~ 45 minutes) (Timby, et al., 2006). In addition, allopregnanolone has a short half-life in the brain, its concentrations are maximal within 10 minutes, and has a mean retention time of less than one hour. These data suggest that while allopregnanolone may preferentially accumulate in the brain, it is unlikely that allopregnanolone given at postnatal day 7, remains in high enough concentration, to continue to elicit GABA_A-mediated effects weeks later. Studies in human beings indicate

that short-term doses of allopregnanolone elicit neurological effects within minutes. Thus, the question about additional mechanisms of allopregnanolone action persist.

Studies by the Ory laboratory provide evidence of these additional mechanisms (Langmade, et al., 2006). The Ory laboratory used a synthetic enantiomer of allopregnanolone to shed light on additional mechanisms of action. They treated NP-C mice at postnatal day 7 with either allopregnanolone or the enantiomer of allopregnanolone, called *ent*-allopregnanolone (Covey, et al., 2000). Remarkably, they showed that treatment of NP-C mice with each of these compounds yielded indistinguishable results! Animals treated with either allopregnanolone or *ent*-allopregnanolone had identical survival profiles and locomotor profiles. Since *ent*-allopregnanolone has $\sim 1/300^{\text{th}}$ the action of the naturally occurring compound at GABA_A receptors (Alakoskela, et al., 2007, Covey, et al., 2000) the results suggest that the beneficial effects of allopregnanolone in NP-C mice are not mediated by GABA_A receptors.

In addition to working through GABA_A receptors, allopregnanolone is a ligand for the promiscuous pregnane-X-receptor (Kliwer, et al., 2002, Lamba, et al., 2004, Moore, et al., 2000, Watkins, et al., 2001). This receptor is found mainly in the liver; however, we have found that PXR is also expressed in the mouse brain (Figure 4), albeit at much lower concentrations than in the liver. Both allopregnanolone and *ent*-allopregnanolone cause increased expression of *cyp3A13* mRNA, a PXR target gene. However, this increased gene expression requires 10-50 μM concentrations of the steroids, in contrast to the 10-100 nM concentration of allopregnanolone needed to augment GABA_A-ergic function. In mice treated with either allopregnanolone or *ent*-allopregnanolone, there is increased brain expression of *cyp3A13* within 24 hours of treatment, which persists 28 days later. Thus, induction of PXR may be an additional mechanism through which allopregnanolone elicits its effects, and results in beneficial treatment of NP-C. It is unknown if other PXR ligands would thus be equally (or even more) effective in treatment of NP-C. Similarly, if PXR is the mechanism through which both allopregnanolone and *ent*-allopregnanolone mediate their effects *in vivo* in NP-C mice, ablation of brain expression of PXR in NP-C mice should result in ineffective treatment of NP-C mice. Studies studying additional PXR and GABA_A receptor ligands are currently ongoing.

8. Conclusions

The results from these studies have demonstrated that a lack of allopregnanolone synthesis in the early neonatal period may contribute to the neuropathology seen in NP-C mice. The treatment studies suggest that allopregnanolone may function in the early postnatal period in the brain of mice, and that allopregnanolone's actions in NP-C mice may be time-specific. These actions may be related to specific cellular development, proliferation, and migration. The action of allopregnanolone on reducing cellular accumulation of gangliosides and cholesterol may likewise contribute to the beneficial effects of allopregnanolone treatment. The mechanism(s) through which allopregnanolone functions in NP-C is unknown. Our studies using the synthetic GABA_A receptor ligand ganaxolone, suggests that this receptor may indeed play a role in the beneficial actions of allopregnanolone. Indeed, we have shown that allopregnanolone can mediate beneficial actions in cultured Purkinje neurons which are mediated through GABA_A-receptors. However, the compelling studies using a GABA_A-inactive *ent*-allopregnanolone suggest that GABA_A-receptors may not be the only receptor involved in the beneficial *in vivo* mechanisms. These results also suggest that the pregnane-X-receptor may mediate some of the effects of allopregnanolone. Hence, studies using additional known GABA_A agonists, such as benzodiazepines, or of other neurosteroids, such as tetrahydrodeoxycorticosterone and corticosterone-derived neuroactive steroids, in addition to studies using known ligands of the pregnane-X or the progesterone receptors may provide insight into the role of these receptors, and roles of neurosteroids, in successful treatment of

neurodegeneration in NP-C. Additional *in vivo* and *in vitro* studies are needed to clarify the exact mechanism of action of allopregnanolone.

Our studies have demonstrated that NP-C represents a prototype of disordered neurosteroidogenesis. Other neurodegenerative diseases may also involve similar reductions in neurosteroid synthesis, either as primary effects, or due to specific loss of neurons that express neurosteroidogenic enzymes. Since many neurodegenerative diseases share common neuropathology, it may be likely that these diseases, like NP-C, may benefit from allopregnanolone treatment. Ultimately, understanding the mechanism of allopregnanolone action will clarify our understanding of the cellular processes that result in neurodegeneration.

Acknowledgements

This work was supported by grants NIH NS 049462 NSF 0090995, March of Dimes FY06-340/FY079-554, grants from The National Niemann Pick Disease Foundation, The Ara Parseghian Medical Research Foundation and The Lysosomal Storage Disease Research Consortium to SHM, and a fellowship from NIH Training Grant DK07161 to MDS. We thank Dr. Kelvin Gee, University of California, Irvine, for the ganaxolone.

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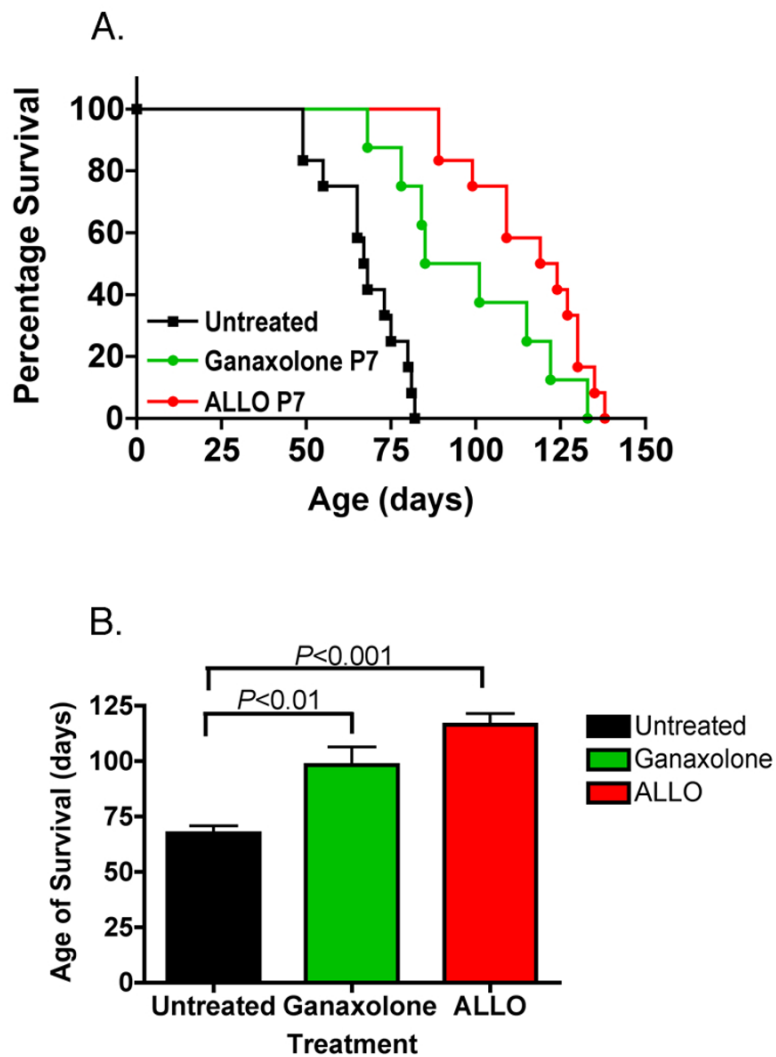
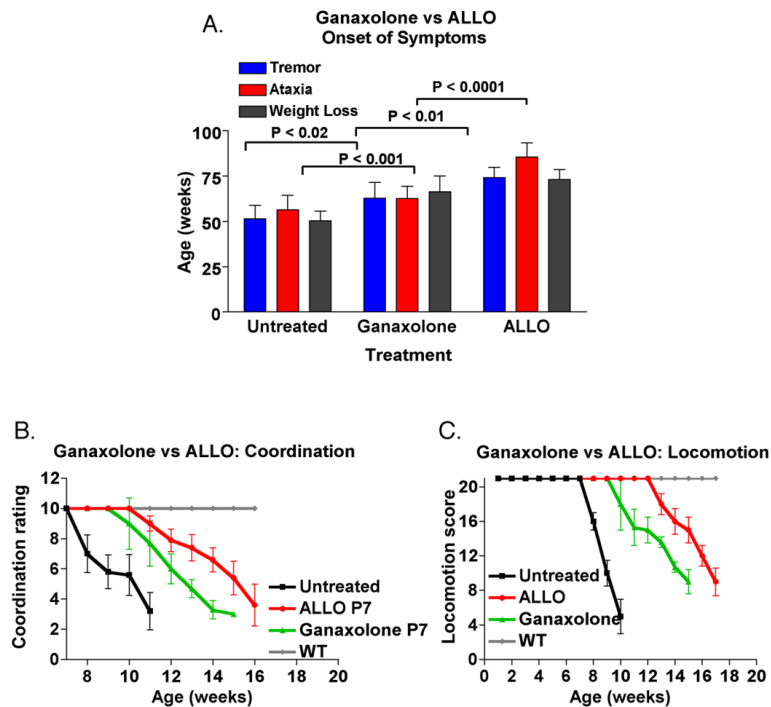


Figure 1. Effect of a single dose of allopregnanolone or ganaxolone on survival of NP-C mice. Allopregnanolone (Allo) or ganaxolone (25 mg/kg in 20% β -cyclodextrin) or nothing (untreated, 20% β -cyclodextrin only) was administered subcutaneously in a single injection at postnatal day 7. A. Survival curves for treatments. B. Average survival time. Data are means \pm S.D. N=12 for untreated and allopregnanolone-treated mice; n=8 for ganaxolone-treated mice.

**Figure 2.**

Effect of a single dose of allopregnanolone (allo) or ganaxolone on onset of symptoms of NP-C mice. A. Onset of symptoms: Animals were assessed weekly for weight, tremor, ataxia, and week of onset was noted. B. Motor coordination and C. Locomotor function: mice were assessed weekly for locomotor function and coordination. These assays used locomotor tests established for assessing spinal cord injuries and recovery (Basso, et al., 1995). The rater was blinded to treatment. Data are means \pm S.D. N=12 for untreated and allopregnanolone-treated mice; n=8 for ganaxolone-treated mice.

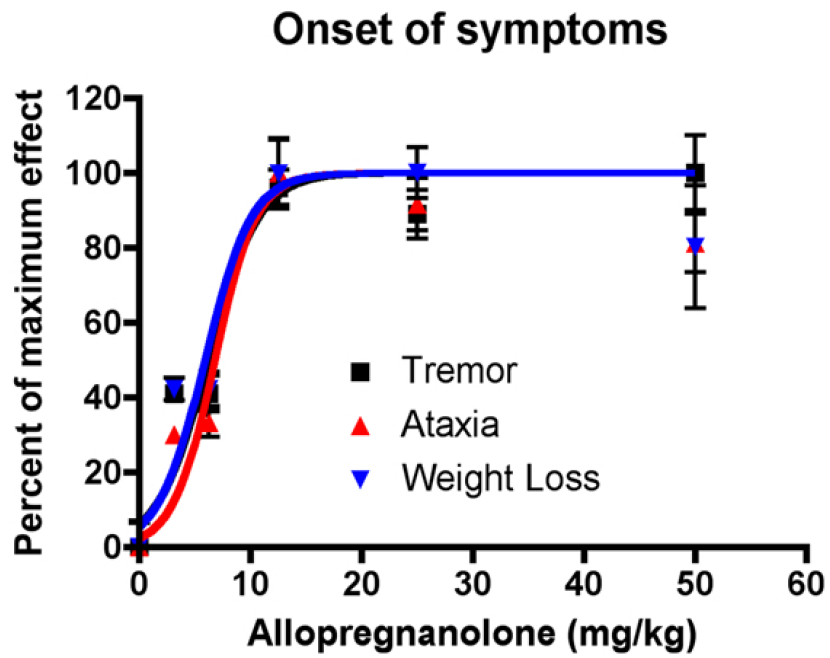


Figure 3. Dose-responsiveness of allopregnanolone treatment. NP-C mice were treated with a single dose of allopregnanolone (0 – 50 mg/kg) at postnatal day 7. Onset of symptoms (tremor, ataxia and weight loss) was assessed weekly. Data are means \pm S.D. N=12 mice for each dose.

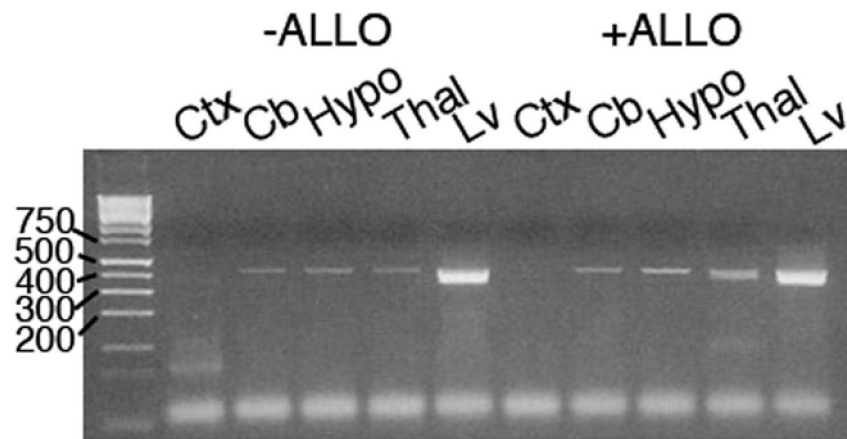


Figure 4.

RT-PCR analysis of pregnane X receptor mRNA expression in the brain. Brains from postnatal day 7 mice were treated with nothing (-ALLO) or with 25 mg/kg allopregnanolone, subcutaneously (+ALLO), and brains were removed 24 hours later. Brains were separated into various regions, RNA was isolated and cDNA was prepared and amplified with primers specific for mPXR. Amplification was for 35 cycles, and PCR products of 418 bp were separated on 2% agarose gels. Ctx, cortex; Cb, cerebellum; Hypo, hypothalamus; Thal, thalamus; Lv, liver.