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Review

Endogenous and synthetic neurosteroids in treatment of Niemann–Pick Type C disease

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A R T I C L E   I N F O

A B S T R A C T

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 P450scc, 3β HSD, as well as those that expressed 3α HSD and 5α 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 life span, 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 (3α-hydroxy-3β-methyl-5α-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.

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Contents

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 1980s, 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, 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,

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, 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; Ghoumari et al., 2005, 2003; Le Goascogne et al., 2000; Schumacher et al., 2000, 2004, 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*alpha* reductase type I and 5*alpha* reductase type II (Mahendroo et al., 2004, 2001, 1997). Among these knockout 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 alpha reductase type I and type II exhibited parturition

and fecundity defects similar to those of animals without 5 alpha-reductase type 1; 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 1278 amino acids (Carstea et al., 1997) that shares homology with other proteins that regulate cholesterol homeostasis, including 3-hydroxy-3-methylglutaryl-Co-A (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 panethnic, 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, 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 P450scc, 3βHSD, 5α reductase and 3α 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 3α 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. 5α reductase and 3α 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 5α reductase activity (conversion of progesterone to dihydroprogesterone) in all brain regions. This reduction in 5α reductase and 3α 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 2 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 life span, a 5-week delay in loss of locomotor function, and a 3- to 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, 2000a,b; 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 Fig. 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; Fig. 1). In addition to increasing longevity, ganaxolone treatment resulted in a significant delay in tremor, ataxia and weight loss in treated NP-C mice (Fig. 2A). When we compared these beneficial

Fig. 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 ± S.D. *N* = 12 for untreated and allopregnanolone-treated mice; *n* = 8 for ganaxolone-treated mice.**

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 (Figs. 2B 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 (5α-pregnan-3,20-dione) by the enzyme 3α 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 3β-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
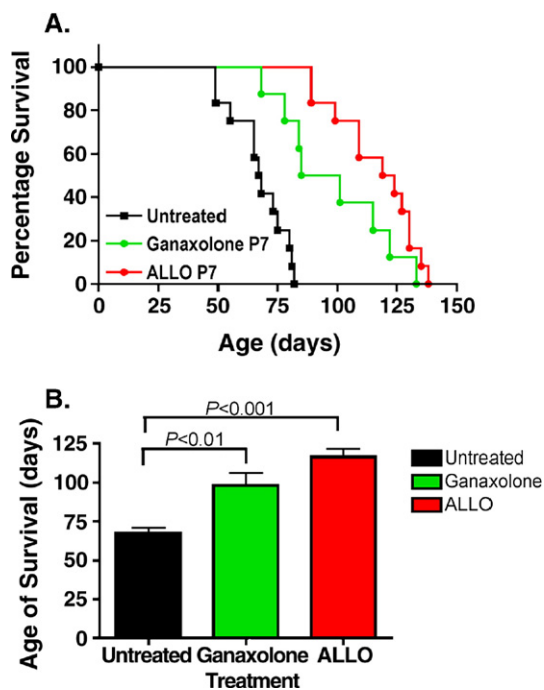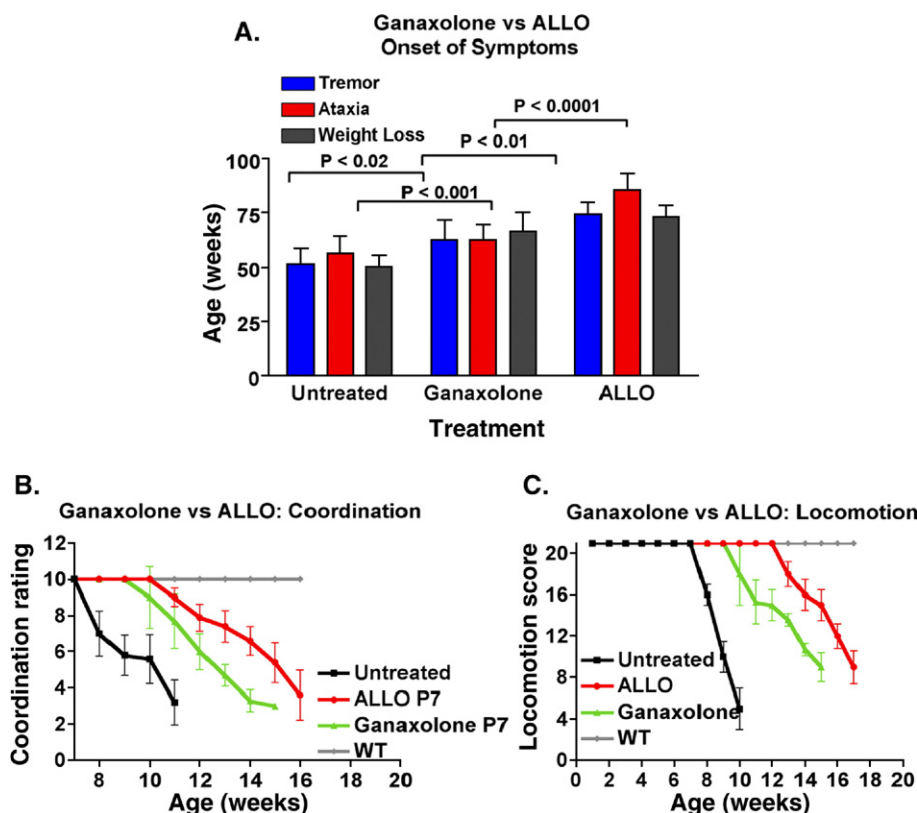
Fig. 2 – Effect of a single dose of allopregnanolone (allo) or ganaxolone on onset of symptoms of NP-C mice. (A) Onset of symptoms: animals were assessed weekly for weight, tremor, ataxia, and week of onset was noted. (B) Motor coordination and (C) locomotor function: mice were assessed weekly for locomotor 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±S.D. *N*=12 for untreated and allopregnanolone-treated mice; *n*=8 for ganaxolone-treated mice.

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.

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 allopreg-

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 (Fig. 3, onset of symptoms). At this dose of treatment, we determined that the maximum concentration of allopregnanolone achieved in the brain is ~25 μM (~8 μg/g tissue). Allopregnanolone has a very short half life in the plasma of wild type and NP-C mice (t$_{1/2}$~30 min) as well as in human beings (t$_{1/2}$~45 min) (Timby et al., 2006). In addition, allopregnanolone has a short half-life in the brain, its concentrations are maximal within 10 min, and has a mean retention time of less than 1 h. These data
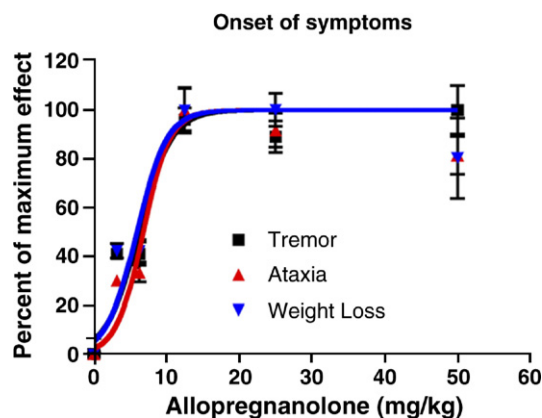

Fig. 3 – Dose-responsiveness of allopregnanolone treatment. NP-C mice were treated with a single dose of allopregnanolone (0–50 mg/kg) at postnatal day 7. Onset of symptoms (tremor, ataxia and weight loss) was assessed weekly. Data are means±S.D. *N*=12 mice for each dose.

nanolone elicit neurological effects within minutes. Thus, the question about additional mechanisms of allopregnanolone action persists.

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 ~1/300th 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 (Kliewer 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 (Fig. 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 h 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 on 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.
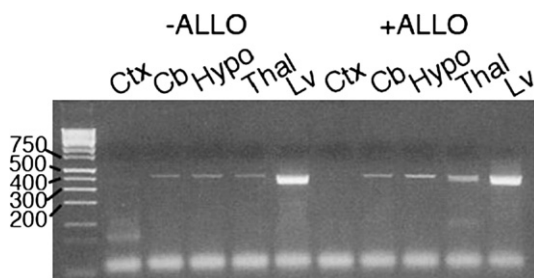

Fig. 4 – RT–PCR analysis of pregnane-X-receptor mRNA expression in the brain. Brains from postnatal day 7 mice were treated with nothing (–ALLO) or with 25 mg/kg allopregnanolone, subcutaneously (+ALLO), and brains were removed 24 hours later. Brains were separated into various regions, RNA was isolated 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.

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