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Neurosteroids as stress modulators and neurotherapeutics: lessons from the retina

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Introduction
Neurosteroids are important modulators of neuronal stress and use a variety of mechanisms to help neurons establish homeostasis following insults. Neurosteroids are synthesized endogenously in the brain from cholesterol or sterol precursors, and are a subset of a broader class of steroids that modulate brain function, referred to as neuroactive steroids (NAS) (Paul and Purdy, 1992). Increasing evidence indicates the importance of NAS as therapeutics for neuropsychiatric illnesses (Zorumski et al., 2013), and has led to FDA approval of brexanolone, for treatment of postpartum depression (Meltzer-Brody and Kanes; 2020). Brexanolone is a formulation of the neurosteroid, allopregnanolone (AlloP), in cyclodextrin (Capisol) for intravenous infusion. Another NAS, zuranolone, is in late-stage human trials for postpartum depression and major depression (Gunduz-Bruce et al., 2019; Deligiannidis et al., 2021). The approval of brexanolone has prompted increasing interest in understanding mechanisms contributing to the action of AlloP and related NAS. For over 25 years, our group has examined models of neuronal injury in preserving retinal structure and function in an in vivo rodent model. These studies in the retina have important implications for the ongoing development of allopregnanolone and other neurosteroids as therapeutics for neuropyschiatric illnesses.

Key Words: allopregnanolone; autophagy; enantiomers; excitotoxicity; gamma-aminobutyric acid type A receptors; glaucoma; optic nerve; oysterols

Abstract
Neurosteroids are rapidly emerging as important new therapies in neuropsychiatry, with one such agent, brexanolone, already approved for treatment of postpartum depression, and others on the horizon. These steroids have unique properties, including neuroprotective effects that could benefit a wide range of brain illnesses including depression, anxiety, epilepsy, and neurodegeneration. Over the past 25 years, our group has developed ex vivo rodent models to examine factors contributing to several forms of neurodegeneration in the retina. In the course of this work, we have developed a model of acute closed angle glaucoma that involves incubation of ex vivo retinas under hyperbaric conditions and results in neuronal and axonal changes that mimic glaucoma. We have used this model to determine neuroprotective mechanisms that could have therapeutic implications. In particular, we have focused on the role of both endogenous and exogenous neurosteroids in modulating the effects of acute high pressure. Endogenous allopregnanolone, a major stress-activated neurosteroid in the brain and retina, helps to prevent severe pressure-induced retinal excitotoxicity but is unable to protect against degenerative changes in ganglion cells and their axons under hyperbaric conditions. However, exogenous allopregnanolone, at a pharmacological concentration, completely preserves retinal structure and does so by combined effects on gamma-aminobutyric acid type A receptors and stimulation of the cellular process of macroautophagy. Surprisingly, the enantiomer of allopregnanolone, which is inactive at gamma-aminobutyric acid type A receptors, is equally retinoprotective and acts primarily via autophagy. Both enantiomers are also equally effective in preserving retinal structure and function in an in vivo glaucoma model. These studies in the retina have important implications for the ongoing development of allopregnanolone and other neurosteroids as therapeutics for neuropyschiatric illnesses.

Relevance of the Retina and Glaucoma to Neuropsychiatry

Multiple neuropsychiatric illnesses involve changes in axons and white matter in the brain. These include neurodegenerative illnesses such as Alzheimer’s and Huntington’s diseases, but also primary psychiatric illnesses including schizophrenia, major depression, and autism, among others (Fields, 2008; Alnaes et al., 2018). Axonal changes contribute to altered connectivity within and across brain regions underlying mental functions of cognition, emotion, and motivation, and can be observed early in the course of illness, particularly in disorders with developmental origins. Given that glaucoma involves damage to retinal ganglion cells (RGCs) and their axons that form the optic nerve, glaucoma could provide insights into understanding axonal dysfunction and its treatment. Glaucoma is the second leading cause of blindness worldwide and the number one cause of blindness among African Americans. Glaucoma is also associated with more than two-fold increase in major depression (Yoshikawa et al., 2019).

A second reason for interest in the retina involves increasing evidence that RGCs mediate effects of light on mood and behavior, including risk of depression. It is now clear that there are multiple subtypes of RGCs, including RGCs that express melatonin and have intrinsic photosensitivity (ip) akin to photoreceptors (Tran et al., 2019). Different subtypes of ipRGCs connect to different subcortical regions and mediate unique behavioral effects (Fernandez et al., 2018; Do, 2019). One subclass connects to the suprachiasmatic nucleus and secondarily to the hippocampus, mediating effects of light on learning and memory (Fernandez et al., 2018; Huang et al., 2021). A

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Neurosteroids as neuroprotectants

Because of the therapeutic potential of AlloP and other NAS, we initially examined whether exogenous AlloP alters injury to RGCs and axons in the nerve fiber layer (NFL) of rat retinas subjected to high pressure and varying concentrations of AlloP resulted in concentration-dependent retinal protection. At 10 mM AlloP, there was no clear change in pressure-induced damage; some neuroprotection was observed at 100 nM AlloP, complete retinal preservation at 1 μM (Ishikawa et al., 2014), a concentration above physiological levels.

In related studies, we examined the role of endogenous AlloP in modulating effects of high pressure. Using liquid chromatography and tandem mass spectrometry to measure AlloP, we found very low levels of the steroid under basal conditions when retinas were incubated for 24 hours at 10 mm Hg. Under normobaric conditions where retinas were incubated at 75 mm Hg resulted in 60–70-fold increases in AlloP compared to normobaric conditions (Ishikawa et al., 2014). These increases in AlloP at high pressure were partially inhibited (approximately 50% reduction) by finasteride at a subthreshold concentration. This inhibition was observed with dutasteride, a broader spectrum 5AR antagonist (Pinacho-Garcia et al., 2020), and with the competitive NMDA receptor antagonist, 2-amino-5-phosphonovalerate (Ishikawa et al., 2014). Using an antibody against 5α-reductase and steroid hormone receptors, Dutasteride blocked the increase in AlloP (Tokuoka et al., 2010, 2011), we also observed immunohistochemical evidence of increases in neurosteroids in RGCs; these changes were dampened by the antagonists described above (Ishikawa et al., 2014).

Changes in neurosteroid levels led us to consider whether endogenous 5α-reduced steroids play a protective role in the retina under stress. When retinae were incubated with 5AR antagonists under normobaric conditions, no significant histological changes were observed. However, 5AR antagonists led to marked damage in multiple retinal layers during exposure to high pressure. These changes included RGC and axonal damage as well as excitotoxic changes in other retinal layers. Excitotoxic changes included marked swelling of neurons resulting in “bulls-eye” appearances of cells in the inner nuclear layer (INL) and “Swiss-cheese” (edematous) changes in the inner segment layer. This excitotoxicity was blocked by the NMDA receptor antagonist, 2-amino-5-phosphonovalerate, and by exogenous AlloP at 1 μM (Ishikawa et al., 2014).

We also found that high pressure increases expression of genes and proteins thought to be involved in neurosteroidogenesis, including Tspo and 5AR. Increases in 5AR, mostly type II, a form of the enzyme inhibited by dutasteride and finasteride (Pinacho-Garcia et al., 2020), were observed in RGCs and INL (Ishikawa et al., 2016), consistent with what we observed with changes in AlloP levels described above. Tspo has a complex relationship to sterol metabolism. While it is clearly an important component of mitochondrial function, its role in neurosteroid synthesis is less certain based on studies in TSPO knockout mice (Selvaraj et al., 2015), although other studies suggest some role in neurosteroidogenesis (Tokuoka et al., 2010; Ishikawa et al., 2016; Mages et al., 2019).

How is AlloP neuroprotective? Roles of gamma-aminobutyric acid-A receptors and autophagy

AlloP, a complex neuromodulator produced on-demand under stressful conditions in the brain and nervous system. It has several known mechanisms that could contribute to neuroprotection. Most prominently, AlloP is a potent and effective positive allosteric modulator (PAM) of gamma-aminobutyric acid (GABA) receptors, the major inhibitory neurotransmitter that mediates the majority of fast inhibitory neurotransmission (Zorumski et al., 2019). Potentiation of GABA-A receptors occurs at mid-nanomolar concentrations; at higher nanomolar to micromolar concentrations, AlloP can directly open GABA-A channels in the absence of GABA (called direct channel gating). GABA-A receptors are heteropentamers with 19 subunits identified (Belelli and Lambert, 2005). Different subtypes of GABA-A receptors are expressed at synapses where they mediate phasic forms of inhibition. Other GABA-A receptors are expressed at extrasympatic sites where they sense ambient levels of AlloP and mediate tone inhibition (Belelli et al., 2009). Importantly, AlloP and related NAS modulate both phasic and tonic inhibition, giving them the unique ability to regulate the balance of excitation and inhibition throughout the brain. Thus, modulation of GABA transmission is a major mechanism likely contributing to the effects of AlloP.

Beyond GABA, AlloP also has effects on other ion channels and intracellular processes. These include inhibition of low voltage-activated (T-type) calcium channels that mediate burst firing in certain neurons and second messenger and neurotransmitter release (Pathiratna et al., 2005). AlloP is a highly lipophilic steroid and accesses intracellular compartments where it modulates inflammation (Balan et al., 2019, 2021), mitochondrial function, and energy utilization (Grimm et al., 2017).

We examined the effects of GABA-A receptors in the effects of AlloP in our glaucoma model using picROTOX, a non-competitive GABA-A receptor antagonist. The protective effects of exogenous AlloP were completely blocked by co-administration of picROTOX, indicating a prominent role for GABA-A receptors in neuroprotection. In addition to overriding protection of RGCs and axons in the INL, picROTOX also protected against the excitotoxic changes in the retina including excitotoxic changes in the INL and inner plexiform layer (Ishikawa et al., 2014).

Because neuroprotective effects of AlloP in other studies involve mechanisms in addition to GABA-A receptor modulation (Langmade et al., 2006), we also...
The enantiomer of AlloP is also neuroprotective in the retina. In an effort to determine the importance of GABA-A receptor PAM activity in neuroprotection by AlloP, we used its unnatural enantiomer (ent-AlloP).

To determine whether effects observed in the ex vivo glaucoma model translate to animals, we used a rat model of increased intraocular pressure (IOP) produced by intracameral injection of polystyrene microbeads. In this model, IOP increases from about 10 mmHg to 30 mmHg over 3 days after bead injection and IOP remains elevated at this level for the remainder of the 3-week experiment (Ishikawa, 2021, 2022). In control rats, elevated IOP by microbead injections. Nonetheless, both enantiomers produced strong neuroprotection based on histology and measures of apoptosis of RGCs, and both promoted autophagy. The autophagy inhibitor, 3-methyladenine, blocked neuroprotection by ent-AlloP, indicating preserved visual function as well as histology (Ishikawa et al., 2022). In control rats, elevated IOP by microbead injections. Nonetheless, both enantiomers produced strong neuroprotection based on histology and measures of apoptosis of RGCs, and both promoted autophagy. The autophagy inhibitor, 3-methyladenine, blocked neuroprotection by ent-AlloP, indicating preserved visual function as well as histology (Ishikawa et al., 2022).

The enantiomer of AlloP was not altered by picrotoxin, unlike natural AlloP (Ishikawa et al., 2022). However, ent-AlloP increased the number of autophagosomes and degenerative autophagic vacuoles in the NFL, and enhanced protein levels of LC3B-II while decreasing levels of p62, indicating that ent-AlloP, like AlloP, promotes autophagy. The autophagy inhibitor, 3-methyladenine, blocked neuroprotection by ent-AlloP. This latter result is intriguing and suggests that it is possible to dissociate the role of GABA-A receptors (or other mechanisms) from autophagy in the effects of natural AlloP. Furthermore, these results indicate that ent-AlloP promotes autophagy (or a major mechanism of retinal protection) that is independent of GABA-A receptors in contrast to AlloP (Figure 3). In direct comparisons using biochemical markers of autophagy, ent-AlloP appears to be more effective than AlloP in activating autophagy at a concentration of each that is fully neuroprotective (Ishikawa et al., 2022). We note that there is presently controversy concerning the role of autophagy in glaucoma and other neurodegenerative illnesses (Spalding et al., 2005; Kroemer et al., 2010, but there is considerable interest in whether agents targeting this mechanism can be developed for use in neuropsychiatric illnesses (Hui et al., 2021). Additionally, the targets and mechanisms by which NAS stimulates autophagy remain undefined, and it is presently unclear whether autophagy is a primary action or the result of another modulated cellular process.

AlloP enantiomers are protective in an in vivo glaucoma model. To determine whether effects observed in the ex vivo glaucoma model translate to animals, we used a rat model of increased IOP by microbead injections. Neither steroid altered the increase in IOP caused by microbead injections. Nonetheless, both enantiomers produced strong neuroprotection based on histology and measures of apoptosis of RGCs, and both promoted autophagy based on measures outlined in the ex vivo studies above. Similarly, both steroids preserved the retinal scotopic threshold response, indicating preserved visual function as well as histology (Ishikawa et al., 2021, 2022).
Lessons Learned from the Retina

Studies in the retina raise several important considerations for therapeutic use of neurosteroids in neuropsychiatric disorders. Below we outline several key points that we take from the studies described. We provide additional commentary for certain points that may be relevant to considering how neurosteroids are used clinically going forward.

1. Neurosteroids, particularly AlloP, are important endogenous modulators that help to prevent neuronal stress and function under stressful conditions. This is likely true throughout the brain as it is in the retina.

2. Extracellular glutamate accumulation and tonic activation of NMDA receptors contributes to the effects of stress on the synthesis of neurosteroids. This has been observed in both retina and hippocampus.

3. Other potential contributors could include effects on cellular stress responses indicating that NAS, and AlloP in particular, have anti-inflammatory effects on work with AlloP analogues that are photoaffinity labels, both endogenous and exogenously applied NAS interact with several known intracellular targets. These are potentially important intracellular targets and contribute to neurodegeneration and neuroprotection. In particular, the ex vivo system provides a useful tool for detailed mechanistic studies.

4. Removal of acute protection by endogenous 5-alpha reduced steroids renders neurons highly vulnerable to excitotoxic neurodegeneration involving mitochondrial protein misfolding. However, there are limits on neuroprotection provided by endogenous AlloP, as evidenced by glaucomatous changes even in the presence of increases in endogenous neurosteroid.

5. Pharmacological effects of neurosteroids are required for full protection even in the presence of elevated endogenous steroid levels. These pharmacological effects may require concentrations above those that are considered physiological as evidenced by full protection with 1 µM but not 100 nM exogenous AlloP. Furthermore, a single injection of neurosteroid at a pharmacological dose can have lasting neuroprotective effects in vivo even when the primary neuronal stressor (high pressure) persists. We note that current use of brexanolone (AlloP) for postpartum depression involves a 12-hour intravenous bolus with the dose is maintained at a high level observed in pregnancy (approximately 100 nM) and maintained at that level for 28 hours before tapering off by the end of infusion (Meltzer-Brodny and Kanes, 2020). Another NAS, zaranolone, is administered orally as a single, 30–50 mg daily dose (Beattie et al., 2019; Deligiorgis et al., 2021). Levels in plasma by these steroids and duration of exposure at high levels in key brain regions may be important in determining clinical efficacy, and high doses may be required for maximal results, based on studies in the retina. It is also intriguing that even transient and perhaps intermittent exposures to GABAergic NAS can have persisting effects both in the glaucoma model and in humans with major depressive syndromes. These observations make it important to understand the transient effects of NAS following transient exposures; studies in the retina can help this effort based on results to date.

6. The enantiomer of AlloP is an intriguing pharmacological tool, providing unexpected neuroprotection via the cellular mechanism of autophagy. Neuroprotection by ent-AlloP does not differ from AlloP, and may thus obviate certain side effects of AlloP including sedation and loss of consciousness, perhaps allowing administration of higher doses. Metabolism of ent-AlloP and other enantiomeric steroids will likely differ from those of AlloP and possibly contribute to longer-lived effects. Side effects of enantiomeric neurosteroids are presently unknown. A diagram of the roles of endogenous and exogenous AlloP in the studies described is shown in Figure 3. We also note that in our in vivo glaucoma model (Ishikawa et al., 2021; 2022; 2023) as a model of development of neurodegeneration (Budelier et al., 2006), both AlloP enantiomers provide neuroprotection following a single administration that persists for weeks in rodents, while having no effect on the causative mechanisms that underlie neuropathology.

Future Directions in Neurosteroid Pharmacology

Neuroprotective effects of both endogenous and exogenous AlloP in the retina are unequivocal. Future studies are required to define the full mechanisms by which neurosteroids produce their protective effects including GABA-A receptor and other alternative targets. It is unknown whether ent-AlloP and its enantiomer stimulate autophagy, but it is clear that GABA-A receptors are not the entire story, particularly for ent-AlloP. Based on work with AlloP analogues that are photoaffinity labels, both endogenous and exogenously applied NAS interact with several known intracellular targets. These steroids interact strongly with Golgi and this interaction, as well as intracellular and membranous accumulation, is not enantioselective (Jiang et al., 2016). Specific intracellular targets identified to date include the GABA-A receptor (β-2/3), tubulin, at Cy3-554 (Chen et al., 2012), and mitochondrial proteins voltage-dependent anion channel-1 and -2 (VDAC) at Glu-73 of VDAC-1 along with four additional sites (Darbandi-Tonkabon et al., 2003, 2004, Budelier et al., 2017, Cheng et al., 2019). The site on β-tubulin is conserved across several species (Sodero et al., 2011) and plays a role in regulating autophagy (Nobrega et al., 2019). Another oxysterol, 25-hydroxycholesterol is also intriguing as a modulator of autophagy, and is synthesized in the brain primarily in microglia (Wong et al., 2020; Izuami et al., 2021), 25-hydroxycholesterol also has weak partial agonist PAM effects on NMDA receptors and can serve as a functional inhibitor of 24-HC (Linsenbardt et al., 2014).

In summary, studies in the retina clarify some of the protective mechanisms of AlloP and offer potential to identify other important effects going forward. It also appears that we are presently only scratching the surface of other neurosteroid-modulated receptors and their potential roles as therapeutic targets in neurosteroids, including how these modulators interact to promote neuronal homeostasis.

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Conflicts of interest: CFZ serves on the Scientific Advisory Board of Sage Therapeutics. DFC was a co-founder of Sage Therapeutics. DFC and CFZ have equity in Sage Therapeutics. Sage Therapeutics did not fund this research. Other authors have no conflicts to declare.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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