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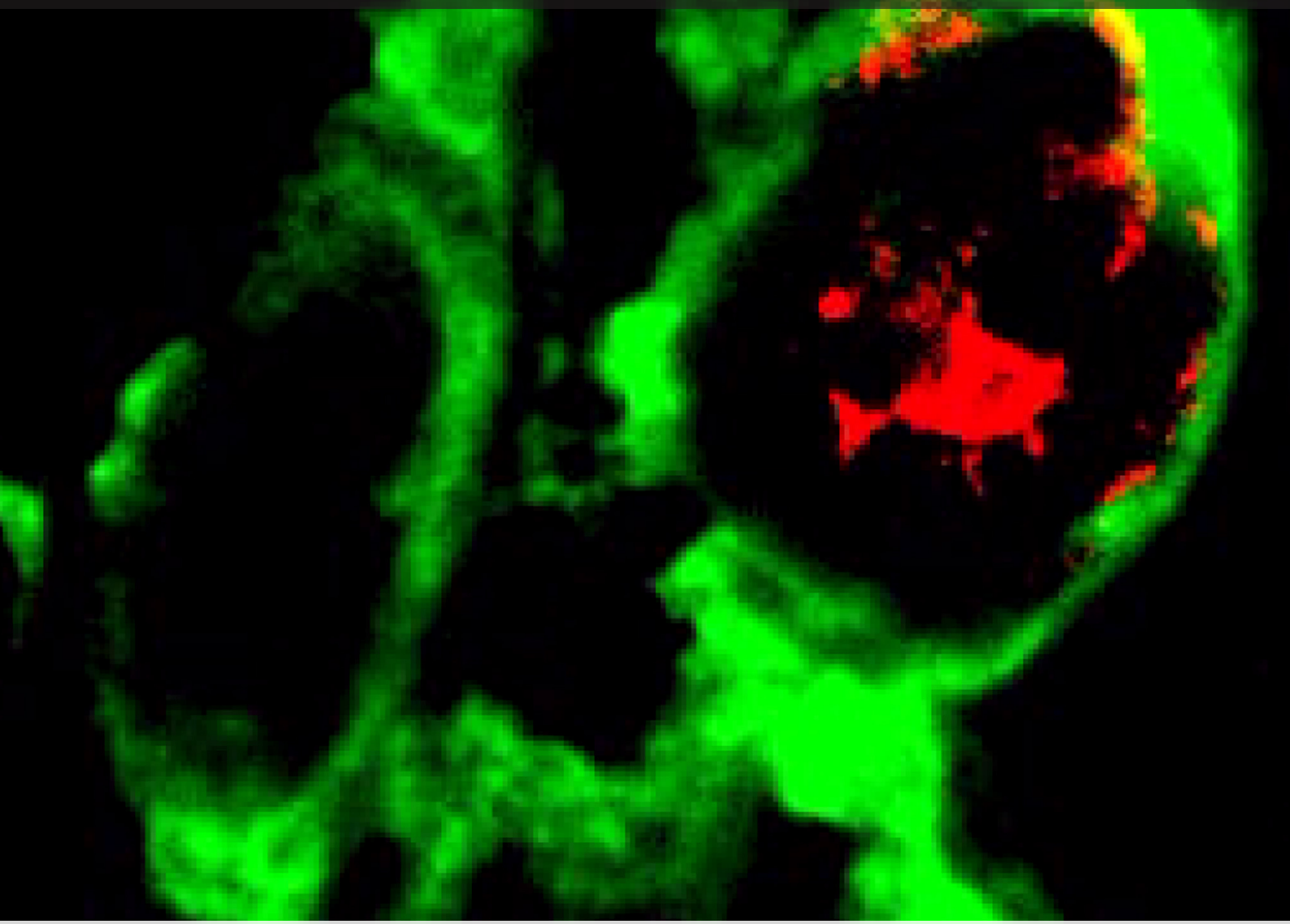

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NEW PERSPECTIVES IN NEUROSTEROIDS ACTION: A SPECIAL PLAYER ALLOPREGNANOLONE

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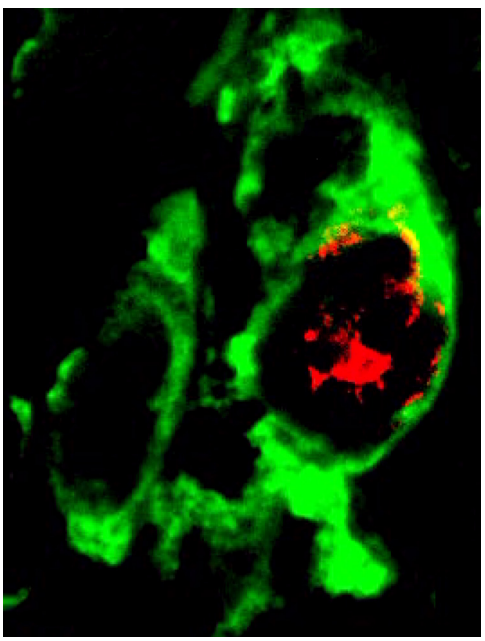

A mouse section of substantia nigra *pars compacta* double labeled for Bromodeoxyuridine (BrdU, red) and Tyrosine Hydroxylase (TH, green). The double positive image shows the BrdU-labeled nucleus and TH labeling in cytoplasm and neurites. Nuclei of some TH positive neurons that do not incorporate BrdU are evident. (from Wang JM, Front Cell Neurosci. 2014;8:224)

Early in the 80's date the first observations on the existence of hormonal steroids that may be synthesized and act in the nervous system. In order to refer to these endogenous steroids, proved important to control central and peripheral nervous system, it was proposed the term "neurosteroids" (NSs). Over the years, their importance in regulating the physiological functions of neuronal and glial cells increased progressively. These steroids can be involved in several pathophysiological conditions such as depression, anxiety, premenstrual syndrome (PMS), schizophrenia and Alzheimer's disease. Among the different classes of NSs, the progestagens revealed particularly important. The progesterone metabolite 5α-pregnan-3α-ol-20-one, also named tetrahydroprogesterone or allopregnanolone (ALLO) was among the first steroids that were originally shown to act as neurosteroid. ALLO is synthesized through the action of the enzymatic complex 5α-reductase-3α-hydroxysteroid-dehydrogenase, which converts progesterone into dihydroprogesterone and subsequently, via a bidirectional reaction, into ALLO. NSs exert complex effects in the nervous system through 'classic', genomic, and 'non-classic', non-genomic actions. ALLO displays a rapid 'non-genomic' effect, which mainly involves the potent modulation of the GABA type A (GABA-A) receptor. Recently a membrane receptor

has been also identified as target for ALLO's effects (i.e. the membrane progesterone receptor, mPR); it is able to activate a intracellular signalling cascade through G protein-coupled dependent mechanisms.

By these ways, ALLO is able to modulate several cell functions, acting as neurogenic molecule on neural progenitor cells, as well as by activating proliferation and differentiation of glial cells, in the central and peripheral nervous system.

In this topic, we review the most recent acquisitions in the field of neurosteroids, focusing our attention on ALLO because its effects on the physiology of neurons and glial cells of the central and peripheral nervous system are intriguing and could potentially lead to the development of new strategies for neuroprotection and/or regeneration of injured nervous tissues, as well as for the treatment of neuropsychiatric disorders.

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EDITORIAL
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Editorial on "New perspectives in neurosteroids action: a special player allopregnanolone"

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Keywords: steroid, progestagens, non-genomic mechanisms of steroid hormones, neuroprotective agents, pain

The hypothesis that neurosteroids are synthesized and active in the nervous system is well-established among neuroscientists. Their presence was shown for the first time more than 20 years ago (Baulieu and Robel, 1990), however we believe that different neurosteroids actions in central (brain and spinal cord) and peripheral nervous system are not still fully elucidated, deserving further investigation.

Allopregnanolone (Allo) is the most important neurosteroid (Baulieu and Robel, 1990), targeting both neurons and glial cells in central and periphery, primarily through rapid "non-genomic" action via γ-amino butyric acid (GABA) type A receptor (GABA-AR). Given these prerogatives, Allo is a promising drug for development of novel neuroprotective and neuroregenerative strategies, as well as for the treatment of neuropsychiatric disorders. Rupprecht overviewed the state of the art of Allo's actions in the brain, suggesting different molecular mechanisms beyond the GABA-AR interaction. He explores the therapeutic use of Allo and its synthetic analogous ganaxolone in human psychiatric disorders, suggesting that the analysis of neurosteroid profile in neuropsychiatric patients is necessary (Rupprecht, 2014). Ganaxolone has been recently proposed for epilepsy and infantile spasms treatments (Riikonen, 2014), however its use for neuropsychiatric diseases is emerging. The posttraumatic stress disorder (PTSD) is a severe neuropsychiatric condition for which efficient therapies are still lacking. Ganaxolone, acting on GABAergic system, is a therapeutic alternative to the serotonin reuptake inhibitors, the only drugs currently approved by the Food and Drug Administration for PTSD. Pinna and Rasmusson suggested that restoring Allo's brain levels is beneficial in PTSD patients. Using an *in vivo* PSTD mice model, they also demonstrated that the increased corticolimbic levels of Allo reduce the PTSD-like behavior. Therefore, they proposed ganaxolone as alternative treatment for patients suffering PTSD or other disorders implying Allo biosynthesis impairment (Pinna and Rasmusson, 2014).

Allo has recently emerged as neurogenic molecule acting on neural progenitor cells. Interestingly, it may also activate glial cells proliferation and differentiation, in the central or in the peripheral nervous system. Focusing on the neural architecture and neurogenesis in the nigrostriatal tract, Wang proposed Allo as a neurotrophic agent able to stimulate the number of total cells and to re-establish the dopaminergic neurons circuitry (Wang, 2014). This striking approach has been so far suggested to cure neurodegenerative diseases, such as Parkinson and/or Alzheimer. Indeed, Allo reduces β-amyloid protein levels and neuroinflammation, revealing as efficient molecule for the treatment of Alzheimer and other neurologic disorders. Brinton and colleagues presented a safe treatment with Allo that has been optimized for neuroregeneration and reduction of Alzheimer symptoms. Moreover, by tailoring doses/regimen to the different etiologies, Brinton proposes Allo as novel reliable approach for multiple sclerosis, Niemann-Pick, diabetic neuropathy and traumatic brain injury (Irwin et al., 2014). The brain of patients with multiple sclerosis presents a dysregulation

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in Allo biosynthesis. The hypothesis discussed by Power and colleagues raised the possibility that changes in Allo biosynthesis may control leukocyte functions and the neuroinflammation associated to multiple sclerosis. They found that Allo administration ameliorates neurobehavioral deficits of animals with autoimmune demyelination, proposing its use for neuroinflammatory pathologies (Noorbakhsh et al., 2014).

The pineal gland is still a neglected structure among neuroendocrinologists. Tsutsui and Haraguchi provided clear evidences that the gland is an important neurosteroidogenic organ. They reported a new neuroprotective role of Allo in the brain. During the development, Allo from the pineal gland prevents Purkinje cells death by suppressing the caspase-3 activity (Tsutsui and Haraguchi, 2014).

Neurosteroids are promising drugs also for the treatment of pain. However, they display some side effects such as sedation, amnesia and tolerance, restricting their therapeutic use. Poisbeau, Schumacher and colleagues overviewed the analgesic effects of endogenous neurosteroids, focusing on pharmacologic strategies aimed at stimulating local production of 3-alpha reduced neurosteroids (Poisbeau et al., 2014). This approach limits the side effects, targeting specific structures equipped with the neurosteroid biosynthetic machinery, including the mitochondrial translocation protein complex TSPO. In the perspective of Puia and Magnaghi the functional cross-talk between Allo, the protein kinase type C (PKC) and GABA-AR was discussed. It was reviewed how GABA-AR is modulated by Allo and/or PKC phosphorylation, through molecular mechanisms that can be mutually interconnected (Puia et al., 2015).

To further complicate the Allo's action, the possibility to figure out alternative molecular mechanisms is becoming increasingly credible. A family of new membrane progesterone receptors (mPRs) has been identified as putative target for this neurosteroid. Frye et al. (2014) faced Allo's effects on behavioral processes, involving rapid modulatory actions via GABA-AR and/or n-methyl-D-aspartate (NMDA) receptors. They also characterize the role of promiscuous nuclear receptor, the pregnane xenobiotic receptor (PXR), which may bind Allo in the central nervous system. This mechanism is supposed to support Allo's effects on the midbrain ventral tegmental area (VTA), controlling lordosis and sexual behavior.

In conclusion, Allo is a novel, promising, alternative and reliable drug with several neuroprotective properties. We very much hope this issue will help readers to understand pros and cons of Allo "neuroactions!"

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New perspectives in neurosteroid action: open questions for future research

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Keywords: neurosteroid, allopregnanolone, TSPO, GABA, ion channel

Neurosteroids are still a hot topic in cellular and systemic neuroscience although the first report on anaesthetic actions of progesterone from Selye was published already in 1941 (Selye, 1941). It is a fascinating concept that endogenous metabolites of progesterone such as allopregnanolone and pregnanolone are powerful allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors. This at a first glance simple principle created by nature raises several questions that still are major challenges for neurosteroid research. What is the exact site of interaction of such neurosteroids with $GABA_A$ receptors? Is it really a binding site with clear saturable binding kinetics or rather an interaction site? Recent studies show that photolabeling of amino acids in the third transmembrane domain of the β3 subunit of the $GABA_A$ receptor by neurosteroid analogs is feasible (Chen et al., 2012) but does this really prove a binding site? What makes the difference in the regulation of GABAergic neurotransmission between the modulation by a 3α-reduced neurosteroid such as allopregnanolone and a benzodiazepine? Both are positive allosteric modulators of $GABA_A$ receptors and enhance GABAergic neurotransmission but there appear to be great differences with regard to abuse liability and tolerance development (Rupprecht et al., 2009). Do they merely target different subunit compositions? An argument against this hypothesis is that allopregnanolone does not necessarily need a refined subunit composition to exert its actions, a β subunit is sufficient (Puia et al., 1990; Rupprecht and Holsboer, 1999). Thus, a more fascinating novel research area could be to identify what neuronal networks ultimately are targeted by either 3α-reduced neurosteroids or benzodiazepines. Do such neurosteroids and benzodiazepines recruit a different composition of post- and extrasynaptic $GABA_A$ receptors? For example, future studies employing voltage sensitive dye imaging might address such questions. To what extent receptors other than $GABA_A$ receptors are involved in neurosteroid action?

As a more systemical approach neuroimaging studies in humans, e.g., by means of functional magnetic resonance tomography (fMRI), might compare the brain areas involved after administration of benzodiazepines (Leicht et al., 2013) with neurosteroids such as allopregnanolone. As such, a major issue of future research in this area should be the elucidation of the mechanisms of action of neurosteroids both at the molecular, cellular and brain network level.

Another important area of research is the role of neurosteroids such as allopregnanolone for normal and pathological behavior in animals and humans and for neuropsychiatric disorders. It is evident from many preclinical studies that neurosteroids modulate anxiety-related behavior but nevertheless many issues are far from being understood. For example, what is the role of various neurosteroids with a different receptor profile acting in concert, e.g., pregnenolone and allopregnanolone? What about concentration and time dependency of neurosteroid effects? It may well be that such phenomena affect both physiological and pathological conditions. For example, it has been shown that negative mood symptoms may occur in women with premenstrual dysphoric disorder (PMDD) during the luteal phase of the menstrual cycle when progesterone and allopregnanolone levels usually are high (Bäckström et al., 2014) which has to be reconciled with the known anxiolytic effects of moderate to high concentrations of allopregnanolone. Moreover, in such patients there is an apparent discordance between the sensitivity to diazepam and allopregnanolone with decreased sensitivity to diazepam, whereas sensitivity to allopregnanolone is increased (Bäckström et al., 2014). A widely neglected research area is the role of isomers which acts as functional antagonists of allopreganolone, for example its 3β epimer (3β, 5α-pregnanolone). All these compounds finally act in concert in the modulation of rodent and human behavior. An example for such an altered equilibrium of steroid composition is the prominent decline in 3α-reduced neurosteroids after challenge with sodium lactate or cholecystokinin tetrapeptide (CCK-4) in patients with panic disorder together with a marked increase in the 3β-reduced isomer (Ströhle et al., 2003), which may result in a decreased GABAergic tone related to pathophysiology of panic attacks. Moreover, studies investigating the composition of neurosteroid profiles in neuropsychiatric disorders during differential psychopathological states are rare and need further elaboration. It is not surprising that neurosteroids such as allopregnanolone play a role in

the pathophysiology of mood disorders (Schüle et al., 2014) and particularly for women (Schiller et al., 2014).

Besides their neuromodulatory potential a major issue is whether endogenous neurosteroids or synthetic neurosteroid derivatives can be used as novel therapeutic agents for the treatment of neuropsychiatric disorders. Ganaxolone is a first example of a synthetic 3α-reduced neurosteroid which is under investigation for the treatment of epilepsy, e.g., infantile spams (Riikonen, 2014). Another attractive area of research is the use of neurosteroidogenic compounds to promote endogenous neurosteroidogenesis. Observations came from both preclinical and clinical studies that for example antidepressants such as selective serotonin reuptake inhibitors (SSRIs) or mirtazapine (Pinna et al., 2006; Schüle et al., 2014) may enhance neurosteroidogenesis probably through interference with neurosteroidogenic enzymes. Moreover, ligands of the translocator protein 18 kDa (TSPO) have recently gained considerable attention as putative novel therapeutic agents in neuropsychopharmacology (Rupprecht et al., 2010). Numerous reports suggest that they promote the transport of cholesterol to the mitochondrial matrix thereby initiating neurosteroidogenesis, although recently the requirement of TSPO for steroidogenesis has been questioned (Morohaku et al., 2014; Tu et al., 2014). TSPO ligands are used as molecular imaging tools for assessing brain damage and microglia activation in positron emission tomography (PET) studies and have been suggested to exert potential beneficial effects in numerous preclinical investigations, for example peripheral nerve lesions (Rupprecht et al., 2010), neuropathic pain (Patte-Mensah et al., 2014), Alzheimer's disease (Chua et al., 2014), and retinal damage (Karlstetter et al., 2014). First clinical studies suggest that TSPO ligands, e.g., olesoxime, represent a therapeutic option in amyotrophic lateral sclerosis (Rupprecht et al., 2010). Moreover, TPSO ligands such as XBD173 or etifoxine may act as anxiolytic agents in clinical studies with a more favorable side effect profile than that of benzodiazepines (Rupprecht et al., 2009, 2010). It is intriguing that etifoxine is available in France since many years for the treatment of adjustment anxiety disorder. This shows that it is feasible to develop TSPO ligands for clinical indications with a favorable side effect profile.

In conclusion, neurosteroids, e.g., allopregnanolone, and neurosteroidogenic compounds such as TSPO ligands still represent a challenging area of research that has the potential to further elucidate the physiology of rodent and human behavior, the pathophysiology of neuropsychiatric diseases and to open the door for novel treatment avenues in neuropsychopharmacology.

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Ganaxolone improves behavioral deficits in a mouse model of post-traumatic stress disorder

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Allopregnanolone and its equipotent stereoisomer, pregnanolone (together termed ALLO), are neuroactive steroids that positively and allosterically modulate the action of gamma-amino-butyric acid (GABA) at $GABA_A$ receptors. Levels of ALLO are reduced in the cerebrospinal fluid of female premenopausal patients with post-traumatic stress disorder (PTSD), a severe, neuropsychiatric condition that affects millions, yet is without a consistently effective therapy. This suggests that restoring downregulated brain ALLO levels in PTSD may be beneficial. ALLO biosynthesis is also decreased in association with the emergence of PTSD-like behaviors in socially isolated (SI) mice. Similar to PTSD patients, SI mice also exhibit changes in the frontocortical and hippocampal expression of $GABA_A$ receptor subunits, resulting in resistance to benzodiazepine-mediated sedation and anxiolysis. ALLO acts at a larger spectrum of $GABA_A$ receptor subunits than benzodiazepines, and increasing corticolimbic ALLO levels in SI mice by injecting ALLO or stimulating ALLO biosynthesis with a *selective brain steroidogenic stimulant*, such as *S*-norfluoxetine, at doses far below those that block serotonin reuptake, reduces PTSD-like behavior in these mice. This suggests that synthetic analogs of ALLO, such as ganaxolone, may also improve anxiety, aggression, and other PTSD-like behaviors in the SI mouse model. Consistent with this hypothesis, ganaxolone (3.75–30 mg/kg, s.c.) injected 60 min before testing of SI mice, induced a dose-dependent reduction in aggression toward a same-sex intruder and anxiety-like behavior in an elevated plus maze. The EC_{50} dose of ganaxolone used in these tests also normalized exaggerated contextual fear conditioning and, remarkably, enhanced fear extinction retention in SI mice. At these doses, ganaxolone failed to change locomotion in an open field test. Therefore, unlike benzodiazepines, ganaxolone at non-sedating concentrations appears to improve dysfunctional emotional behavior associated with deficits in ALLO in mice and may provide an alternative treatment for PTSD patients with deficits in the synthesis of ALLO. Selective serotonin reuptake inhibitors (SSRIs) are the only medications currently approved by the FDA for treatment of PTSD, although they are ineffective in a substantial proportion of PTSD patients. Hence, an ALLO analog such as ganaxolone may offer a therapeutic GABAergic alternative to SSRIs for the treatment of PTSD or other disorders in which ALLO biosynthesis may be impaired.

Keywords: ganaxolone, allopregnanolone, selective brain steroidogenic stimulants, 5α-reductase type I, PTSD, PTSD therapy, anxiety disorders, $GABA_A$ receptor

INTRODUCTION

Traumatic life events involving the threat of injury or death, such as combat exposure, sexual assault, witnessing of terroristic attacks, motor vehicle accidents, or involvement in natural disasters may lead to post-traumatic stress disorder (PTSD). PTSD symptoms appear following the traumatic event and fail to extinguish or may worsen over time. PTSD symptoms defined by the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5; American Psychiatric Association, 2013) include intrusive memories of the event, recurrent flashbacks and nightmares, emotional and physiological reactions to trauma reminders, difficulty sleeping, trouble concentrating, irritability and aggression, increased startle, hypervigilance, strong negative emotions and beliefs related to the trauma, emotional numbing and avoidance of reminders of the event. An estimated 7–8% of Americans will experience PTSD at some point in their lives, and about 3.6% of U.S. adults aged 18–54 (5.2 million people) will have PTSD during the course of a given year. The prevalence of PTSD in women (10.4%) is about twice that in men (5.0%), representing a relatively small portion of individuals who have experienced at least one traumatic event (60.7% of men and 51.2% of women). However, exposure to certain types of trauma, such as sexual assault and combat, is associated with a substantially higher (15–30%) risk for PTSD. PTSD is also associated with increased rates of other psychiatric and medical comorbidities including depression, anxiety disorders, traumatic brain injury, chronic pain, cardiovascular

disorders, metabolic syndrome, and substance abuse, particularly tobacco and alcohol dependence (Rasmusson et al., 2010; Carlson et al., 2011; Friedman et al., 2014; Rasmusson and Shalev, 2014; Scioli-Salter et al., 2014).

Notwithstanding the prevalence of this debilitating psychiatric disorder in the general population, the only FDA-approved drugs for the treatment of PTSD are the selective serotonin reuptake inhibitors (SSRIs; Brady et al., 2000; Davidson et al., 2001; Marshall et al., 2001; Tucker et al., 2001). The response rate to these drugs, however, is relatively small, and some studies have shown that male combat veterans, in particular, may be resistant to their therapeutic effects, although ethnic differences may play a role in veteran response rates (Hertzberg et al., 2000; Zohar et al., 2002; Friedman et al., 2007; Panahi et al., 2011). The search for neurobiological biomarkers for PTSD is therefore a current focus of investigation in the hope that a better understanding of individually variable neurobiological risk factors for PTSD will spur development of more specific and individually effective therapies.

Stress-induced alterations in the composition of $GABA_A$/benzodiazepine receptor complexes are involved in the lack of response to classical benzodiazepine ligands as well as in the production of dysfunctional behaviors following stress or traumatic events, as documented in both preclinical and clinical studies. In postmortem studies, alterations in $GABA_A$ receptor binding and receptor subunit composition, as well as in in GABA synthesis and transport are associated with anxiety disorders and depression in humans (Vaiva et al., 2004; Geuze et al., 2008). In studies of patients with PTSD, GABA levels are reduced (Kugaya et al., 2003), as are $GABA_A$/benzodiazepine receptor binding (Bremner et al., 2000). Similarly, in rodents, chronic stress and fear conditioning have been shown to diminish GABA-mediated neurotransmission within the amygdala (Martijena et al., 2002), by decreasing expression of genes for GABA synthesizing enzymes, decreasing NE α_1-stimulated GABA release from interneurons within the basolateral nucleus of the amygdala (BLA; Braga et al., 2004), downregulating gephyrin, a protein that anchors synaptic $GABA_A$ receptors, and downregulating synaptic $GABA_A$ receptors themselves (Chhatwal et al., 2005; Heldt and Ressler, 2007). Together, these studies suggest why benzodiazepines have not been found to be beneficial in treating the core symptoms of PTSD (Geuze et al., 2008). In addition, recent work shows that reductions in GABA synthesis by knockdown of GAD67 in the amygdala, as well as specific knockdown of the $GABA_A$ receptor α1 subunit that confers benzodiazepine sensitivity on corticotropin releasing factor (CRF) neurons, disrupts extinction (Gafford et al., 2012; Heldt et al., 2012).

Levels of neurosteroids that positively and allosterically modulate GABA action at $GABA_A$ receptors (Puia et al., 1990, 1991; Belelli and Lambert, 2005) also have been found to be low in PTSD patients (Rasmusson et al., 2006). In premenopausal women, cerebrospinal fluid (CSF) levels of ALLO and its equipotent stereoisomer pregnanolone (together termed ALLO) were 40% of the levels seen in healthy subjects and were inversely correlated with PTSD re-experiencing and comorbid depressive symptoms (Rasmusson et al., 2006). In fact, levels were lowest in those PTSD patients with current comorbid depression. In addition, the ratio of ALLO to its steroid precursor, 5α-dihydroprogesterone (5α-DHP), was decreased among the PTSD patients, suggesting dysfunction of the enzymes involved in ALLO synthesis (Rasmusson et al., 2006). Similarly, among recently deployed male veterans, the ratio of ALLO to progesterone, the precursor for 5α-DHP, was lowest in those veterans with the most severe PTSD and depression symptoms (Kilts et al., 2010).

Although neurosteroids such as ALLO have activity at all subtypes of $GABA_A$ receptors, they have highest affinity for a benzodiazepine-resistant subset of extrasynaptic $GABA_A$ receptors composed of α_4 and δ subunit combinations or α_6, γ, and β subunit combinations (Lambert et al., 2003; Belelli and Lambert, 2005). These extrasynaptic receptors are activated by concentrations of GABA lower than that required for activation of synaptically located $GABA_A$ receptors. As a consequence, extrasynaptic $GABA_A$ receptors are thought to maintain a tonic inhibitory conductance that modulates gain in neuronal output during periods of increased input (Mitchell and Silver, 2003; Semyanov et al., 2003, 2004; Mody and Pearce, 2004; Sun et al., 2004), as occurs during stress. Of note, α_4, δ, and α_6 $GABA_A$ receptor subunits increase under conditions in which ALLO levels are decreased (Smith et al., 1998; Follesa et al., 2001; Gulinello et al., 2002; Sundstrom-Poromaa et al., 2002; Pinna et al., 2006b). In hippocampus (at least, as other areas have not yet been studied), extrasynaptic $GABA_A$ receptors also appear to be reciprocally upregulated when synaptic $GABA_A$ receptors are downregulated. This suggests that after fear conditioning when synaptic $GABA_A$ receptors are downregulated in the amygdala, maintenance of adequate GABA tone in the amygdala may depend on positive modulation of extrasynaptic $GABA_A$ receptors by neurosteroids, such as ALLO, that are synthesized and released locally or that enter the brain after release from the adrenal gland. Thus, pharmacological interventions aimed at normalizing brain ALLO levels in PTSD patients with deficiencies in ALLO synthesis, might be expected to restore GABAergic neurotransmission and enhance recovery from PTSD.

We previously sought to investigate this hypothesis in mice subjected to four weeks of social isolation, which results in a 70% reduction in ALLO and 5α-DHP biosynthesis (Matsumoto et al., 1999; Dong et al., 2001). Importantly, the largest decrease of ALLO induced by social isolation was found in the amygdala and hippocampus, followed by the olfactory bulb and frontal cortex (Pibiri et al., 2008). ALLO levels failed to change in the cerebellum and striatum (Pibiri et al., 2008). *In situ* immunohistochemical studies further demonstrated that 5α-reductase conversion of 5α-DHP to ALLO, the rate-limiting enzymatic step in ALLO biosynthesis, was specifically decreased in cortical pyramidal neurons of layers V–VI, hippocampal CA3 pyramidal neurons, glutamatergic granular cells of the dentate gyrus, and pyramidal-like neurons of the basolateral amygdala (Agís-Balboa et al., 2007). Notably, brain interconnections arising from these corticolimbic areas play a primary role in the regulation of emotional behavior, including fear responses, as demonstrated by both human and basic research studies (Myers and Davis, 2007). Accordingly, in SI mice, downregulation of ALLO biosynthesis was associated with the emergence of neurobehavioral dysfunction including anxiety-like behavior and aggression towards same-sex

intruders (Matsumoto et al., 1999; Pinna et al., 2003, 2006a, 2008; Pibiri et al., 2008). Furthermore, SI mice exposed in a novel environment (i.e., the context) to the administration of acoustic tones preceding unconditioned footshock stimuli, exhibited exaggerated conditioned contextual fear response and impaired fear extinction (Pibiri et al., 2008; Pinna et al., 2008). Thus, protracted social isolation combined with fear-conditioning could be a suitable mouse model to study emotional behaviors and neurochemical alterations related to PTSD (Pibiri et al., 2008; Pinna, 2010).

Similar to PTSD patients, SI mice also show resistance to classical benzodiazepine ligands such as diazepam and zolpidem in association with changes in mRNA and protein expression for several $GABA_A$ receptor subunits in the frontal cortex and hippocampus (Pinna et al., 2006b; Nin Schuler et al., 2011). Expression of $GABA_A$ receptor subunits α1, α2, and γ2 were reduced by approximately 50%, whereas the mRNAs encoding α5 and α4 subunits, which confer increased sensitivity to neuroactive steroids such as ALLO, were increased by approximately 130% compared to levels in group-housed control mice (Pinna et al., 2006b). In the SI mice, the systemic administration of ALLO or infusion of ALLO directly into the basolateral amygdala had a strong anti-aggressive effect (Nelson and Pinna, 2011). These results were replicated by the administration of *S*-norfluoxetine at doses that failed to have serotonergic effects but potently increased ALLO biosynthesis in target corticolimbic areas, including the hippocampus, basolateral amygdala, and frontal cortex (Pinna et al., 2006a; Nelson and Pinna, 2011).

The present translational study was undertaken to evaluate whether ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one), a 3β-methylated synthetic analog of allopregnanolone (ALLO) that cannot be converted back into its progesterone precursors, has a similar capacity to improve anxiety and PTSD-like behaviors manifested by SI mice, including increased aggression and exaggerated contextual fear responses. Ganaxolone has shown efficacy as an anticonvulsant in a number of animal models (e.g., Reddy and Rogawski, 2010), and is currently being investigated for the treatment of refractory epilepsy (Bialer et al., 2013) and PTSD in human clinical trials[1].

MATERIALS AND METHODS

SUBJECTS

Adult male Swiss–Webster mice (Harlan Breeders, Indianapolis), 18–20 g body weight, were maintained under a 12-h dark/light cycle and provided food and water *ad libitum* in a vivarium with temperature and humidity kept near 24°C and 65%, respectively. SI mice were housed individually in a 24 × 17 × 12 cm cage for 3–4 weeks, while group-housed control mice were housed in groups of 5. Ganaxolone was obtained from Marinus Pharmaceuticals, Inc[2]. Ganaxolone, pregnanolone, or vehicle (corn oil) in a volume of 100 μl/10 g was injected subcutaneously (s.c.) 60 min before behavioral tests of locomotor activity, anxiety-like behavior, and aggressive behavior toward an intruder in the home cage. In a study of ganaxolone effects on fear extinction and retention,

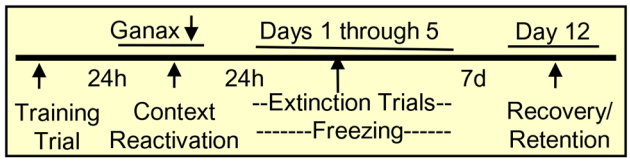

FIGURE 1 | Contextual fear conditioning protocol. Mice were trained in the conditioning chamber by tone plus footshock, which was repeated three times every 2 min. The total time in the conditioning chamber was 8 min. To induce retrieval/reactivation of the training memory, mice were placed in the conditioning chamber for 5 min and immediately after the reactivation session, they received a single injection of vehicle or ganaxolone. For the extinction trial (5 days), mice were placed in the chamber for 5 min without footshock, and freezing was measured as an indication of contextual fear. After an interval of 7 days (day 12), mice were reexposed to the chamber without footshock and freezing was measured as an indicator of the spontaneous reinstatement of contextual fear, or inversely, as extinction retention.

ganaxolone or vehicle was administered just once, immediately after the first session in which the mice were reexposed to the context in which fear conditioning was performed the day before (i.e., subsequent to the first reactivation or extinction session; **Figure 1**). All experimental protocols were approved by the Office of Animal Care and Institutional Biosafety Committee and the Office of the Vice Chancellor for Research of the University of Illinois at Chicago.

BEHAVIORAL TESTING

ELEVATED PLUS MAZE

Behavioral testing was performed between 10.00 and 14.00 h in a light- and sound-controlled room using an elevated plus-shaped maze constructed of black acrylic and elevated 50 cm above the floor (Uz et al., 2004). In this test, mice chose between entering the two relatively anxiogenic 45 × 10 cm open arms and the two relatively safe 45 × 10 × 12 cm closed arms that extended from a 10 × 10 cm central platform. Mice were initially placed facing the closed arm. Entry onto an arm with less than four legs was counted as a crossing. An arm entry was scored when all four legs were within the arm. Behavior in the maze was recorded and scored for 10 min, 60 min after the single s.c., injection of ganaxolone (3.75–30 mg/kg) or vehicle (corn oil). Time spent on the open arm and the number of open arm crossings, closed arm crossings, and closed arm entries were analyzed. After each test, the maze was wiped with ethanol/water (50% v/v).

RESIDENT–INTRUDER TEST

To test aggression, a male intruder mouse of the same strain as the resident mouse, was placed in a resident home cage (24 × 17 × 12) and resident–intruder interactions were videotaped for 10 min. Aggressive behavior of SI mice was characterized by an initial pattern of exploratory activity around the intruder, followed by rearing and tail rattle, accompanied within a few seconds by wrestling and/or a violent biting attack. The total duration of wrestling and attack behavior during the 10 min observation period was measured as previously described (Pinna et al., 2003, 2005), 60 min after administration of a single dose of ganaxolone (3.75–30 mg/kg, s.c.). To establish whether ganaxolone

[1]http://clinicaltrials.gov

[2]www.marinuspharma.com

is superior to ALLO in decreasing aggressiveness of SI mice, an EC_{50} dose of ganaxolone (10 mg/kg, s.c.) was used in a comparison experiment with the same dose of pregnanolone (10 mg/kg, s.c.). Behavioral testing was performed between 10.00 and 14.00 h.

CONTEXTUAL FEAR CONDITIONING

Apparatus

The conditioning and extinction chamber (25 cm wide, 18 cm high, and 21 cm deep) had a cage floor made of stainless steel rods connected to an electric shock generator (San Diego Instrument, Inc., San Diego, CA). It was surrounded by a frame that emitted 16 infrared photo beams. A computer controlled the delivery of electric footshocks and recorded beam interruptions and latencies to beam interruptions (freezing time).

Conditioning trial

The group-housed and SI mice were placed in the chamber and allowed to explore for 2 min before exposure to a 30 s, 85 DB acoustic tone (conditioned stimulus, CS) that co-terminated with a 2 s, 0.5 mA electric footshock (unconditioned stimulus, US). The tone plus footshock was repeated three times randomly within each subsequent 2 min epoch. One minute after the last tone-footshock delivery, mice were returned to their home cages. The total time in the conditioning chamber was 8 min.

Reactivation

Mice were returned to the chamber 24 h later for 5 min without footshock presentation to induce retrieval/reactivation of the training memory. Immediately after the reactivation session, each mouse received a single s.c., injection of vehicle or EC_{50} dose of ganaxolone (as established in the previous tests of aggression).

Contextual fear

Twenty-four hours after the reactivation/first extinction trial, the mice were placed in the chamber for 5 min without footshock, and freezing was measured as an indication of contextual fear.

Extinction and extinction retention

Mice were placed in the chamber for the next 5 days in a row starting 24 h after the reactivation session. After a subsequent interval of 7 days (day 12), mice were reexposed to the chamber without footshock and freezing was measured as an indicator of the spontaneous reinstatement of contextual fear, or inversely as extinction retention (**Figure 1**). Freezing was defined as the absence of movement except respiration while the mice remained in a crouched posture (Pibiri et al., 2008).

MEASUREMENT OF EXPLORATORY ACTIVITY IN A NOVEL CAGE

A computerized AccuScan 12 Animal Activity Monitoring System (Columbus Instruments, Columbus, OH, USA) assisted by VERSAMAX software (AccuScan Instruments, Columbus, OH, USA) was used to quantify locomotor activity (Pinna et al., 1997, 2006b). Each activity cage consisted of a 20 × 20 × 20 cm Perspex box surrounded by horizontal and vertical infrared sensor beams. Horizontal sensors beam interruptions were taken as a measure of horizontal activity, whereas vertical sensor beam interruptions counted as rearing activity. Activity was recorded from group-housed and SI mice between 13.00 and 15.00 h for 15 min beginning 60 min after a single injection of vehicle or various doses of ganaxolone (3.75–30 mg/kg, s.c.).

STATISTICAL ANALYSES

Results are presented as means ± SEMs unless otherwise indicated. Comparisons between the control group and each of the treatment groups were performed using one-way ANOVA followed by LSD's test or repeated measures ANOVA followed by a Greenhouse–Geisser correction. Significance was set at $P < 0.05$. Ganaxolone EC_{50} values were calculated from dose–response curves analyzed by the "quantal dose–response: probits test" using the computer program of Tallarida and Murray equipped with a statistical package. Statistical comparisons among the different EC_{50}s were performed with the "cohort package software[3]."

RESULTS

DOSE-DEPENDENT GANAXOLONE EFFECTS ON AGGRESSIVE BEHAVIOR IN SI MICE

Administration of ganaxolone (3.75–30 mg/kg, s.c.) resulted in a dose-dependent decrease of aggressive behavior directed by SI resident mice toward same-sex intruders (**Figure 2**). There was a highly significant main effect of ganaxolone treatment on aggressive behavior ($F_{4,36} = 6.89$, $P < 0.001$). The dose of 30 mg/kg was not more efficacious than the 15 mg/kg dose in decreasing aggression. Equimolar doses of ganaxolone and pregnanolone were equipotent in ameliorating the social isolation-induced aggression. The analyses of the dose–response curve resulted in an EC_{50} of 9.7 mg/kg of ganaxolone, which was the dose used in the evaluation of the contextual fear conditioning response experiments.

ANXIETY-LIKE BEHAVIOR IN GROUP-HOUSED AND SI MICE TREATED WITH GANAXOLONE

This study confirmed findings of previous experiments demonstrating increased anxiety-like behavior in a plus maze in SI mice compared with group-housed mice (Pinna et al., 2006a; Nin Schuler et al., 2011). There was a significant main effect of ganaxolone treatment within SI mice and a dose-dependent effect of ganaxolone treatment on several anxiety-like measures (ratio of open to closed arm total time: $F_{4,41} = 2.80$, $P = 0.038$; ratio of open to closed arm rest time $F_{4,41} = 2.66$, $P = 0.04$; **Figures 3** and **4**). The lowest dose of ganaxolone (3.75 mg/kg) only showed a trend towards improvement of anxiety-like behavior expressed as the ratio of open arm to closed arm total time ($P = 0.08$; **Figure 3**). Ganaxolone treatment at the 7.5 mg/kg dose significantly increased the ratios of open arm to closed arm rest time as well as total time spent in the open arms ($P = 0.02$ and $P = 0.01$, respectively). The most effective 15 mg/kg dose of ganaxolone induced anxiolytic effects as determined by the ratios of open arm to closed arm rest time and total time ($P = 0.007$ for both measures). The dose of 30 mg/kg did not elicit an improvement of social isolation-induced anxiety-like behavior (**Figures 3** and **4**).

[3]www.cohort.com

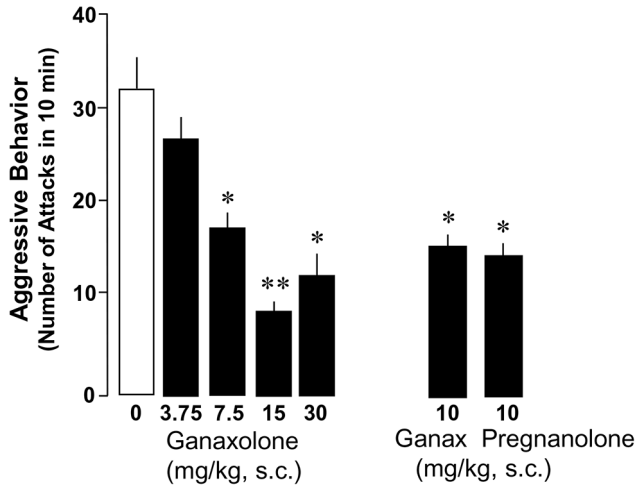

FIGURE 2 | Ganaxolone dose-dependently decreases social isolation-induced aggression of resident mice toward a same-sex intruder. Dose–response curve resulted in an EC_{50} dose of 10 mg/kg of ganaxolone. Equimolar doses of ganaxolone and the $GABA_A$ receptor active, ALLO isoform, pregnanolone were equipotent in decreasing aggressive behavior in SI mice. Data represent the mean ± SEM of 8–10 SI mice. $*P < 0.01$; $**P < 0.001$, when compared with vehicle-treated (0) mice.

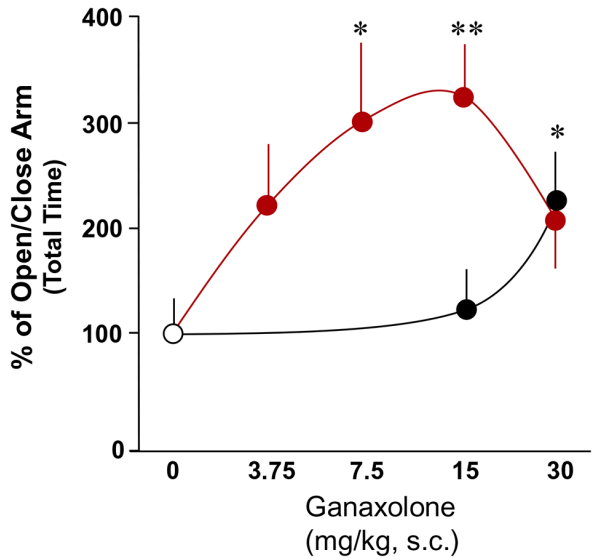

FIGURE 3 | The effects of ganaxolone on anxiety-like behavior in SI mice (red circles) results in a bell shaped dose–response curve, which is shifted to the right in group-housed mice (black circles). Ganaxolone in the dose range of 3.75–30 mg/kg improves anxiety-like behavior of SI mice exposed to an elevated plus maze and assessed by open to close arm total time, and improved anxiety-like behavior of group-housed mice at the high dose of 30 mg/kg, s.c. Data represent the mean ± SEM of eight to fourteen mice. $*P < 0.05$; $**P < 0.01$ when compared with vehicle-treated (0) mice.

In group-housed mice, there was a significant main effect of ganaxolone treatment (ratio of open to closed arm total time: $F_{2,22} = 4.46$, $P = 0.027$). Ganaxolone at a dose of 15 mg/kg, s.c., did not affect anxiety-like measures. The highest 30 mg/kg ganaxolone dose did, however, induce an anxiolytic effect as mice treated with this dose showed an increase in the ratio of open arm to closed arm total time ($P = 0.04$; **Figure 3**) and in the ratio of open arm to closed arm distance traveled ($P = 0.027$; not shown).

CONTEXTUAL FEAR RESPONSES IN SI MICE THAT RECEIVED AN EC_{50} DOSE OF GANAXOLONE

SI mice compared to group-housed mice exposed to contextual fear conditioning exhibited increased freezing and reduced extinction over a period of five extinction trials (**Figure 5**). Repeated-measures ANOVA with a Greenhouse–Geisser correction showed a significant group by drug treatment by extinction session interaction for freezing across extinction sessions day 1–3, the time interval over which extinction continued to decline ($F_{1.995,43.885} = 3.618$; $P < 0.035$). *Post hoc* testing revealed that ganaxolone treatment compared to vehicle treatment resulted in less freezing in the SI mice. Ganaxolone did not affect freezing time in the group-housed mice. Importantly, the single EC_{50} dose (10 mg/kg) of ganaxolone administered after the first fear reactivation/extinction session prevented the spontaneous reemergence of contextual fear responses after the passage of time—or from another perspective, enhanced extinction retention ($T_{1,23} = 5.809$, $P = 0.025$; **Figure 5**).

EFFECTS OF GANAXOLONE ON EXPLORATORY ACTIVITY IN SI AND GROUP-HOUSED MICE

Ganaxolone did not reduce exploratory activity in either SI or group-housed mice, even at the highest dose (30 mg/kg) tested. There was a trend for the lowest doses of ganaxolone (3.75 and 7.5 mg/kg) to stimulate both horizontal and vertical locomotor activity in SI mice (**Figures 6** and **7**).

DISCUSSION

This study assessed the effects of a synthetic ALLO analog, the neuroactive steroid ganaxolone, on anxiety-like behavior, aggression, and contextual fear conditioning and extinction, as well as locomotor activity in male mice. Importantly, ganaxolone administered s.c. at 3.75–30 mg/kg did not impair exploratory activity as assessed by characterization of horizontal and vertical locomotion patterns. Ganaxolone did, however, show a strong anxiolytic effect in mice tested in the elevated plus maze, with lower doses effective in SI mice with deficits in ALLO, and higher doses effective in group-housed mice with normal ALLO levels. Ganaxolone also dose-dependently decreased aggression in SI mice to a same-sex intruder at doses comparable to ALLO doses with comparable effects. Most intriguingly, an EC_{50} dose (10 mg/kg, s.c.) of ganaxolone, given immediately after reactivation of contextual fear 1 day after fear conditioning, substantially diminished contextual fear on subsequent test days in SI mice. In addition, it blocked the spontaneous reemergence of contextual fear a week after extinction was complete – or from another perspective, corrected deficits in extinction retention exhibited by SI mice. Of note, such deficits in extinction retention have been observed in studies of PTSD in humans (e.g., Milad et al., 2008), thus reinforcing the idea that deficiencies in GABAergic neurotransmission associated with deficient ALLO biosynthesis constitute a vulnerability to the development

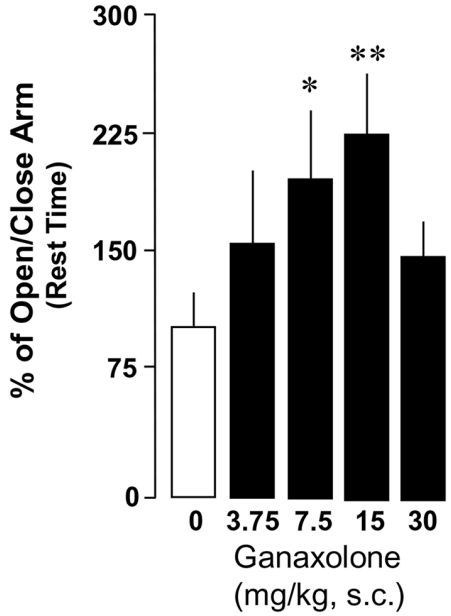

FIGURE 4 | Ganaxolone dose-dependently decreases social isolation-induced anxiety-like behavior of mice exposed to an elevated plus maze, determined by the ratios of open arm to closed arm rest time. Data represent the mean ± SEM of 8–14 mice. *$P < 0.05$; **$P < 0.01$ when compared with vehicle-treated (0) mice.

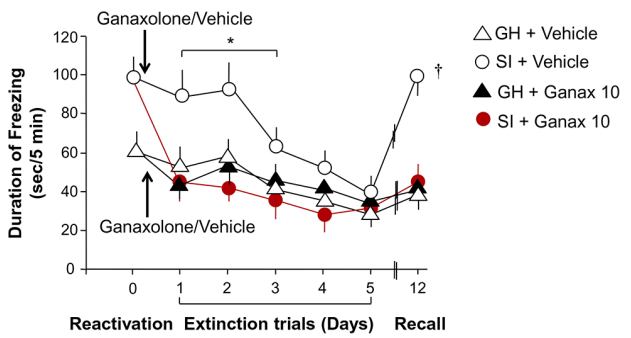

FIGURE 5 | Ganaxolone facilitates fear extinction and blocks contextual fear reconsolidation. SI mice (empty circles) exhibit increased freezing and reduced extinction compared to group-housed mice over a period of five extinction trials. Ganaxolone treatment administered immediately after a reactivation session (black arrow) compared to vehicle treatment resulted in less freezing in the SI mice (red circles). Ganaxolone did not affect freezing time in the group-housed mice (black triangle). Importantly, ganaxolone prevented the spontaneous reemergence of contextual fear responses after the passage of time – or from another perspective, enhanced extinction retention in SI mice. Data represent the mean ± SEM of 10–12 mice. *$P = 0.035$ when compared to SI + Ganaxolone; †$P = 0.025$ when compared to SI + ganaxolone on recall (day 12).

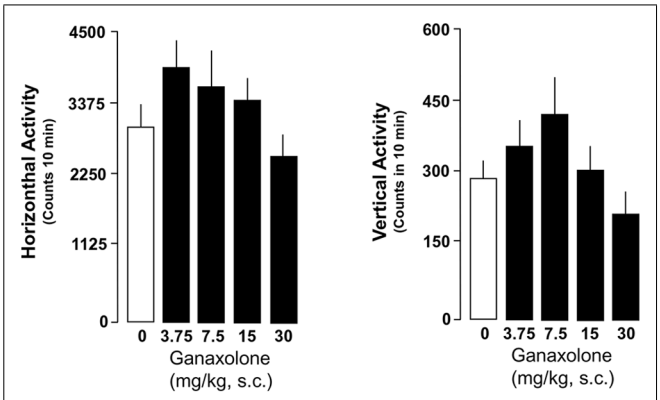

FIGURE 6 | Ganaxolone did not alter exploratory activity determined as means of horizontal and vertical activity in SI mice even at the highest dose (30 mg/kg) tested. The lowest doses of ganaxolone (3.75 and 7.5 mg/kg, s.c.) exhibit a trend to increase both horizontal and vertical locomotor activity in SI mice. Data represent the mean ± SEM of six to eight SI mice.

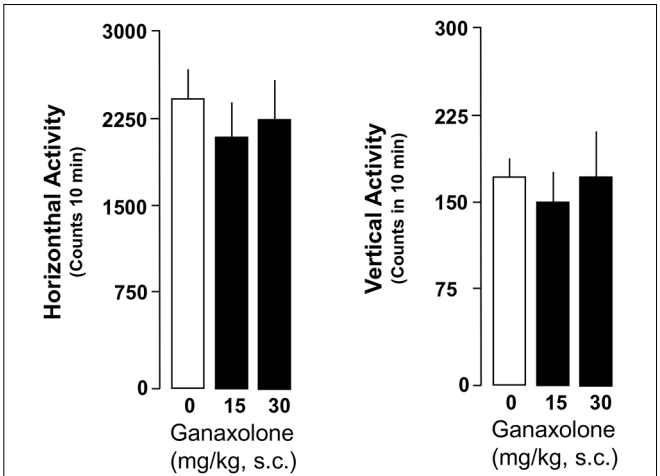

FIGURE 7 | Ganaxolone fails to change locomotor measures assessed as horizontal and vertical activity in group-housed mice. Data represent the mean ± SEM of six group-housed mice.

of PTSD-like behaviors in response to threat, modeled in this study by exposure to footshock in a Pavlovian fear conditioning paradigm.

These data are in agreement with previous reports demonstrating strong anxiolytic effects of ganaxolone at 10 mg/kg i.p. in rats (Kudagi et al., 2012) and wild-type or $GABA_A$ receptor delta subunit knockout mice (Mihalek et al., 1999). These results thus suggest that ganaxolone may be useful in clinical practice for a subpopulation of patients in whom anxiety or PTSD symptoms are related to deficient ALLO biosynthesis. It is possible that ganaxolone also may find application in other disorders characterized by a downregulation of brain ALLO levels, including depression (Uzunova et al., 1998; Agis-Balboa et al., 2014).

CURRENT PTSD TREATMENT OPTIONS

Currently, there is no specific pharmacological treatment for PTSD. The only FDA approved medications for the management of this debilitating disorder are the serotonin selective reuptake inhibitors (SSRIs), paroxetine and sertraline. Although SSRIs improve symptoms of PTSD in some patients, meta-analyses have demonstrated that response rates rarely exceed 60% and

that only 20–30% of patients achieve a full remission of symptoms (Westenberg, 1996; Walderhaug et al., 2010; Ipser and Stein, 2012). Venlafaxine, a serotonin–norepinephrine reuptake inhibitor (SNRI) was shown to induce a positive clinical response in 78% of PTSD patients (Davidson et al., 2006). However, only 40% of patients who completed the treatment achieved PTSD remission and the drug was not effective for PTSD hyperarousal symptoms (Davidson et al., 2006).

The finding that low non-serotonergic doses of fluoxetine and congeners increase ALLO levels as their primary mechanism of action, suggests that SSRIs acting as *selective brain steroidogenic stimulants* (SBSSs; Pinna et al., 2003, 2006a; Pinna, 2014) may thereby improve psychiatric symptoms and be of use in PTSD (reviewed in Pinna and Rasmusson, 2012). However, the high rate of resistance to current medications of this class suggests that deficits in the activity of enzymes involved in ALLO synthesis may not be amenable to correction by SSRIs in PTSD—and/or that the pathophysiology of PTSD is more complex and/or varies among individuals. The study by Rasmusson et al. (2006), suggested that 3α-hydroxysteroid dehydrogenase (3α-HSD) activity is downregulated in premenopausal women with PTSD. Work by Gillespie et al. (2013), on the other hand, showed that a polymorphism of the 5α-reductase type I gene predicted an increase in the risk for PTSD in men. This suggests the possibility that the specific enzyme site responsible for ALLO deficiency in PTSD may differ between men and women, as supported by the work of Pinna et al. (2008) showing that social isolation downregulates corticolimbic ALLO levels in male rodents at 5α-reductase, but not female rodents, unless the females are oophorectomized and replaced with testosterone (Pinna et al., 2005). Hence, it is possible that SSRIs and SNRIs currently in use for the treatment of PTSD do not adequately enhance gene expression or enzymatic function at these sites in individuals resistant to their therapeutic effects. Therefore, an alternative strategy might be to directly activate $GABA_A$ receptors with an ALLO analog such as ganaxolone (Gulinello et al., 2003; Kaminski et al., 2004; Pinna, 2014).

OTHER NEUROSTEROIDOGENIC DRUGS FOR THE POTENTIAL TREATMENT OF PTSD-LIKE SYMPTOMS

There are several other neurosteroidogenic biomarkers with the potential to serve as targets for the next generation of anxiolytic, antidepressant, or PTSD relevant drugs. One of the best studied is the 18 kDa translocase protein (TSPO; Papadopoulos et al., 2006; Rupprecht et al., 2009, 2010; Schüle et al., 2011, 2014), formally known as the peripheral benzodiazepine receptor (Costa and Guidotti, 1991; Costa et al., 1994). TSPO regulates the availability of neurosteroids in the brain by facilitating access of cholesterol to the inner mitochondrial membrane and its subsequent conversion to pregnenolone by the rate-limiting step enzyme, P450scc, located within the inner mitochondrial membrane (Papadopoulos et al., 2006; Rupprecht et al., 2009, 2010). TSPO agents have been shown to potently increase ALLO levels in brain regions that regulate emotional behavior, such as the hippocampus and cortex, and to induce anxiolytic effects (Kita et al., 2004). Several TSPO ligands have recently been shown to be effective in rodent models of PTSD, including AC-5216/XBD173 and YL-IPA08 (Qiu et al., 2013).

Another neurosteroidogenic target is the pregnane xenobiotic receptor (PXR), a well-characterized, ubiquitous and promiscuous nuclear receptor important for metabolism and xenobiotic clearance in liver, kidney and intestine (Geick et al., 2001; Dussault and Forman, 2002; Francis et al., 2002; Kliewer et al., 2002). The recent discovery of PXR expression in brain has suggested a potential role for PXR in neural plasticity, as well. For example, PXR gene expression fluctuates across the estrous cycle in female rats and increases in the midbrain following mating, while knockdown of PXR expression in the ventral–tegmental area (VTA) reduces biosynthesis of ALLO in response to mating (reviewed in Frye, 2011; Frye et al., 2012, 2013). Inhibition of TSPO with the selective antagonist, PK11195, also reduces ALLO levels in midbrain, and reduces lordosis, effects reversed by ALLO administration. Together these data suggest that PXR may be upstream of TSPO (Frye et al., 2014).

The endocannabinoid system also has attracted attention as a steroidogenic target. The primary active ingredient of *Cannabis sativa*, Δ9-tetrahydrocannabinol (THC), increases pregnenolone synthesis in brain via activation of the type 1 cannabinoid receptor (CB1; Vallée et al., 2014). Other cannabinoid ligands thus are being studied for their potential as anxiety and PTSD therapies. There are interesting similarities between the cannabinoid system and ALLO in the regulation of emotion. Levels of ALLO and the endocannabinoid, anandamide (AEA) are decreased in models of stress-induced anxiety and depression (Matsumoto et al., 1999; Dong et al., 2001; Pibiri et al., 2008; Rademacher et al., 2008; Hill et al., 2009), and both ALLO and drugs that increase ALLO or AEA levels have similar effects on fear responses (Costanzi et al., 2003; Pibiri et al., 2008; Pinna et al., 2008; Lin et al., 2009).

The potential role of the endocannabinoid system in regulating emotional experience is further supported by the density of endocannabinoid receptors on glutamatergic neurons in emotion relevant areas such as the amygdala, hippocampus, and cortex (Slanina and Schweitzer, 2005; Katona, 2009). In addition, cannabinoids regulate intracellular peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptorsuperfamily (Forman et al., 1996; O'Sullivan, 2007; Pistis and Melis, 2010). The endocannabinoids, AEA and palmitoylethanolamide (PEA) are PPAR-α agonists, and PEA's action at PPAR-α induces analgesia by enhancing ALLO biosynthesis (Sasso et al., 2012). A PEA-related increase in brain stem ALLO levels also potentiates pentobarbital hypnosis, an effect mimicked by PPAR-α agonists and prevented by ALLO biosynthetic enzyme blockers (Sasso et al., 2010, 2012). Also of note, PEA administration shows antidepressant effects equal to those of fluoxetine (Umathe et al., 2011; Yu et al., 2011) that activate ALLO biosynthesis (Pinna et al., 2003, 2006a).

The finding that activation of CB1 and PPAR-α receptors is capable of inducing ALLO biosynthesis, together with the pivotal role of ALLO in facilitating the action of GABA at $GABA_A$ receptors, invites speculation about whether cannabinoid-related anxiolytic and anti-fear effects are due to the induction of corticolimbic ALLO biosynthesis. Cannabidiol, a non-sedating phytocannabinoid with a remarkably safe profile in humans, as well as other cannabinoids (Lin et al., 2006; Kobilo et al., 2007; Suzuki et al., 2008; Stern et al., 2012) have recently been shown

to disrupt recent and older contextual fear memories by interfering with their reconsolidation. This effect of cannabidiol is long lasting and can be prevented by pharmacological antagonism of CB1 receptors (Stern et al., 2012). Interestingly then, the anti-fear effects of cannabidiol resulting in reconsolidation blockade were similar to the effects of midazolam, which like ALLO, activates $GABA_A$ receptors (Stern et al., 2012).

The findings of the current study also support a role for $GABA_A$ receptors in reconsolidation blockade and recovery from conditioned fear (Duvarci and Nader, 2004; Bustos et al., 2006). Administration of the ALLO-like compound, ganaxolone, during a critical time-limited window following exposure to conditioned contextual cues (Stern et al., 2012), markedly reduced the expression of fear in subsequent extinction trials and prevented the spontaneous recovery of fear (**Figure 5**). Given that PTSD is associated with benzodiazepine resistance, synaptic $GABA_A$/benzodiazepine receptor complexes in humans with PTSD are decreased, and synaptic $GABA_A$ receptors in the amygdala decrease after fear conditioning in rodents (Mou et al., 2011), it is tempting to speculate that blockade of reconsolidation may result from activation of extrasynaptic receptors, which are highly sensitive to neurosteroids (Belelli and Lambert, 2005). Furthermore, given that synaptic $GABA_A$ receptors in the amygdala are restored after extinction of fear in rodents (Heldt and Ressler, 2007), it is possible that such restoration is a functional consequence of activation of extrasynaptic $GABA_A$ receptors by GABAergic neurosteroids such as ALLO during extinction.

CONCLUSION

Post-traumatic stress disorder appears to be a multifactorial disorder with several symptom clusters and involving neurochemical deficits that may vary among individuals with PTSD. Current treatments for PTSD are only efficacious in some patients or in some symptom clusters and not in others. Accumulated knowledge about the heterogeneous pathophysiology of PTSD thus suggests that treatments of the future should be "individually designed" rather than one-size fits all. In the case of PTSD patients who exhibit deficient ALLO biosynthesis and related deficits in GABAergic neurotransmission, ganaxolone administration may facilitate recovery. Perhaps then, future clinical trials of ganaxolone should be guided by pre-treatment ascertainment of ALLO levels and other relevant GABAergic system biomarkers as possible predictors of treatment efficacy.

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Allopregnanolone and neurogenesis in the nigrostriatal tract

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Reinstalling the neurobiological circuits to effectively change the debilitating course of neurodegenerative diseases is of utmost importance. This reinstallation requires generation of new cells which are able to differentiate into specific types of neurons and modification of the local environment suitable for integration of these new neurons into the neuronal circuits. Allopregnanolone (APα) seems to be involved in both of these processes, and therefore, is a potential neurotrophic agent. Loss of dopamine neurons in the substantia nigra (SN) is one of the main pathological features of Parkinson's and also in, at least, a subset of Alzheimer's patients. Therefore, reinstallation of the dopamine neurons in nigrostriatal tract is of unique importance for these neurodegenerative diseases. However, for the neurogenic status and the roles of allopregnanolone in the nigrostriatal tract, the evidence is accumulating and debating. This review summarizes recent studies regarding the neurogenic status in the nigrostriatal tract. Furthermore, special attention is placed on evidence suggesting that reductions in allopregnenalone levels are one of the major pathological features in PD and AD. This evidence has also been confirmed in brains of mice that were lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or those bearing neurodegenerative mutations. Lastly, we highlight studies showing that allopregnanalone can augment the number of total cells and dopaminergic neurons via peripheral exogenous administration.

Keywords: allopregnanolone, neurogenesis, substantia nigra, nigrostriatal, tyrosine hydoxylase, neural circuits, motor performance

INTRODUCTION

Research data consistently suggests that a small molecule, the neurosteroid allopregnanolone (APα) capable of permeating the brain-blood-barrier, is a latent restorative therapeutic agent for reestablishing neuronal circuits in hippocampus and also the nigrostrital tract. Supportive data demonstrated that APα functioned as a neurotrophic factor for human, rat, and mouse neural progenitor cells (Keller et al., 2004; Wang et al., 2005, 2010; Charalampopoulos et al., 2008) and augmented the number of cells in the hippocampus and reversed deficits in learning and memory in a mouse model for Alzheimer's disease (3xTgAD, a triple transgenic with APPSwe, PS1M146V, tauP301L) (Wang et al., 2010; Chen et al., 2011; Singh et al., 2012), for review see Brinton (2013) and Irwin and Brinton (2014). In contrast, APα has been reported to inhibit the learning and memory when chronically treated for 3 months (Bengtsson et al., 2012, 2013) and the potential mechanisms for this discrepancy have been discussed elsewhere (Brinton, 2013; Wang, 2013; Irwin and Brinton, 2014). In addition, APα also plays a role in regulating depressive episodes (Schüle et al., 2011, 2014; Evans et al., 2012; Hellgren et al., 2014) and the antidepressant effects of APα is probably mediated via neurogenesis in dentate gyrus in hippocampus (Evans et al., 2012). Recently, accumulated data indicated that APα increased the number of total cells, tyrosine hydroxylase (TH) positive cells, and newly formed (BrdU positive) TH expressing cells in the substantia nigra (SN), and improved the balance and coordination of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mice, an animal model for Parkinson's disease (PD; Adeosun et al., 2012). The augmentation of TH positive neurons by APα in the SN of 3xTgAD mice (Sun et al., 2012a) clarified that APα accomplished its role through the reestablishment of DA neuronal architecture, rather than blockading the neurotoxic effects of MPTP. This review summarizes and highlights the current discoveries involving the generation of new neurons in the nigrostriatal tract and the therapeutic potential for the related neuronal disorders of APα.

THE SIGNIFICANCE FOR A THERAPEUTIC STRATEGY TO REINSTALL THE FUNCTIONAL DA NEURONS IN NIGROSTRIATAL TRACT

AD and PD are devastating, degenerating neural disorders which currently cannot be cured. More than 5 million and nearly 1 million Americans have AD or PD, respectively, and every minute a new case is added in this cohort. These diseases not only bring about suffering for the patients themselves, it is

also a heavy financial burden and high labor cost for both the families and society. The severity of these diseases is closely related to the number of neurons that are lost in specific brain regions.

For example, the symptoms of PD are closely associated with the depletion of striatal dopamine (DA), brought on by the impairment of normal neurobiological architecture of neural cells in the nigrostriatal tract, resulting in the degeneration and death of DA neurons. The role of nigrastriatal tract in AD has been reported in studies using post-mortem brains from patient with AD, transgenic mice with human AD mutations, and also from those studying the dopamine effects in AD (Uchihara et al., 1992; Love et al., 1996; Perez et al., 2005; Nardone et al., 2014). Diffuse plaques in the striatum and neurofibrillary tangles in the SN were consistent findings in all of the Alzheimer brains tested (Uchihara et al., 1992; Love et al., 1996). Furfuremore, a 41% significant neuronal loss was observed in SN of AD subjects compared to that in the age matched controls (Uchihara et al., 1992). Although in the study by Love et al. (1996), quantitation did not reveal a statistically significant correlation between the density of striatal plaques and the numbers of either neurofibrillary tangles or neurons in the SN in "pure" AD (i.e., without clinical or neuropathological evidence of Parkinson's or cortical Lewy body disease), the mean number of neurons in the SN of Alzheimer brains was lower than that in controls (Love et al., 1996). Pharmacologically, L-Dopa significantly increased Short-latency afferent inhibition (SAI) in the AD patients, while it failed to restore SAI abnormality in patients with Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts (Nardone et al., 2014). Therefore, L-Dopa-mediated changes on SAI in AD patients seem to be a specific effect. The striatum and the SN of transgenic mice harboring familial AD (FAD)-linked APPswe/PS1DeltaE9 mutants exhibit morphological alterations accompanied by amyloid-beta (Aβ) deposition 6 months of age, and the extent of deposition increases in an age-dependent manner (Perez et al., 2005). In addition, a reduction in the dopamine metabolite DOPAC was also observed in the striatum of these mice (Perez et al., 2005). These findings suggested a close association between amyloid deposition and nigrostriatal pathology and suggest that altered familial AD-linked amyloid metabolism impairs, at least in part, the function of dopaminergic neurons.

L-DOPA treatment and deep brain stimulation only provide symptomatic relief by increasing brain DA levels without altering the course of the disease. Scientists have been attempting to adjust the developmental course of the disease by restoring region-specific DA neuron architecture (Soderstrom et al., 2006). Initial trials to replenish DA neurons used grafts of DA-producing adult adrenomedullary tissue and then fetal mesencephalic tissue (Collier et al., 2002; Williams and Lavik, 2009; Lindvall and Kokaia, 2010), but encountered many obstacles (Björklund, 1993). Recent discoveries overcame these hurdles by generating patient-derived pluripotent and growth factor-enhanced fibroblasts to increase the supply of tissue for grafting and to prevent transplant rejection. However, new problems have materialized. It is still not clear whether grafted cells will survive in a pathological environment with a deteriorated milieu and be appropriately integrated into a >50-year-old local neuronal network. In fact, patients in clinical trials with grafted cells emerged with dyskinesia (Freed, 2002; Maries et al., 2006). This data implicates that the grafted new cells were not integrated into the existing neuronal network and were not capable of performing the expected functions. Interestingly, PD pathological markers, Lewy bodies and α-synuclein aggregates, had been observed in the grafted cells (Li et al., 2008; Hansen et al., 2011). Thus, there is an urgent need for a therapeutic strategy to restore functional DA neurons, which could effectively integrate into the existing neuronal network.

APα IMPROVES BALANCE AND COORDINATION AND INCREASES THE NUMBER OF NEW TYROSINE HYDROXYLASE CELLS IN SNpc OF MPTP-LESIONED MICE

MPTP-lesion impairs the motor performance, particularly in the modalities of balance and coordination, in C57BL/6J mice (Antzoulatos et al., 2010). The balance and coordination of MPTP-lesioned mice were improved in a rotarod performance task in which mice were forced to move correctly to prevent them from falling. Mice that received peripheral administration of APα almost completely regained their ability to walk on the rod (Adeosun et al., 2012). Correlated to the improvement of motor performance, the number of tyrosine hydroxylase immunoreactive (TH-IR) cells in the SN in APα-treated, MPTP-lesioned mice was increased. This data suggests that APα promotes the reinstallation of functional neural circuits in the nigrostriatal pathway either by reversal (recovery) of the MPTP-induced degeneration of TH neurons, and/or the generation of new (or differentiated) TH-expressing neurons in this brain region. In addition to the increase of TH-IR neurons, APα also increased the number of Nissl stained cells, which were both reduced in mice only received the MPTP neurotoxin. These results, in addition to the increase of BrdU/TH double positive cells in APα-treated mice, suggest that new cells, not only TH-IR neurons but also the non-neuronal cells, were added into the SN of the MPTP-lesioned mice (Adeosun et al., 2012; **Figure 1**).

It is still in debate whether neurogenesis also occurs in the SN. Studies from different groups demonstrated that new cells were born in the healthy SN (Lie et al., 2002; Zhao et al., 2003). The precursor cells isolated from the SN had the ability to differentiate into neurons *in vitro* (Lie et al., 2002) or the generation of new mature nigral DA neurons under physiological conditions by colocalization of BrdU and TH (Zhao et al., 2003). In contrast, opposite report indicated that there is no evidence for neurogenesis in SN (Frielingsdorf et al., 2004) and argued that the BrdU and TH co-localization was an overlay of a BrdU positive glia on an adjacent neuron (Borta and Hoglinger, 2007). However, a number of works have also described the expression of polysialylated-neural cell adhesion molecule (PSA-NCAM), a molecular expressed in multipotent progenitor cells, in the cells of SN (Nomura et al., 2000; Yoshimi et al., 2005) and a small number of cells are PSA-NCAM double positive (Yoshimi et al., 2005). Borta and Hoglinger (2007) discussed that PSA-NCAM is also expressed in other cells undergoing plastic changes, and therefore, these results do not support the hypothesis of dopaminergic neurogenesis in the SN. Peng et al. reported that fibroblast growth

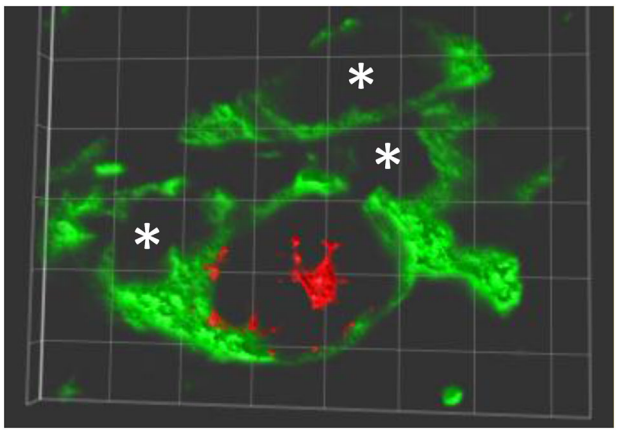

FIGURE 1 | New TH expressing neurons in SNpc of allopreganolone treated mice lesioned by MPTP. A 3-dimensionally rotated double-immunolabeling image shows a new neuron (red, BrdU positive in nuclear) expressing TH (green in cytoplasm and neurites), and a few TH positive only neurons marked with *. Similar image can be found in Adeosun et al. (2012).

factor 2 increased the number of BrdU and doublecortin double positive cells in SN of MPTP-lesioned mice (Peng et al., 2008). Others reported that either physical activity or Unilateral lesion of the subthalamic nucleus increased the oligodendrogenesis and astrogliogenesis in the SN after 6-OHDA lesion (Steiner et al., 2008; Klaissle et al., 2012). Recently, it was also reported that the majority of newly generated cells in the adult mouse SN express low levels of doublecortin (Worlitzer et al., 2013). Taken together, these data support the generation of new cells in SN, but whether these new cells will differentiate into functional DA neurons is not clear. Perhaps by reestablishing the extracellular milieu and local environment in SN to a level suitable for new neuron differentiation, maturation and integration into the existing neuronal circuits will be a hopeful solution. In addition, appropriate labeling protocols may be needed to identify the newly generated neurons by optimized amount of BrdU (Zhao and Janson Lang, 2009), or by tracing the ratio of C^{14} in DNA of cells in striatum of human brains (Ernst et al., 2014).

Accumulated evidence suggests that there are multiple neurogenic niches in the brain apart from the hippocampal dentate gyrus sub granular zone (SGZ) and the cerebral sub ventricular zone (SVZ). These include the hypothalamus (Lee et al., 2012), cerebellum (Keller et al., 2004; Ponti et al., 2005, 2006, 2008, 2010; Bonfanti and Ponti, 2008; Hajihosseini et al., 2008), striatum (Tattersfield et al., 2004; Ninomiya et al., 2006; Luzzati et al., 2007; Snyder et al., 2010; Danilov et al., 2012; Delavaran et al., 2013; Ernst et al., 2014; Kempermann, 2014), and SN (Bayer et al., 1995; Zhao et al., 2003; Chen et al., 2005; Van Kampen and Robertson, 2005; Yoshimi et al., 2005; Arias-Carrión et al., 2006, 2009; Freundlieb et al., 2006; Shan et al., 2006; Steiner et al., 2006; Esposito et al., 2007; Mandel et al., 2007; Di Giovanni et al., 2009; Ries et al., 2009; Park et al., 2012; Sun et al., 2012a,b; Worlitzer et al., 2013). Therefore, APα may promote the generation of new cells locally in SN. One such possibility is that APα increases proliferation of glial fibrillary acidic protein (GFAP), or Ng2 expressing glia cells, which maintain their mitotic status, and drives the differentiation of these new cells into DA neurons in the SN. This hypothesis is supported by the recent studies demonstrating that the primary progenitors in adult neurogenesis are astrocyte-like cells that express GFAP and that surviving cells exhibit neurites 7 days after proliferation (Cabras et al., 2010; Ming and Song, 2011). Furthermore, it has also been reported that, in the presence of sonic hedgehog, GFAP-expressing mesencephalic progenitor cells can be differentiated into TH-IR neurons within 4 days (Matsuura et al., 2001). Parallelly, the new adult subependyma cells (BrdU positive) of the lateral ventricle can differentiate into TH-expressing neurons after 24-h exposure to fibroblast growth factor (bFGF2) and glial cell conditioned media (Daadi and Weiss, 1999). Therefore, it is possible that in the SN, the proliferating glial-like cells have the capacity to differentiate into both neurons and glial cells as regulated by their microenvironment. This is further supported by a recent study which demonstrate that glia cells can differentiate into neurons in the presence of neuronal differentiation 1, a basic helix-loop-helix transcription factor (Guo et al., 2014).

APα IS A POTENTIAL NEUROGENIC AGENT IN NIGROSTRIATAL TRACT

The neurotrophic feature of APα is widely supported by the literature. APα is produced in pluripotent progenitor cells (Lauber and Lichtensteiger, 1996; Gago et al., 2004) and neurons (Pinna et al., 2004; Agís-Balboa et al., 2006) throughout the embryonic period. In late gestation, a developing period in which large amount of CNS neurons are generated and functional circuits are formed, APα concentration is 20–30 times higher than any other time in life (Pomata et al., 2000). In pathological conditions, the concentration of APα is significantly reduced in the brains of humans with AD (Marx et al., 2006; Smith et al., 2006; Naylor et al., 2010), with PD (di Michele et al., 2003; Luchetti et al., 2010) as well as from the brains of a transgenic mouse model of AD (Wang et al., 2007, 2010). More interestingly, the lower the APα concentration, the more severe these neurodegenerative diseases are and the pathology appears to be inversely correlated with the levels of APα (Naylor et al., 2010).

In mice lesioned with MPTP, APα reversed the cell number decline of TH-expressing and Nissl positive cells in both SN and Locus coeruleus (LC; Adeosun et al., 2012). This data suggest that the generation of new cells by APα is not cell type, brain region, or mouse model specific, as we previously reported the neurogenic property of APα in the SGZ and SVZ in a mouse model of AD (Wang et al., 2010; Chen et al., 2011; Singh et al., 2012). The fact that APα increased the proliferation of cerebellar neurogenic cells supports the observation that APα is not only a neurogenic agent in known neurogenic areas such as SGZ and SVZ, but also in brain regions such as the cerebellum (Keller et al., 2004) and the SNpc (Adeosun et al., 2012; Sun et al., 2012a).

Interestingly, it appears that the neurogenic effects of APα need to be enhanced or maintained with physical activities (Adeosun et al., 2012). In support, utilizing running wheels or forced

treadmill for several weeks increased the TH expression (Gerecke et al., 2010; Tajiri et al., 2010). Moreover, a significant increase in numbers of newborn NG2-positive and GFAP-positive cells was observed in the SN of 6-OHDA lesioned animals living in enriched environment with physical activity for 7 weeks. These mice showed improved motor behavior compared to controls under standard conditions (Steiner et al., 2006). Therefore, it is likely that forced physical activity helps the survival and differentiation of newly formed cells induced by APα. This point of view is supported by the fact that newly formed neural progenitors can differentiate into TH-expressing neurons within 24 h when exposed to glial cell conditioned media or basic bFGF2 (Daadi and Weiss, 1999), and in line with report that bFGF2 expression is increased after physical exercise (Gómez-Pinilla et al., 1997).

APα FUNCTIONS IN THE NIGROSTRIATAL TRACT OF MICE WITH AD MUTATIONS

Is APα only a blockade for MPTP-lesion, or a neurogenic agent in the SN? Recent work demonstrating that APα also increases TH positive neurons and total cell numbers in the SN of a triple transgenic mouse for AD (3xTgAD) Sun et al. (2012a) clarified that APα played its role through reestablishment of DA neuronal architecture, rather than by the blockade of the neurotoxic function of MPTP. Further support is from reports that genetic risk factors found in familial AD (i.e., mutations in APP, PS1 and tau phosphorylation genes) also play a role in SNpc neuropathology and atrophy.

Besides the occurrence of plaques, tangles and hippocampal atrophy, atrophy in brain nuclei containing TH expressing neurons is also a neuropathological feature of late-onset AD (Chui et al., 1986; LaFerla et al., 1997; Zarow et al., 2003). For example, a meta-analysis concluded a consistently high TH neuron loss (52–76%) in LC, and a variable neuron loss (4–50%) in the SNpc in post-mortem brains of late onset AD subjects (Zarow et al., 2003). These data indicate that reduction of TH expressing and total neurons in SN of animals bearing AD mutations (Sun et al., 2012a) occurs, and is in agreement with those early studies from AD subjects (Zarow et al., 2003).

Supportive evidence was also emerged from the transgenic APP/PS1 mouse model of AD, in which hyper-accumulated Aβ-42 residues lead to the early appearance of amyloid plaque formation when compared to mice with only the single transgene APP (Perez et al., 2005; O'Neil et al., 2007). In the APP/PS1 double mutant mice there was a significant (24%) reduction in TH-positive neurons in the LC in comparison to their background controls (O'Neil et al., 2007). Interestingly, the loss of TH expressing neurons was not observed in the transgenic mouse model with APP23 (Szot et al., 2009) nor PADPP (German et al., 2005). These findings suggest that the loss of TH positive neurons may be a result of the double APP/PS1 mutations, rather than a single APP mutation.

It has been proposed that tau protein abnormalities play a more important role in the loss of neurons in AD, and that deposition of amyloid plaques does not correlate well with neuron loss (Mudher and Lovestone, 2002; Mudher et al., 2004; Schmitz et al., 2004). Neurofibrillary tangle formation is composed of hyperphosphorylated microtubule-associated protein tau that appears to accumulate within vulnerable neurons and may eventually kill the cell, leaving behind only a ghost tangle and no neuron (Ramsden et al., 2005; Iqbal and Grundke-Iqbal, 2006; Gong and Iqbal, 2008). The 3xTg mice carry, in addition to two mutations in human familial AD genes (APP_{Swe}, $PS1_{M146V}$), one frontal temporal dementia-linked tau mutation (tau_{P301L}) and mimic multiple aspects of AD neuropathology in relevant brain regions (Oddo et al., 2003a,b). The reduction of TH-immunoreactive neurons in the SNpc of 3xTgAD male mice at 3 months old, extend the previous report and supports the hypothesis that early neurogenic deficits lead to the reduction of total neuron numbers in multiple brain regions of AD subjects (Wang and Sun, 2010) including SNpc. SN lesions are frequently present in AD and include pigmented neuronal cell loss, gliosis, Lewy bodies, α-synuclein-stained structures, and hyperphosphorylated tau accumulation in neurofibrillary tangles as well as neuritis (Kazee et al., 1995; Klunk et al., 2004; Burns et al., 2005), suggesting that AD is a significant risk factor for SN lesions (Kazee et al., 1995; Kazee and Han, 1995). APα-induced neurogenesis is dose-dependent and the most effective dose *in vitro* also has neurogenic effects *in vivo* that are accompanied with a reversal of the cognitive deficits in 3xTgAD mice (Wang et al., 2005, 2010). Previous studies indicated biphasic dose-dependent efficacy of APα on neurogenesis (Wang et al., 2005, 2010). At 100, 250, and 500 nM concentrations, APα significantly increased BrdU incorporation (lower concentrations were not statistically different from the control). At 1000 nM, a reversal of the dose–response was first apparent, with higher doses shifting the response to significant repression of proliferation at 100–1000 nM. A recent study titrated the optimal regimen for therapeutic efficacy of APα treatment *in vivo* in 3xTgAd mice (Chen et al., 2011). In both APα treatment regimens of a single exposure of 1/month and of repeated expose (1/week/6 months), APα treatment significantly increased the survival of cells that were generated at the first exposure to APα. The repeated exposure (1/week/6 months) APα treatment regimen had greater regenerative efficacy. However, the 3/week/3 months regimen significantly reduced regenerative efficacy (Chen et al., 2011). This once per week regimen suggested there might be a 7-day cycle which could help reach the best effects of APα and this seems consistent with the role of APα in SN (Adeosun et al., 2012; Sun et al., 2012a).

In contrast, recent work by Bengtsson and colleagues demonstrated that constant infusion of APα (via ALZET mini-pumps) for 3 months increase GABAergic function/inhibition in brain. These levels of APα showed a negative impact on both learning and memory and neuropathology of amyloid beta deposition (Bengtsson et al., 2012, 2013; Wang, 2013). Results of these investigations are in line with learning and memory deficits experienced by people who are chronically treated with high levels of anti-seizure medications. Mechanistically, this may be due to the accumulation of APα in the brain with a final concentration high enough to enter the second phase (inhibition) of the dose response of APα on neurogenesis (Wang et al., 2005; Brinton, 2013; Irwin and Brinton, 2014).

Wang and Brinton reported that APα transiently increases intracellular calcium concentration in primary cultured hippocampal neurons. This intracellular calcium increase is mediated by $GABA_A$ receptor and L-type Calcium Channel (Wang and Brinton, 2008) and this calcium increase is related to neural progenitor cell proliferation *in vitro* (Wang et al., 2005) and *in vivo* (Wang et al., 2010). The APα induced transient increase of intracellular calcium concentration and the subsequent proliferation of progenitor cells was abolished by inhibitors for $GABA_A$ receptor and voltage gated calcium channel blockers (Wang and Brinton, 2008). Therefore, it is likely the effects of APα on the increase of new neurons and cells are also mediated by $GABA_A$ receptor regulated transient increase of calcium concentration, however, more experimental evidence is needed.

SUMMARY AND POTENTIAL EXPECTATION

In summary, research demonstrates that the levels of APα, are reduced in the brains of subjects with AD or PD. The promising role of this APα therapy in AD and PD is supported by the recent work that peripheral administration of APα, with its ability to permeate the blood brain barrier, could improve cognitive and motor performance and increase the number of DA neurons in the SN of mice lesioned by MPTP and mice with AD mutations. These results support that APα accomplishes its role through the reestablishment of dopamine neuronal architecture, rather than blockading the neurotoxic effects of MPTP.

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Frontiers in therapeutic development of allopregnanolone for Alzheimer's disease and other neurological disorders

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Allopregnanolone (Allo), a neurosteroid, has emerged as a promising promoter of endogenous regeneration in brain. In a mouse model of Alzheimer's disease, Allo induced neurogenesis, oligodendrogenesis, white matter generation and cholesterol homeostasis while simultaneously reducing β-amyloid and neuroinflammatory burden. Allo activates signaling pathways and gene expression required for regeneration of neural stem cells and their differentiation into neurons. In parallel, Allo activates systems to sustain cholesterol homeostasis and reduce β-amyloid generation. To advance Allo into studies for chronic human neurological conditions, we examined translational and clinical parameters: dose, regimen, route, formulation, outcome measures, and safety regulations. A treatment regimen of once per week at sub-sedative doses of Allo was optimal for regeneration and reduction in Alzheimer's pathology. This regimen had a high safety profile following chronic exposure in aged normal and Alzheimer's mice. Formulation of Allo for multiple routes of administration has been developed for both preclinical and clinical testing. Preclinical evidence for therapeutic efficacy of Allo spans multiple neurological diseases including Alzheimer's, Parkinson's, multiple sclerosis, Niemann-Pick, diabetic neuropathy, status epilepticus, and traumatic brain injury. To successfully translate Allo as a therapeutic for multiple neurological disorders, it will be necessary to tailor dose and regimen to the targeted therapeutic mechanisms and disease etiology. Treatment paradigms conducted in accelerated disease models in young animals have a low probability of successful translation to chronic diseases in adult and aged humans. Gender, genetic risks, stage and burden of disease are critical determinants of efficacy. This review focuses on recent advances in development of Allo for Alzheimer's disease (AD) that have the potential to accelerate therapeutic translation for multiple unmet neurological needs.

Keywords: allopregnanolone, Alzheimer's disease, β-amyloid, neurogenesis, regeneration, cholesterol homeostasis, myelin, treatment regimen

INTRODUCTION

Neurosteroids, including allopregnanolone (Allo), are a class of neural messengers that regulate multiple processes in brain from ion channel properties to systems regeneration. Therapeutically it is possible to develop neurosteroid analogs to selectively target one action or to use endogenous neurosteroid molecules as systems biology regulators. Each strategy has its strengths and weaknesses. Depending upon the targeted disease and mechanism therein, it is possible to selectively activate a subset of responses or the full complement of potential responses. Therapeutic application of neurosteroids to achieve efficacy in a disease state requires both the understanding of the disease and the systems pharmacology of the neurosteroid (Brinton, 2013).

This review, while largely focused on therapeutic development of Allo for Alzheimer's disease (AD), raises issues that are applicable more broadly to other diseases for which Allo may have therapeutic benefit. Thus the review is structured around a framework of *considerations that matter*. To substantially advance translational research that is predictive of clinical outcome, we address multiple issues that will ultimately determine translational feasibility and success of Allo across multiple disease conditions. Lastly, most neurodegenerative diseases have no cure although in some instances palliative care extends life but not function. Therapeutics to prevent neurodegenerative disease in populations at risk, delay progression of disease in those affected and restore function in those with late stage disease remain elusive. It is our goal to enable others in the field to advance with greater speed and greater success by considering key aspects of translational research that really matter.

MOLECULAR PHARMACOLOGY MATTERS

Mechanisms of drug action should be optimally identified, confirmed and characterized in preclinical studies before

progressing to clinical trials (Becker et al., 2014). Metabolites of progesterone with reduced A-ring steroid structures are potent endogenous agonist modulators of gamma-aminobutyric acid (GABA) type A receptors ($GABA_AR$s). The $GABA_AR$ is a ligand-gated ion channel and is primarily associated with inhibition of or fine-tuning of excitatory neurotransmission. The neurosteroid progesterone metabolites that modulate the $GABA_AR$ include 3α-hydroxy-5β-pregnan-20-one (pregnanolone); 3α-hydroxy-5β-pregnan-20-one (allopregnanolone; Allo; 3α,5β-tetrahydroprogesterone) and its 21-hydroxylated derivative: tetrahydro-DOC (THDOC) derived from A-ring reduction of deoxycorticosterone. Of these metabolites, Allo is amongst the most potent endogenous allosteric modulators of the $GABA_AR$ (Belelli and Lambert, 2005). Within the mammalian brain, Allo has been shown to modulate anxiety, depression, seizure activity, sedative-hypnotic activity, and the immune system. Adding to this list, we have shown that Allo promotes the neuroregenerative system and modifies the course of neurodegenerative disease.

Neurosteroid molecular structures and their structure-activity relationship with their cognate receptors have undergone eons of co-evolutionary selection. Steroid binding site-containing $GABA_AR$s could have evolved during early chordate evolution, possibly between the branch points of Cephalochordata (lancelets) and Agnatha (lampreys) (Paul and Purdy, 1992). Much earlier, phylogenetic studies conclude that ligand gated ion channels evolved from protoreceptors in unicellular organisms (Pierobon et al., 2004).

Ionotropic $GABA_AR$s primarily transport chloride and bicarbonate ions. The potencies and efficacies of neurosteroids including Allo depend on the subunit composition of $GABA_AR$s. GABAergic neurotransmission can be fine-tuned by allosteric modulation at $GABA_AR$ binding sites for barbiturates, benzodiazepines, anesthetic alcohols, and neurosteroids. At low concentrations, neurosteroids bind to $GABA_AR$ at distinct sites to act as positive or negative modulators of $GABA_AR$ function (Gee et al., 1987, 1988). Allo is a potent positive allosteric activator of $GABA_AR$ channels that at nanomolar concentrations enhances the apparent affinity of GABA for $GABA_AR$ and, at micromolar concentrations, can directly activate $GABA_AR$ chloride channels. Allo binds to two transmembrane sites of the heteropentameric $GABA_AR$ assembled from eight subunit families (Hosie et al., 2006). $GABA_AR$ binding sites have the general subunit stoichiometry 2α:2β:1γ. $GABA_AR$ channel complexes that occur at the synaptic cleft have a higher threshold for activation and display phasic conductance. In contrast to synaptic $GABA_AR$s, a subset of extrasynaptic $GABA_AR$s, contain the neurosteroid-sensitive δ subunit making them pharmacologically distinct and display a tonic conductance pattern (Meldrum and Rogawski, 2007). $GABA_AR$s have been identified by electron microscopy in adult hippocampal subgranular zone (SGZ) progenitor cells (Mayo et al., 2005). Surrounding local interneurons that project towards the neurogenic niche and release GABA to adult dentate granule cells are subjected to tonic GABAergic signaling via δ-subunit-containing $GABA_AR$s (Overstreet Wadiche et al., 2005). Functionally, GABA plays a key role in the generation of spontaneous network activity within immature dentate granule cells (Owens and Kriegstein, 2002; Sipila et al., 2004).

GABAergic signaling likewise controls proliferation of adult progenitor cells within the subventricular zone (SVZ) neurogenic niche (Liu et al., 2005). Progenitor cells in the SVZ co-express $GABA_AR$ β_2, β_3 receptor subunits, GAD65/67 and GFAPδ (Dieriks et al., 2013). The expression of GAD65/67 was detected at lower amounts in the SVZ than in the caudate nucleus, and co-labeling was observed with $GABA_AR$ β_2, β_3, and PCNA, suggesting that cells with these markers utilized GABA from early neurogenesis until maturity. $GABA_AR$ γ_2 was the most abundant and highly localized to the SVZ. $GABA_AR$s are found throughout the SVZ on all major cell types, however $GABA_AR$ γ_2 shows the highest specific expression in the SVZ (Dieriks et al., 2013).

Through a PKC-dependent signaling mechanism, the neurosteroid THDOC selectively potentiated phosphorylation and membrane insertion of the α_4/δ subunit-containing extrasynaptic $GABA_AR$ subtypes mediate tonic conductance in the dentate gyrus (Abramian et al., 2014). This effect of THDOC was specific as it did not phosphorylate α_5/δ or KCC2 (Abramian et al., 2014).

The mechanism of action for Allo activated cell cycle gene expression in neural stem cells is mediated by binding to $GABA_AR$ to elicit an efflux of chloride and a concomitant influx of calcium that contributes to the induction of cell signaling events which lead to gene transcription of mitotic genes and downregulation of anti-mitotic genes (Brinton, 2013). A rapid rise in intracellular calcium, and subsequent activation of the cell cycle, initiates neurogenesis (Wang et al., 2005; Wang and Brinton, 2008; Brinton, 2013). Upon exogenous administration, a threshold brain concentration of Allo in neural progenitor cells of the neurogenic niches, activates a signaling cascade to trigger cell proliferation (Wang et al., 2005; Wang and Brinton, 2008; Brinton, 2013). Allo in blood and brain is subsequently enzymatically cleared within a timeframe of minutes to hours (Zhu et al., 2001; Timby et al., 2006; Irwin and Brinton, 2014) sufficient to allow regenerative system required for Allo-induced neurogenesis to continue (Brinton, 2013).

Collectively these data provide a window into the richness of the $GABA_AR$ system and how Allo modulates the function of these receptors to affect both the excitability of the brain and its regenerative capacity. The unanticipated link of the $GABA_AR$ to the regeneration of neural stem/progenitor cells suggests the possibility that other unanticipated and exciting relationships have yet to be identified.

CHOLESTEROL AND THE STEROIDOGENIC SYSTEM MATTER

The brain must synthesize its own supply of cholesterol from acetyl-CoA independent of peripherally circulating cholesterol. Because all steroids are generated from cholesterol, changes in cholesterol homeostasis will inevitably affect steroidogenesis.

Cholesterol must be delivered to and from cells by lipoproteins which include low-density lipoproteins (LDL) and high-density lipoproteins (HDL). The lipoprotein component ApoE, shuttles cholesterol between cells through the interstitial fluid and therefore regulates the distribution and redistribution of cholesterol to each cell type (Mahley, 1988). Cholesterol is transported within

the cell via steroidogenic acute regulatory protein (StAR) to the mitochondrial membrane translocator protein (TSPO). TSPO is a mitochondrial rate-limiting control checkpoint regulating cholesterol uptake and thus the synthesis of neuroactive steroids (Rupprecht et al., 2010; Irwin and Brinton, 2014). TSPO forms a cholesterol transport pore in the mitochondrial inner membrane with other proteins that include the StAR, voltage-dependent anion channel protein (VDAC), and adenine nucleotide transporter protein (ANT).

The cholesterol transport pore transports cholesterol to the mitochondrial matrix to be converted into pregnenolone by the cytochrome P450 side-chain cleavage (CYP450scc) enzyme (Liu et al., 2006). Pregnenolone diffuses out of the mitochondrial compartment and is converted in the cytosol to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Two enzymes, 5α-reductase (5α-R) type-I and 5α-hydroxysteroid dehydrogenase (3α-HSD) are required to synthesize Allo from progesterone (Mellon et al., 2001; Mellon, 2007).

Allo is a reduced metabolite of progesterone, synthesized in the gonads, adrenal cortex, and the central nervous system (Genazzani et al., 2000). In the central and peripheral nervous systems, Allo synthesis occurs primarily in glial cells—astrocytes, oligodendrocytes, and Schwann cells and in many neuronal cell types including neural progenitors (Melcangi et al., 1996; Griffin and Mellon, 2001; Mellon and Vaudry, 2001; Benarroch, 2007).

An expression pattern of progesterone converting enzymes is evident in both hippocampus and cortex. Endogenous Allo production is controlled by the rate-limiting reduction of progesterone to 5α-dihydroprogesterone (5α-DHP) by 5α-R. Progesterone is converted to Allo by the sequential action of 5α-R type-I, to 5α-DHP, which is then converted by 3α-HSD to form Allo. 5α-reductase and 3α-hydroxysteroid dehydrogenase are functionally expressed in pluripotent progenitors, neural progenitor cells, and subsets of hippocampal neurons that contain 5α-R and 3α-HSD (Melcangi et al., 1996). Subsequently, 3α-HSD catalyzes conversion of 5α-DHP into Allo. Amyloid-β-binding alcohol dehydrogenase (ABAD) is an enzyme that is associated with mitochondria and facilitates back conversion of Allo to 5α-DHP (Yang et al., 2005). Interestingly, anti-depressants, such as fluoxetine, were demonstrated to increase Allo production and although not directly correlated, increased neurogenesis (Malberg et al., 2000; Uzunova et al., 2004, 2006).

Increasing evidence indicates that altered cholesterol homeostasis is linked to neuropathologies including AD (Schumacher et al., 2004; Mellon et al., 2008; Brinton, 2013). In addition to the mechanism of action whereby Allo induces neurogenesis (Brinton, 2013), Allo regulates cholesterol homeostasis via mechanisms that increases liver-X-receptor (LXR) and pregnane-X-receptor (PXR; Chen et al., 2011). LXR is a nuclear hormone receptor abundant in the brain, primarily expressed in glial cells and acts as a molecular sensor of cholesterol levels and initiates cholesterol clearance (Whitney et al., 2002; Jakobsson et al., 2012). Loss of either LXRα or LXRβ subtype expression exacerbated AD-related pathology in APP/PS1 double transgenic mice (Zelcer et al., 2007). Loss of LXR has been shown to repress cortical neurogenesis particularly during late-embryonic stage development of layer II/III (Fan et al., 2008). LXR activation increases cholesterol efflux through increased ABCA1 and ApoE expression, and prevents overactivation of γ-secretase and overproduction of Aβ (Whitney et al., 2002; Shenoy et al., 2004; Jiang et al., 2008). LXR activation improved cognitive function in multiple mouse models of amyloidogenesis (Schultz et al., 2000; Whitney et al., 2002; Yang et al., 2006; Xiong et al., 2008; Donkin et al., 2010; Leduc et al., 2010a).

LXRs are recruited to ABCA1 gene promoter regions and ApoE expression to decrease Aβ plaque formation and increase Aβ clearance (Koldamova et al., 2010) through phagocytosis by microglia (Terwel et al., 2011). Further, LXRs are recruited to the ABCG1 promoter in a ligand-dependent manner to alter epigenetic histone methylation allowing for a relaxed chromatin structure accessible to further gene expression and cholesterol efflux (Jakobsson et al., 2012). LXRs reduce neuroinflammation by inhibition of inflammatory genes (Zelcer et al., 2007). Inflammatory cytokines reach high levels in AD and when suppressed by LXR activation, enhance the phagocytic activity of microglia and thus Aβ clearance. LXR ligands activate PXR (Riddell et al., 2007). In parallel with an Allo-induced increase in LXR expression in the pre-pathology condition, Allo also increased PXR expression in the pre-pathology 3xTgAD mouse brain (Chen et al., 2011). PXR activation, primarily in neurons, induces cytochrome P450 3A (CYP3A) enzymes including CYP3A4 and CYP3A13 and subsequent cholesterol hydroxylation and activation of organic anion transporters (OATs) for cholesterol extrusion (Sun et al., 2003). In addition to increased LXR and PXR expression, Allo treatment initiated in pre-Aβ pathology 3-month-old 3xTgAD mice treated once per week for 6 months increased expression of 3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMG-CoA-R) (Chen et al., 2011). Although HMG-CoA-R is the rate-limiting enzyme in cholesterol synthesis, it is also required for production of oxysterols that activate LXR and PXR-mediated gene transcription of cholesterol- and lipid-transport proteins (Leduc et al., 2010b). These data predict that an Allo-induced increase in brain LXR and PXR leads to increased cholesterol efflux, thereby reducing gamma-secretase activation by cholesterol-laden lipid rafts. Allo-stimulated cholesterol efflux is a plausible mechanism for the observed reduction of 27 kD and 56 kD intraneuronal Aβ oligomers after 6 months of once per week treatment (Chen et al., 2011).

In vivo, brain cholesterol homeostasis and intraneuronal Aβ are tightly coupled with Allo efficacy (Chen et al., 2011). Deposition of Aβ in the extracellular compartment disconnected this coupled pathway and led to a loss of Allo efficacy in advanced stages of AD-like pathology in the 3xTgAD model. Allo significantly reduced Aβ generation in hippocampus, cortex, and amygdala, which was paralleled by decreased mitochondrial ABAD and reduced microglia activation assessed as reduced expression of Iba-1 (Chen et al., 2011). A reduction in ABAD expression lowers mitochondrial dysfunction and simultaneously decreases the amount of Allo that is enzymatically back-converted to its precursor steroid 5αDHP. Allo may stimulate oligodendrocyte progenitor cells in addition to neural progenitor cells and since Allo is a metabolite of progesterone, the observed increases in oligodendrogenesis with progesterone treatment could be due to Allo (Schumacher et al., 2012). The

myelin marker CNPase, a myelination marker, was increased by once per week Allo, indicating myelinating capabilities in the 3xTgAD mouse model (Chen et al., 2011). In the pre-Aβ pathology cohort, 3-month-old 3xTgAD mice displayed increased expression of liver-X-receptor, pregnane-X-receptor, and 3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMG-CoA-R), three key proteins that regulate cholesterol homeostasis (Chen et al., 2011). Collectively, Allo is a systems biology regulator that promotes the neuroregenerative system with a simultaneous reduction of AD pathology in the 3xTgAD male mouse model.

NEUROSTEROID FORMULATION MATTERS

The physico-chemical properties of Allo create a challenge for aqueous formulation. Allo's low molecular weight (318.49 g/mol) and low number of hydrogen bond donors (one) and acceptors (two) are advantageous brain-targeting properties. However, the logP-value for Allo, 5.042, poses a solubility challenge for aqueous formulation and thus hinders its use as an orally administered drug (Luchetti et al., 2010). Further challenges exist for enteral absorption of Allo after absorption. To avoid the issues with Allo absorption through the oral route, Allo formulations were developed for parenteral routes of administration.

The physico-chemical properties of SBECD aid in solubilization of drug molecules with low aqueous solubility including Allo. SBECD is a chiral molecule composed of 7 α-D glucopyranose units with a molecular weight of approximately 2163 (molecular formula $C_{42}H_{70-n}O_{35}\bullet(C_4H_8SO_3Na)_n\bullet xH_2O$ [n = ~6.6]; **Figure 1**). As does its parent molecule, 2-hydroxypropyl β-cyclodextrin (HBCD; HPBCD), sulfobutylether β-cyclodextrin (SBECD) has a primary face diameter of 7.8 Å (or 0.78 nanometers) and a secondary face diameter of 15.3 Å (or 1.53 nanometers). The SBECD chemical structure differs in the side chain hydroxyl groups of HBCD, replaced by sulfo-butyl-ethers thus improving its solubility properties. Multiple SBECD molecules surround each Allo molecule to enable aqueous solubility and enhance delivery properties (**Figure 1**).

The pharmacokinetics of SBECD revealed a low volume of distribution (Vd) corresponding to extracellular water and a short elimination half-life ($t_{1/2}$) (Luke et al., 2010). Renal clearance of SBECD was at a rate corresponding to the glomerular filtration rate in all species investigated. A single IV dose of 600 mg/kg SBECD was administered to male mice. Clearance and Vd were 20.5 mL/min/kg and 0.98 L/kg, respectively; the $t_{1/2}$ was 0.6 h (Luke et al., 2010). In the rabbit, clearance was 5.5 mL/min/kg, Vd was 0.24 L/kg, and $t_{1/2}$ was 0.5 h. A single IV dose of 240 mg/kg SBECD in the dog resulted in a clearance of 4.7 mL/min/kg, Vd of 0.43 L/kg, and $t_{1/2}$ of 1.1 h (Luke et al., 2010). No clinical evidence of toxicity was found in dogs at daily doses up to 1500 mg/kg (Luke et al., 2010). The no observed adverse event level (NOAEL) for IV SBECD, associated with vacuole uptake of renal proximal tubule epithelium, was set at 80 and 30 mg/kg in rats and dogs, following bolus injection. The NOAEL associated with foamy or lipid-laden macrophages in the lung was established at 160 mg/kg in rats and 200 mg/kg in dogs. SBECD is renally excreted intact and in all studies, no evidence of metabolism of SBECD exists (Luke et al., 2010).

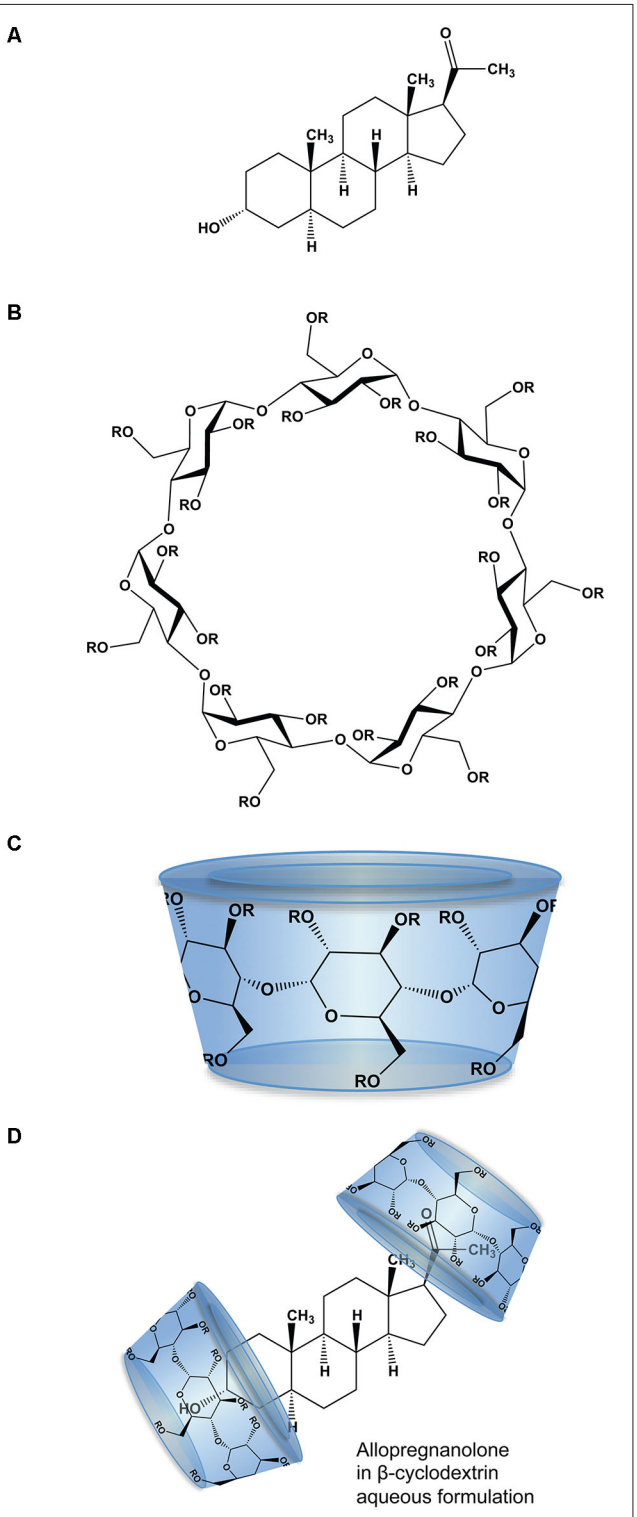


FIGURE 1 | Molecular structure and formulation of allopregnanolone. **(A)** Chemical structure of allopregnanolone; molecular weight 318.49 g/mol. **(B)** Chemical structure of β-cyclodextrin derivatives, where R is hydrogen or 2-hydroxypropyl ($CH_2CHOHCH_3$) for HBCD or sulfobutylether ($(CH_2)_4$ SO_3 Na) for SBECD. HBCD has an average molecular weight ~1460 g/mol, with 0.7–3.15 average degrees of substitution for hydrogen. SBECD molecular weight ~2163 g/mol with average degrees of substitution estimated

(*Continued*)

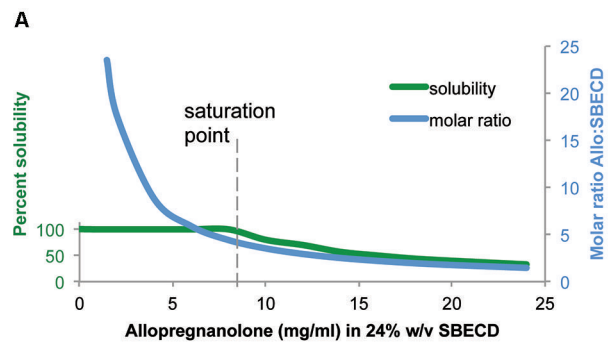
FIGURE 1 | Continued
between 6.3–6.6. **(C)** The β-cyclodextrins form three-dimensional cyclic oligosaccharide toroidal structures comprised of 1,4-linked glucopyranose molecules with a hydrophilic outer surface and a hydrophobic inner surface. **(D)** Allopregnanolone in β-cyclodextrin formulation to render allopregnanolone water soluble for rapid release *in vivo*. Two or more molecules of β-cyclodextrin, represented as toroid-shaped hydrophilic caps, form an inclusion complex with each relatively insoluble Allo molecule.

SBECD is approved for use in marketed drug products including intravenous voriconazole, amiodarone, ziprasidone, aripiprazole, and maropitant (Luke et al., 2010). Human exposure data based on Pfizer's regulatory submission were derived from four clinical studies where IV SBECD was administered (Luke et al., 2010). A total of 49 healthy male volunteers received IV infusions of SBECD alone. SBECD doses between 25 and 200 mg/kg/day were used to assess the safety and pharmacokinetics. In patients with renal problems, steady-state conditions indicated that even with daily hemodialysis, SBECD was effectively eliminated during 6 h of renal replacement therapy (Hafner et al., 2010). In older patients such as those in AD clinical trials, renal function is an important consideration when selecting a formulation and monitoring its safety and clearance. SBECD is relatively safe and with intermittent exposure is unlikely to accumulate based on human pharmacokinetic studies.

The therapeutic dose of Allo for humans is likely within the dose-range explored in previous clinical studies with IV Allo 0.05–0.9 mg/kg (Timby et al., 2006; van Broekhoven et al., 2007; Grant et al., 2008; Kask et al., 2008, 2009). With a 30% SBECD formulation of Allo for example, the amount of SBECD would be less than the NOAEL with species allometric scaling. Hypothetically, IV Allo at 0.9 mg/kg or 6.3 mg/70 kg human, in a 5 mg/ml soluble formulation of 30% SBECD, would amount to 378 mg of SBECD or 5.4 mg/kg. Citing the NOAEL of 160 mg/kg in rats (Luke et al., 2010), allometric scaling to humans would approximate to 27 mg/kg, whereas the dose in this hypothetical tolerable dose situation would be approximately 5.4 mg/kg SBECD to deliver 0.9 mg/kg Allo.

The complexation ratio of the combination of the excipient (SBECD) and the neurosteroid (Allo) has limitations based on solubility properties (**Figure 1**). The complexation ratio of Allo with cyclodextrins such as SBECD is a major determinant of release of Allo into the blood and brain (**Figure 2**). To develop formulations with clinical utility, we tested multiple Allo/SBECD complexation ratios on behavior in adult rats (Irwin et al., 2013). The optimal Allo:SBECD formulation (molar ratio of 5.89) was fully soluble and bioavailable as indicated by rapidly induced and prolonged sedation at the maximally tolerated dose for sedation 8 mg/kg subcutaneous in rats. When the ratio was increased (molar ratio of 23.56), the rate of Allo release into the brain was reduced as indicated by a lack of sedation (**Figure 2B**). Likewise, when the ratio was decreased (molar ratio of 1.47) relative to optimal, the rate of Allo release into the brain was also reduced (**Figure 2B**). The volume of soluble Allo administered in the saturated suspension formulation is limited when dosing 8 mg/kg to rats and results in mild sedative effects likely due to the relatively small fully soluble fraction giving the suspension formulation dual properties. Based on these data, it was postulated that a small but fully soluble fraction was rapidly delivered to the brain at a low dose followed by a slowly absorbed suspension fraction that does not possess the release rate required for sedation at the 8 mg/kg dose level (Irwin et al., 2013).

Efforts to synthesize water-soluble analogs of neurosteroids, including Allo and progesterone have been made with the goal to maintain structure-activity relationships (MacNevin et al., 2009). Excipients with prior regulatory approval are most desirable for formulations since they have undergone extensive toxicology testing and are more likely to gain regulatory acceptance when formulated with new active ingredients required to undergo their own extensive quality and safety assessments.



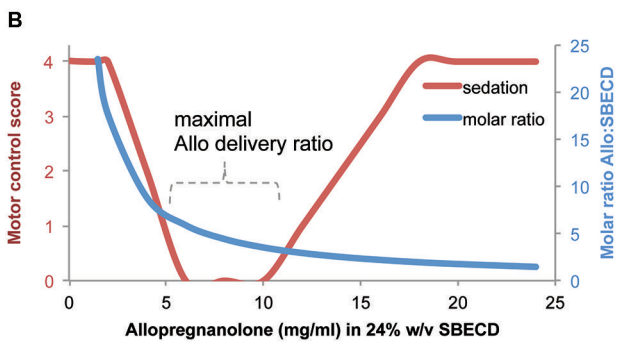


FIGURE 2 | Formulation of allopregnanolone matters. **(A)** Percent solubility of allopregnanolone (Allo) in 24% w/v SBECD. Allo reached an optimal formulation of 6:1 at Allo 6 mg/ml in 24% SBECD. Solubility began to decline, observed by precipitation, when Allo concentration was between 8–10 mg/ml coinciding with a molar ratio of between 4–5 SBECD molecules per Allo molecule in water at room temperature without pH adjustment. **(B)** Motor control/sedation at 25 min after subcutaneous Allo 8 mg/kg to male rats measured by balance beam task on a five point scale where 4 = reaches platform; 3 = takes steps; 2 = all paws on top; 1 = clasp; 0 = fall off beam (Irwin et al., 2013). Molar ratio was plotted to illustrate relationship between Allo-induced loss of motor control and SBECD:Allo complexation ratio. Via subcutaneous injection, soluble formulations with SBECD:Allo between 7:1 and 3:1 maximally delivered Allo to systemic circulation resulting in rapid brain uptake observed by altered motor control. The cyclodextrin vehicle SBECD does not cross the blood brain barrier but facilitates Allo release to steroid carrier proteins in blood. The motor control/sedation test was used as a safety biomarker of maximally tolerable Allo target engagement in brain.

DOSE AND ROUTE OF ADMINISTRATION MATTER

For therapeutic use of Allo, it is imperative to determine the optimal dose, formulation, and dosing regimen (Brinton, 2013). The dose of Allo matters within the context of the targeted biological system. Safety and efficacy must be balanced to avoid unnecessary overexposure and remain consistent with a balanced neuroendocrine system (**Figure 3**). For most efficacy outcomes, Allo, like other neurosteroids, has an inverted U-shaped dose response profile—too high or too low of an Allo dose leads to suboptimal responses (Wang et al., 2005). Allo induces neurogenesis through potentiation of $GABA_AR$ chloride channels in neural progenitor cells in a dose-dependent manner: 10, 100, and 250 nM doses were efficacious whereas neurogenic efficacy diminished at higher doses (Wang et al., 2005; Wang and Brinton, 2008).

In vivo, we have shown that both dose and route matter to determine the therapeutic range and tolerability of Allo (Irwin et al., 2011; Irwin and Brinton, 2014). In our previous *in vivo* studies, subcutaneous Allo was administered in the range of 1–20 mg/kg to mice (Wang et al., 2010). The dose of Allo 10 mg/kg via subcutaneous route was selected for subsequent studies based on hippocampal BrdU incorporation as an indicator of neurogenesis (Wang et al., 2010). Subcutaneous Allo injections were also shown to be effective in a mouse model of Niemann-Pick Type C (NPC; Griffin et al., 2004) and in addition to increased myelin formation and decreased demyelination, the efficacy of Allo was partly due to a reduction of oxidative stress (Zampieri et al., 2009).

For human translation, subcutaneous or intramuscular injections are advantageous due to ease of administration, patient compliance, and tolerability. Allo pharmacokinetics and pharmacodynamics were determined by a combination of brain and blood levels associated with degree of sedation and amount of hippocampal neurogenesis (Irwin et al., 2013). Our recent studies found that sedation following subcutaneously administered Allo in the rat was more variable than the intramuscularly administered response (Irwin et al., 2013). Our analyses indicated that an intramuscular dose was approximately twice as potent at inducing sedation as subcutaneous delivered in a rapid release formulation (Irwin et al., 2013). The no-observable-adverse-effect or subsedative dose level was finely tuned by conducting a series of rat sedation studies within the dose range of our mouse efficacy studies. Subsedative doses were determined to optimally increase markers of neurogenesis including significant increases in BrdU labeled nuclei in the hippocampus (Irwin et al., 2013). Pharmacokinetic studies often begin with the intravenous route to provide maximal bioavailability and inform further studies by alternative routes. An intravenous Allo no-observable-adverse-effect dose of 0.5 mg/kg in mice or 0.2 mg/kg to rats is predicted by allometric species conversion calculations to be equivalent to a human intravenous dose of approximately 0.42 mg/kg or ∼3 mg for a 70 kg adult. Allo has been tested in preclinical models via multiple routes of administration to establish safe dosage ranges for each route.

When selecting an Allo dose or reviewing the available literature (Irwin et al., 2011; Irwin and Brinton, 2014), it is important to compare and contrast the treatment methods and animal models within each study. Evaluation of therapeutic potential requires a deep understanding of the dose and route of administration that will influence pharmacokinetic and pharmacodynamic outcomes.

TREATMENT REGIMEN MATTERS

ALZHEIMER'S DISEASE

Neurogenesis occurs over the course of hours in the case of transitioning through the cell cycle, to months in the case of full integration into hippocampal circuitry and networks (Brinton, 2013). Activating both regenerative and repair systems to maximize therapeutic benefit in the ageing or degenerating brain requires dual assessment of pathology and proliferation simultaneously.

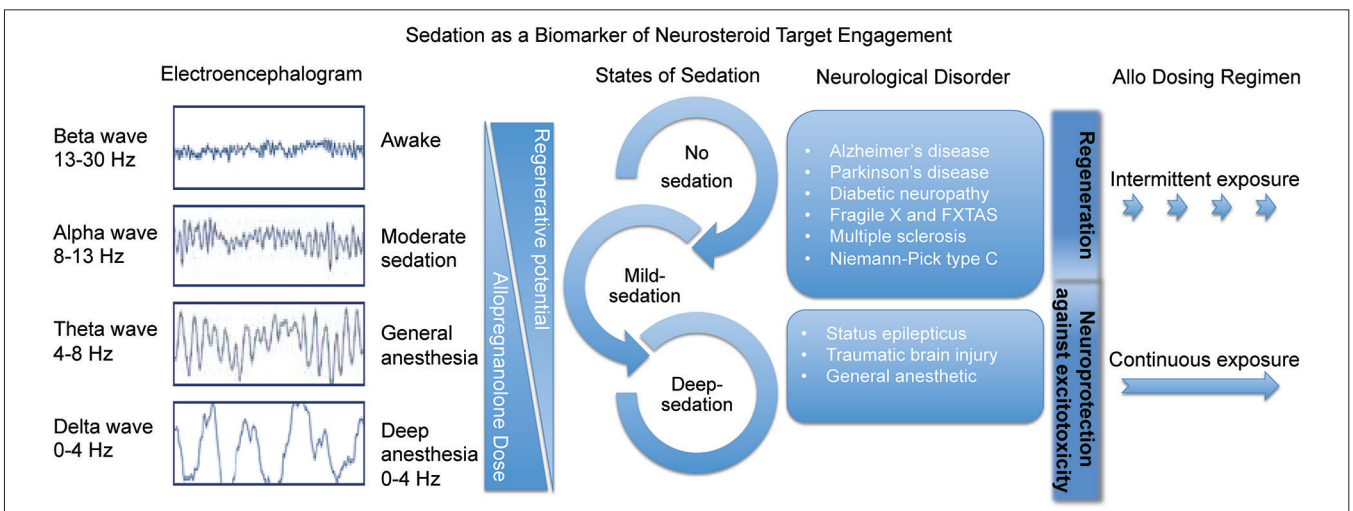


FIGURE 3 | Sedation as a biomarker of neurosteroid target engagement. The sedative component of $GABA_A$ receptor activation in the brain can be used a biomarker outcome of Allo delivery and tolerability. Through activation of the regenerative system in intermittent pulses, Allo is beneficial for many neurological disorders. In status epilepticus and traumatic brain injury it is important to deliver sedative doses of continuous Allo to protect the brain from further excitotoxicity.

Further, multiple dosing regimens should be tested in order to conclude that regimen does matter and to determine an optimal therapeutic regimen. Our studies followed this plan to determine that Allo treatment regimen matters, testing administration of Allo every day, every other day, every week, and every month. Results of these studies indicate that a once per week treatment regimen with a single dose of Allo (10 mg/kg) was optimal to promote neurogenesis while also activating systems that reduced Alzheimer disease pathology (Chen et al., 2011).

We previously demonstrated that Allo induces neural stem cell cycle gene expression (Wang et al., 2005) and induces key regulators of cholesterol homeostasis (Chen et al., 2011) to provide mechanistic plausibility for its therapeutic efficacy to promote neurogenesis and cognitive function while reducing AD pathology following intermittent dosing. Allo promotes neurogenesis (Wang et al., 2005, 2010), recovery of learning and memory function (Wang et al., 2010; Singh et al., 2012), and reduction of AD pathology burden (Chen et al., 2011) in the 3xTgAD mouse model. We have demonstrated that regeneration is achieved with either once per month or once per week regimen of Allo (Chen et al., 2011). In the same mouse model, the reduction of AD pathology was achieved with once per week or every other day regimens (Chen et al., 2011; Irwin et al., 2011; Brinton, 2013). A combination of regeneration and reduction of pathology was achievable with an intermittent, once per week, Allo treatment regimen (**Figure 3**).

Intermittent Allo treatment regimens have shown benefit in a spectrum of preclinical neurodegenerative disease models including NPC (Griffin et al., 2004), diabetic neuropathy (Leonelli et al., 2007), peripheral nerve injury (Meyer et al., 2008), multiple sclerosis (Noorbakhsh et al., 2011), and Parkinson's disease (Adeosun et al., 2012). Frequent or constant exposure to Allo is not better for regenerative processes although more often than once per week improved reduction of β-amyloid-burden disease-modifying effects (Chen et al., 2011). By contrast, a constant infusion treatment regimen over the course of months was anti-regenerative and resulted in adverse outcomes (Bengtsson et al., 2012, 2013; Irwin and Brinton, 2014). Unnecessary constant infusion of Allo is not biologically relevant and therefore should not be a therapeutic option for neurological disorders other than epileptic seizure and traumatic injury (**Figure 3**).

Allo concentrations in blood and brain are stress responsive and serve to restore normal GABAergic and hypothalamic-pituitary-adrenal (HPA) function following stress (Crowley and Girdler, 2014). An elevation of neurosteroids in response to stress is an adaptive anxiolytic response in acute stress situations. However, during chronic stress and depression, a condition collectively termed as allostatic load, system-wide decreases in brain and plasma neurosteroid concentrations occur and overall the response to acute stressors becomes dysfunctional (Genazzani et al., 1998; Bernardi et al., 2000; Dong et al., 2001; di Michele et al., 2003). Thus, a disruption in the biologic stress response system can exacerbate stress response disorders (Crowley and Girdler, 2014). Like the regenerative system, the stress response system requires a recovery period. Because of the temporal constraints of the regenerative system in brain, other regenerative factors are likely to have greater efficacy when administered with intermittent treatment regimens. In contrast, those emergency neurological conditions including traumatic brain injury and status epilepticus (Rogawski et al., 2013; Zolkowska et al., 2013) with intense seizure susceptibility and immediate risk of massive glutamate excitotoxicity and hypoxia require constant infusion regimens to dampen neuroexcitation to protect the central nervous system. We postulate that during the early stages of AD pathology development, administering pulsatile doses of Allo is therapeutically relevant to biological systems including the stress response system and the neuroregenerative system. Safe and tolerable Allo dose exposure serves as a neuroendocrine signal to initiate neuroregeneration.

Based on the therapeutic efficacy of Allo in a preclinical AD mouse model and in normal aged mice, we predict that Allo has potential therapeutic benefit in humans to delay progression in persons with familial early-onset AD and to prevent and delay disease in late onset AD. In these populations, Allo could be an effective therapy to promote the regenerative potential and myelination capacity of the brain to prevent or delay progression of mild cognitive impairment to clinically diagnosed AD. In summary, targeting a unique mechanism of action, Allo promotes the innate regenerative capability of the brain by increasing the number and survival of newly generated neurons. However, for therapeutic efficacy of Allo, it is imperative to determine the appropriate dosing regimen specific to each indication.

SAFETY AND TOLERABILITY MATTER

Earlier we reviewed the existing preclinical and clinical safety data in support of Allo therapeutic development (Irwin et al., 2011; Irwin and Brinton, 2014). Allo is a blood brain barrier penetrant molecule with previous safety data in humans (Timby et al., 2006; van Broekhoven et al., 2007; Grant et al., 2008; Kask et al., 2008, 2009). A cumulative dose of 0.9 mg/kg or approximately 6 mg for a 70 kg human, was administered to 9 men and 9 women with mean ages of 24.6 and 21.8 years respectively. Allo was generally well-tolerated with peak blood levels of 100 nM for women and 150 nM for men (van Broekhoven et al., 2007). Self-reported sedation and drowsiness were followed by and recovery followed the metabolic half-life of circulating Allo which was eliminated within hours (van Broekhoven et al., 2007). Physiological exposure to Allo is highest in the third trimester of pregnancy when levels reach 50 ng/ml or 157 nM (Luisi et al., 2000).

In human Phase 1 studies for AD, fully bioavailable intravenous injection of Allo in a dose escalation design will reach a limit with mild sedation to establish the maximally tolerated dose following intravenous administration. Currently, the upper physiological Allo blood concentration during the third trimester of pregnancy sets the safe exposure boundary until chronic toxicology studies conducted under Good Laboratory Practice (GLP) standards in rodent and non-rodent species are completed. Regulatory agencies require extensive and pivotal toxicology studies prior to large clinical trials and Allo drug product approval. These studies are necessary to demonstrate the safety within a specified dosage range and duration of exposure. GLP is a standardized quality system of management controls to ensure that in non-clinical laboratory studies the

integrity of drug products such as Allo are planned, performed, monitored, recorded, reported and archived in a uniform, consistent, reliable, reproducible manner. GLP studies include extensive non-clinical safety tests that include specification of physicochemical properties generated under Good Manufacturing Practices (cGMP) and acute dose-range finding studies to chronic toxicity tests in rodents and non-rodent species to meet or exceed the duration of the clinical trial (Steinmetz and Spack, 2009).

In the US, all preclinical safety studies that contain investigational active pharmaceutical ingredients should seek regulatory guidance and oversight by the FDA. Regulatory agencies of many countries follow the International Conference on Harmonization guidelines. Pre-meeting with the regulatory agency will greatly improve important communication and a focused track to develop the Investigational New Drug (IND) application. IND approval in the US, or its equivalent application review process in other countries, must be obtained prior to initiation of standard clinical trials to establish safety and tolerability (Phase I) and efficacy (Phase II). Application for compassionate use IND requirements may be abbreviated for certain life threatening disease conditions outside of clinical trials that require emergency care decisions for expanded use discussed between physicians and regulatory agencies.

Safety reporting rules and timelines must also be maintained and monitored throughout the trial. In cases where existing preclinical toxicology and human exposure data are available, or highest physiological exposure has been studied, as is the case with Allo (Luisi et al., 2000), regulatory agencies may consider the active pharmaceutical ingredient to be sufficiently safe at a specified dosage range to move forward with safety and efficacy studies in humans. The entire process of drug development including time investment and funding must be kept in mind when planning viable therapeutic strategies for Allo. Along the Allo drug development timeline, leverage points are gained such as completion of chronic toxicology studies and should be shared when permissible to accelerate expanded access for neurological diseases similar dose, route, and treatment regimens overlap.

Toxicology studies define the safe exposure limits in animals and the outcomes are then extrapolated to predict maximally safe and tolerable exposure limits for initial human trials. Disease-modifying claims are stringently reviewed and may require additional safety studies beyond the standard toxicology battery. For example, recent AD beta-amyloid modifying therapies have shown in clinical studies to increase the risk for vascular edema, detected by magnetic resonance imaging (Salloway et al., 2009; Sperling et al., 2011b, 2012). Increased risk for microbleeds prompted regulatory agencies to require new safety measures for candidate AD drugs in pre-clinical stages. Drug candidates that claim to modify amyloidogenic mechanisms may require additional preclinical assessment of the associated risk for cerebral microhemorrhages in an appropriate animal model. Currently several AD mouse models are considered appropriate however these studies are costly in terms of time and money. Microhemorrhage risk assessment studies require cohorts of ∼2 year old transgenic AD mice (Pfeifer et al., 2002; Racke et al., 2005; Demattos et al., 2012).

At the stage of dose range finding in rodents and non-rodents, tolerability predictions can be made ranging from the no-observed-adverse-effect level up to the maximally tolerated dose. For neurological disorders, a well-known biomarker of Allo target engagement and tolerability is sedation (Damianisch et al., 2001). Inhibition of the tuberomammillary nucleus has a key role in the sedative or sleep-inducing response to anesthetics that act on the $GABA_AR$ (Nelson et al., 2002). The sedation response to Allo can be objectively measured by detection of saccadic eye movement or brain activity via electroencephalogram (**Figure 3**; van Broekhoven et al., 2007) as was done with benzodiazepine drug development (Van Steveninck et al., 1993). Sedation level due to neurosteroids can also be subjectively determined by clinical observation and by visual analog scales (van Broekhoven et al., 2007). Typically, a combination of objective and subjective measures of sedation are used to assess tolerability. Allo induces dose-related sleep changes including a reduced sleep onset latency and increased pre-rapid-eye-movement sleep (Lancel, 1999). Allo has a safer tolerance profile than most $GABA_AR$ agonists including benzodiazepine hypnotics (Damianisch et al., 2001).

A neural network comprised of at least three discrete brain regions promotes sedation non-rapid eye movement sleep. GABAergic neurons of which Allo targets are in the ventrolateral preoptic nucleus are under tonic inhibition from noradrenergic neurons of the locus coeruleus. Inhibition of locus coeruleus neurons results in activation of the ventrolateral preoptic nucleus to induce sedation. GABAergic ventrolateral preoptic nucleus neurons of which Allo acts, innervate the ipsilateral tuberomammillary nucleus, a posterior hypothalamic cell group important in promoting arousal. The tuberomammillary nucleus, located on the ventrolateral edge of the posterior hypothalamus, contains neurons that co-express histamine and the inhibitory neurotransmitter GABA, and which project to the cerebral cortex, thalamus, and basal forebrain (Haas and Panula, 2003). These arousal-promoting, histaminergic tuberomammillary nucleus neurons are wake-active and are inhibited by the release of GABA and galanin by ventrolateral preoptic nucleus neurons. In short, the activated ventrolateral preoptic nucleus releases GABA to the $GABA_AR$-containing sites of the tuberomammillary nucleus thus inhibiting release of arousal-promoting histamine into the cortex and forebrain to induce sedation. Through a wake-sleep neuronal network, at suprathreshold doses Allo allosterically potentiates GABA's apparent affinity for the $GABA_AR$ to increase the chloride current of histaminergic neurons of the tuberomammillary nucleus causes sedation. The sedative response is not the primary regenerative target and sedation is less tolerable for treatment of chronic disease. A subsedative dose of Allo that retains activity at Allo-sensitive neural progenitor cells is optimal for regenerative responses and therapeutic development (**Figure 3**).

In a human clinical trial, a sedation-inducing dose of Allo briefly impaired episodic memory 10 min after the end of Allo intravenous infusion when peak blood levels were highest (Kask et al., 2008). Not surprisingly, a $GABA_AR$ allosteric agonist induced temporary memory impairment in a way similar to benzodiazepines. Chronic treatment paradigms that mimic stress

conditions have also been shown to inhibit memory (Turkmen et al., 2006). Allo administered twice daily at high doses to male rats for several consecutive days decreased performance on the Morris water maze, escape latency, path length and thigmotaxis (Turkmen et al., 2006).

Collectively, the data indicate that the sedative properties of Allo are dose dependent and duration of exposure dependent. Allo administered by multiple routes of administration exhibits an acceptable margin of safety.

REGENERATIVE POTENTIAL OF THE HUMAN BRAIN MATTERS

Most studies in AD mouse models, have reported decreased neurogenesis primarily in the hippocampal SGZ of the dentate gyrus and the SVZ lateral ventricles with association with cortical regions including the rostral migratory stream (Lazarov and Marr, 2010). There is strong evidence in AD animal models including the 3xTgAD mouse that demonstrate reduced neurogenesis with degree of AD pathology (Wang et al., 2007, 2010; Rodriguez et al., 2008, 2009; Chen et al., 2011; Singh et al., 2012). Unexpectedly, in post-mortem brain sections from AD victims, doublecortin, a microtubule-associated protein expressed by neuronal precursors and immature neurons, was increased relative to control brain sections (Jin et al., 2004). Neural progenitors have been isolated *in vitro* from post-mortem 11-week post-natal and adult human brain demonstrating that neural progenitors are present throughout life (Palmer et al., 2001). Further studies are required to determine whether the doublecortin immunostaining findings were reproducible with other antibodies or preferably with BrdU or ^{14}C labels. It has been demonstrated that in humans without known neurological disorders, hippocampal neurogenesis occurs throughout adulthood and tapers modestly with advanced age (Spalding et al., 2013).

Humans exhibit substantial hippocampal neurogenesis within the SGZ (Spalding et al., 2013) and striatal neurogenesis associated with the SVZ lateral ventricles (Ernst et al., 2014). Humans, rather uniquely for mammals, do not demonstrate olfactory bulb neurogenesis, as the neuroblasts originating in the neurogenic niche of the lateral ventricle wall do not migrate to the olfactory bulb. Carbon-dated DNA within adult hippocampal neurons revealed that a substantial fraction of neurons were born during adulthood (Spalding et al., 2013; Ernst et al., 2014). Each year approximately 1.75% of the neurons turned over within the self-renewing fraction with only a modest decline during aging. A best-fit scenario model predicted that approximately 35% of the hippocampal cells were cycling corresponding to slightly less than the proportion that constitute the entire dentate gyrus region. From these studies it was estimated that the hippocampal dentate gyrus of human brain produces around 700 new neurons per day. Enough neurons could be replaced in the hippocampus to theoretically regenerate the entire hippocampal neurogenic region over the lifespan suggesting the importance of neurogenesis. Compared to rodents, humans may rely on neurogenesis even more during the aging process (Spalding et al., 2013). In healthy aging, the decline of hippocampal neurogenesis is less than the rate of decline when adjusted for lifespan and compared with rodent models.

Following results demonstrating hippocampal neurogenesis throughout the lifespan, Frisen and colleagues utilized the same ^{14}C dating method to reveal abundant neuroblasts in the human striatum adjacent to the lateral ventricle wall and revealed a constant turnover/generation of striatal interneurons with and annual turnover rate of 2.7% within the renewing fraction (Ernst et al., 2014). This recent finding highlighted a major difference between human neurogenic niches vs. animal models. Rodents for example have well developed rostral migratory streams that send newborn SVZ neurons towards the olfactory bulb. Human brains were found to shunt the equivalent neurogenic niche cells to the striatum. In the brains of Huntington's disease patients, a disease related to the degeneration of striatal neurons (Zuccato et al., 2010; Walker et al., 2011), the patient cohort in the advanced state of the disease lacked these post-natally generated neurons (Ernst et al., 2014).

Once new neurons develop, the cells require approximately 2 months to mature both morphologically and physiologically, suggesting to us that an intermittent treatment regimen (**Figure 3**) would be required to stimulate regeneration in this neurological disorder. The timing of therapeutic intervention must accommodate the development period from which newborn cells acquire GABAergic and glutamatergic inputs and receptors. During this development period the young neurons are highly excitable with increased synaptic dynamics compared to mature neurons (Gage and Temple, 2013). This time course of regeneration, migration, differentiation, creates a temporal map that is coincident with Allo-induced proliferation, increased learning capacity and increased memory function (Brinton, 2013).

GENDER MATTERS

Neurological diseases that have varying prevalence, progression and severity between men and women include AD, Parkinson's disease, attention deficit/hyperactivity disorders, and schizophrenia (Gillies and Mcarthur, 2010). Sex differences have been noted in human safety studies of Allo, where the same intravenous dose in men and women resulted in maximum blood levels that were higher in men 150 nmol/L vs. 100 nmol/L in women, although volume of distribution, elimination half-life, and the area under the curve (AUC) adjusted for body weight did not differ (van Broekhoven et al., 2007).

Clinical differences between females and males may result from differences in brain morphology, neurochemistry, and functional outcomes. It is hypothesized that there is a critical hormone treatment window in the post-menopausal brain. The importance of regimen is again demonstrated when it was shown that continuous vs. cyclic progesterone administration resulted in disparate gene expression profiles in the brain (Zhao et al., 2012). Changes in neural gene expression profiles after alternative progesterone therapies highlights the importance of mimicking physiological profiles of neurosteroid exposure in order to maintain and improve neurological health and function (Zhao et al., 2012). Changes in pathophysiology due to gender may be important in evaluating both efficacy and side-effect profiles during therapeutic development (**Figure 4**). Early in 2014, a drug response gender difference prompted the FDA to issue a safety announcement and reduce by half the zolpidem (Ambien) dosage for women

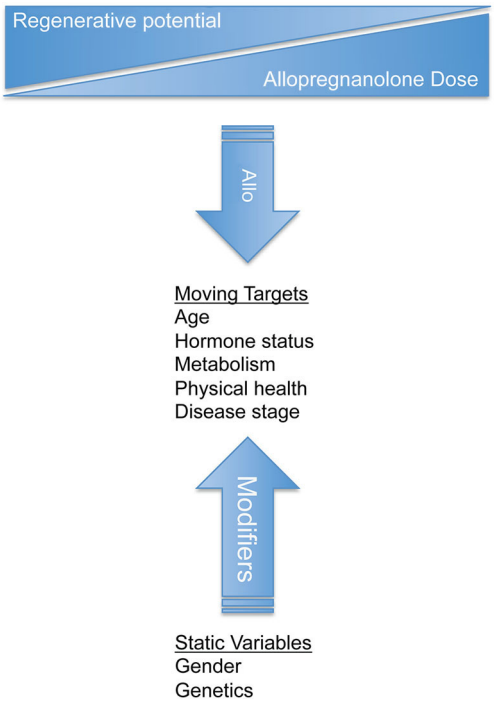


FIGURE 4 | Design of clinical trials for allopregnanolone should consider both static variables and moving targets to treat neurological disorders. Gender and genetics are static variables that influence disease and response to treatment. Static variables impact the moving targets such as age, hormone status, metabolism, health, and disease progression. Successful clinical therapeutic strategies should be tailored for each disease state and should be monitored as the disease and treatment progresses. Dynamic biological processes require an understanding of disease system pathogenesis to select and modify the appropriate treatment regimen.

compared to men.[1] As a result, zolpidem is now the only prescription drug with a different dose for men and women. Zolpidem, like Allo, is a $GABA_A$ agonist with sedative hypnotic actions in the wake-sleep neurocircuitry. Women metabolize and eliminate zolpidem slower than men, making them more susceptible to next morning impairment side effects.

Preclinically, a difference between sexes in Allo response was observed in our studies whereby male rats administered bolus injections of Allo had increased sensitivity to Allo's sedative effects, with greater time and levels of sedation compared to age-matched female rats (Irwin et al., 2013). Allo may exert its effects on males and females differently, i.e., other than through binding postsynaptic $GABA_A$Rs. The female brain may have a greater sensitivity to Allo's potentiation of GABA neurotransmission. In a global ischemia mouse model, females were neuroprotected at a dose of Allo four times lower than the required dose to protect males (Kelley et al., 2011). These data are surprising in light of evidence indicating that male brains have a greater number of $GABA_A$R binding sites relative to female brains (Juptner and Hiemke, 1990).

[1]http://www.fda.gov/drugs/drugsafety/ucm334033.htm

Cyclical changes of steroids during the estrous cycle can lead to changes in $GABA_A$Rs and during late diestrus new receptors have α_4, β_1 and δ $GABA_A$ receptor subunit expression (Lovick, 2008). As β1 subunit containing receptors are extrasynaptic and carry tonic currents, upregulation should lead to a decrease in GABAergic inhibition (Lovick, 2008). Studies in male rats have shown that alterations in Allo plasma levels can, within minutes, modulate the amount of GABAergic inhibition in the periaqueductal gray matter. Function changes resulting from altered intrinsic excitability levels may affect therapeutic responsiveness (Lovick, 2008).

Overall, there is a recognized need for a better understanding of sex differences related to neurotherapeutic response and the clinical relevance of such differences to inform and improve drug development.

DISEASE TARGET AND THERAPEUTIC GOALS MATTER

ALZHEIMER'S DISEASE

Disease target

Regenerative system of the brain, cognition neural circuitry, and etiology of pathology.

Therapeutic response to Allo

Increased hippocampal neurogenesis, increase neural progenitor cell survival, and reverse learning and memory deficits, decreased microglial activation, and decreased amyloid-beta pathology (Brinton, 1994, 2013; Wang et al., 2010; Irwin et al., 2011; Singh et al., 2012; Lo et al., 2014).

Targeted mechanism of action

Potentiation of GABA at $GABA_A$R to depolarize neural stem cells; regulation of LXR and PXR in cholesterol homeostasis.

Dose and treatment regimen

Intermittent exposure in mice: 10 mg/kg once weekly for 6 months (Chen et al., 2011); 1 mg/kg SC single dose, 10 mg/kg SC single dose, 20 mg/kg single dose (Wang et al., 2010); 10 mg/kg SC 3 times per week, for 3 months (Chen et al., 2011); 10 mg/kg once per month, single dose (Singh et al., 2012).

Allo and other trophic factors are decreased in blood and brain of AD patients compared to age-matched controls (Weill-Engerer et al., 2002; Marx et al., 2006; Naylor et al., 2010). Early AD is characterized by loss of episodic and semantic memory (Perry et al., 2000) and signifies hippocampal dysfunction. Diagnostic imaging studies using volumetric MRI revealed a decreased hippocampal volume due to neurodegeneration of gray matter in people diagnosed with amnestic mild cognitive impairment that will progress to AD (Whitwell et al., 2007). In AD, restoration of the dysfunctional neurogenic niche with intermittent pulses of Allo may regain neurological order while avoiding overstimulation.

We previously demonstrated a correlation between Allo-induced neural progenitor cell survival and improved memory function in the triple transgenic mouse model of AD (Brinton, 1994, 2013; Wang et al., 2010; Irwin et al., 2011; Singh et al., 2012). Consistent with the human AD brain neurosteroid profile (Weill-Engerer et al., 2002; Marx et al., 2006; Naylor et al.,

2010), basal concentration of Allo in blood plasma of wild-type mice of was significantly lower than in cortex indicating higher brain accumulation of Allo. Higher level of brain Allo could be attributed to locally synthesized Allo in specific brain regions required for synaptic function. We also found that 3xTgAD mice had lower basal levels of Allo in the cerebral cortex (3xTgAD, 6.49 ± 2.02 ng/g vs. nonTg, 10.36 ± 1.43 ng/g), suggesting that there was either impairment of upstream Allo enzymatic production or accelerated Allo metabolism in 3xTgAD mice brain (Wang et al., 2010).

Within the SGZ and SVZ in male and female 3xTgAD mice a decline in neurogenesis is correlated with age-related AD-like pathology progression (Brinton and Wang, 2006; Rodriguez et al., 2008, 2009; Wang et al., 2010). Our studies have demonstrated that Allo promoted neurogenesis in the hippocampal SGZ to reverse learning and memory deficits (Wang et al., 2010). A study conducted in 3xTgAD mice subjected to an associative learning and memory task were analyzed and found increased neural progenitor cell survival of an intermittent treatment regimen with subcutaneous Allo 10 mg/kg, 3 weeks after a single Allo treatment and post-behavioral analyses. After 3 weeks, Allo-treatment vs. vehicle control demonstrated that surviving BrdU-labeled cells were located deep within the granular cell layer, consistent with the migration pattern of newly formed cells from the SGZ to the granule cell layer (Wang et al., 2010). In an *in vivo* dose-response study, 10mg/kg SC Allo exerted the greatest neurogenic efficacy and was the dose chosen for chronic preclinical efficacy assessment (Wang et al., 2010).

To further assess the preclinical efficacy of Allo for AD, our group has conducted studies of long-term intermittent exposure to Allo. These studies with Allo were also initiated with 3-months of age mice, prior to overt intraneuronal Aβ. In addition to neurogenic efficacy, these long-term studies were allowed us to determine the disease modifying effects afforded by the therapeutic regimen. Our group tested three treatment regimens—once per month, once per week, and every other day (Chen et al., 2011). Overall, we found that the optimal treatment paradigm with subcutaneous Allo, administered once-per-week for 6-months was maximally efficacious for both neurogenic and anti-Aβ endpoints (Importance of regimen discussed in greater detail above). Additionally, these studies demonstrated that together with the dosing frequency, the magnitude of pathology at the start of treatment intervention is critical to the window of therapeutic opportunity for Allo. Administration of Allo prior to and during the early stages of AD pathology significantly increased the regenerative response in brain while additionally reducing burden of pathology in an AD mouse model (Chen et al., 2011). In contrast, Allo treatment initiated at the point of Aβ plaque generation was not efficacious indicating that Allo targets regenerative and pathology reducing mechanisms present during the early to mid stages of the disease (Chen et al., 2011). After intraneuronal Aβ is extracellularly distributed, Allo's efficacy becomes markedly diminished. Based on a collective body of preclinical data evaluating dose and route (Irwin et al., 2011; Brinton, 2013; Irwin and Brinton, 2014), we have selected the once per week regimen to move forward to FDA-compliant chronic toxicology studies and early phase clinical trials.

PARKINSON'S DISEASE

Disease target

Regenerate dopaminergic system of the brain and etiology of pathology.

Therapeutic response to Allo

Neurogenesis in the substantia nigra, functional improvement in motor control (Adeosun et al., 2012), modulates dopamine release (Rouge-Pont et al., 2002).

Dose and treatment regimen

Intermittent exposure: 10 mg/kg once a week for 2 weeks in mice (Adeosun et al., 2012).

Clinical studies investigating the levels of endogenous Allo in PD patients demonstrated that in males, the levels of endogenous Allo were decreased both peripherally and in the CNS (di Michele et al., 2003, 2013). While Allo concentrations in specific human brain regions affected by PD has not been studied, in healthy women, the highest Allo levels were observed in the substantia nigra (SN) and basal hypothalamus (Bixo et al., 1997). The therapeutic goals for exogenous Allo are the modulation of dopamine release, restoration of neuroprotection, modulation of basal GABAergic tone, and neuroregeneration (Luchetti et al., 2010, 2011; Adeosun et al., 2012; di Michele et al., 2013). As in AD, the dose and therapeutic regimen must be carefully considered, as low doses Allo increases dopamine release, but at high doses decreases release (Rouge-Pont et al., 2002). In preclinical *in vivo* studies, once weekly Allo administration has demonstrated functional improvement (Adeosun et al., 2012).

MULTIPLE SCLEROSIS

Disease target

White matter regeneration in the peripheral and central nervous systems, reduce inflammation, etiological mechanism of disease, and prevention of relapse.

Therapeutic response to Allo

Oligogenesis (Garay et al., 2012; Schumacher et al., 2014), neurogenesis (Ghoumari et al., 2003; Gago et al., 2004), cytoprotective, promyelination (Melcangi et al., 1999; Noorbakhsh et al., 2011), anti-inflammatory (Noorbakhsh et al., 2011), reduced disease severity (Noorbakhsh et al., 2011), innate immune function modulator (Noorbakhsh et al., 2014).

Dose and treatment regimen

Continuous exposure: daily IP 10 mg/kg for 28 days in mice (Melcangi et al., 1999). Intermittent paradigm: 1 mg SC every 4 days for 1 month in rats (Noorbakhsh et al., 2011).

The peripheral nervous system synthesizes steroid hormones, including progesterone, and possesses the enzymes required to convert these molecules to neuroactive metabolites, including Allo (Melcangi et al., 1999). In cases where the endogenous levels are insufficient, treatment with exogenous Allo could produce therapeutic benefit (Melcangi et al., 1999). Analyses of endogenous Allo showed that in MS patients, the Allo concentration in white matter was significantly decreased, demonstrating the potential for Allo as a potential therapeutic for this as yet unmet,

clinical need (Noorbakhsh et al., 2011). A number of preclinical *in vivo* and *in vitro* studies have demonstrated the potential for Allo as a MS therapeutic through its anti-inflammatory effects, neurogenesis, and induction of myelin production (Melcangi et al., 1999; Ghoumari et al., 2003; Gago et al., 2004; Noorbakhsh et al., 2011). Common themes, seen in Allo studies for the disease states presented above, also apply to its utilization for MS. Efficacy is limited in late-stage disease, indicating that the system must retain neurogenic potential for therapeutic effect (Melcangi et al., 1999). Allo dosage and treatment regimen have been shown to be critical factors in study design. Positive effects of treatment were limited in *in vitro* studies when length of treatment was less than 24 h (Melcangi et al., 1999). As in AD, more Allo is not beneficial, and *in vitro* dose-response curve studies have demonstrated that the positive proliferative effects of Allo peak at 10 nM doses, and decrease with increasing doses (Gago et al., 2004).

NIEMANN-PICK TYPE C

Disease target

Cholesterol trafficking and clearance system of the brain and etiology of disease.

Therapeutic response to Allo

Increased survival, decreased rate of motor control decline, increased neuronal survival, decreased cholesterol accumulation (Griffin et al., 2004), and reduced oxidative stress (Zampieri et al., 2009).

Dose and treatment regimen

Single dose exposure: 25 mg/kg SC, single injection in mice (Griffin et al., 2004). Continuous exposure: 0.5–2 mg in drinking water (ascending dose; lifetime treatment); 250 mg/90 days SC implant (lifetime treatment) in mice (Griffin et al., 2004).

NPC is a fatal, neurodegenerative, lysosomal storage disorder that affects cholesterol metabolism due to autosomal recessive mutations in the NPC1 and LPC2 loci. Allo has been studied for its therapeutic effect on NPC, which is characterized by defective trafficking of intracellular cholesterol and lysosomal accumulation of unesterified cholesterol gangliosides and other lipids leading to neurological deterioration and degenerating motor and cognitive function. Characterization of the NPC mouse model suggested dysfunctional steroidogenesis from cholesterol, giving credence to the indication for Allo (Griffin et al., 2004). *In vivo* studies have demonstrated that Allo, administered using continuous exposure paradigms results in improved outcomes, including survival and a decreased rate of decline of locomotion and motor coordination (Griffin et al., 2004). In addition to these functional improvements, histological examination demonstrated increased cerebellar neuron survival and decreased accumulation of cortical gangliosides (Griffin et al., 2004). Interestingly, in this disease state, continuous Allo administration via treated water resulted in superior survival outcomes vs. a single Allo SC dose. As has been shown in other disease states treated with Allo, timing of the single-dose injection is vital. Outcomes are best when Allo is administered early postnatally as efficacy diminishes and is quickly lost with advancing disease state (Griffin et al., 2004). *In vitro* studies have demonstrated a significant antioxidant function of Allo, but this remains to be translated to *in vivo* preclinical models (Zampieri et al., 2009).

FXTAS AND FRAGILE X SYNDROME

Disease target

Regenerative system of the brain and etiology of disease.

Therapeutic response to Allo

Improved functional electrical impairments *in vitro* culture of neurons from a mouse model of the disease (Cao et al., 2012).

Dose and treatment regimen

0.01–1 μM (*in vitro*) (Cao et al., 2012).

Preclinical studies demonstrate that Allo is efficacious in neural cells isolated from a fragile X-associated tremor/ataxia syndrome (FXTAS) mouse model (Cao et al., 2012). This mouse model has defects in neuronal morphology and migration. FXTAS related defects occur in basal electrical activity exhibited by permutation CGG repeat expansion-carrying neurons associated with a gain-of-function in type-I mGluRs and/or a loss-of-function in $GABA_AR$ signaling. Allo acutely improved the functional impairments as measured by electrical burst firing, in this preclinical model.

Furthermore, an Allo analog, ganaxolone, formulated in an oral suspension, given in three divided doses (ClinicalTrials.gov identifier: NCT01725152) is currently in Phase 2 proof-of-concept study in children with fragile X syndrome. Fragile X syndrome is the most common inherited form of cognitive impairment results from a single-gene disorder associated with autism. The aim of the study is to assess the safety, tolerability and efficacy of ganaxolone for treatment of anxiety and attention deficits in subjects with fragile X syndrome. The clinical trial is designed to test ganaxolone treatment compared to placebo on measures of anxiety and attention via several neuropsychological and psychometric tests.

DIABETIC NEUROPATHY

Disease target

Regenerate peripheral nerve, reduce pain, increase conductivity of peripheral nerves, and etiological mechanism of disease.

Therapeutic response to Allo

Decreased expression of apoptosis mediators, increased nociception threshold (Afrazi et al., 2014), increased nerve conduction velocity, and restored intra-epidermal nerve fiber density (Leonelli et al., 2007).

Dose and treatment regimen

Intermittent exposure in rats: 5 mg or 20 mg/kg, gastric lavage, for 8 weeks (Afrazi et al., 2014); 3.3 mg/kg SC every 4 days for 8 doses; 3.3 mg/kg SC every 2 days for 16 doses (Leonelli et al., 2007).

Diabetic neuropathy is a unifying term for a heterogenous assembly of symptoms resulting from long-term glucose instability. Neuronal damage, dysfunction and apoptosis can present in patients in a myriad of ways including spontaneous pain, hypoesthesia, allodynia and hyperalegsia. Neurosteroid levels have been

shown to fall in neuropathic pain conditions (Patte-Mensah et al., 2005; Saredi et al., 2005), validating the assessment of Allo for this indication. *In vivo* studies have demonstrated the effect of Allo on improving nociceptive threshold and decreased expression of apoptosis mediators (Afrazi et al., 2014). The theme of an optimal dosage regimen is continued here; Allo demonstrated superior restoration of nerve conduction velocity and intra-epidermal nerve fiber density when administered every other day for 30 days vs. every 4 days for 28 days (Leonelli et al., 2007). As in AD and MS, a higher Allo dose is not beneficial, and an *in vitro* dose-response curve study have demonstrated that the positive cell viability effects of Allo in glucose induced cell toxicity peak at the 2.5 μM dose, and decrease with increasing doses (Afrazi et al., 2014).

STATUS EPILEPTICUS

Disease target

Spontaneous seizure activity of the brain.

Therapeutic response to Allo

Dampens epileptic seizure activity and reduce neuroexcitotoxicity (Rogawski et al., 2013).

Dose and treatment regimen

Immediate and continuous treatment regimen in 6 Hz seizure model, 1.5 mg/kg Allo IV conferred seizure protection within 1 min after dosing in mice (Rogawski et al., 2013; Zolkowska et al., 2013).

Most seizures are spontaneously terminated within a short period of time because of endogenous inhibitory mechanisms including actions of Allo on $GABA_A$Rs. However, when seizures do not stop spontaneously, this results in status epilepticus, a life-threatening neurological emergency condition. Allo is currently in clinical trials on emergency basis for certain cases of status epilepticus (Rogawski et al., 2013). Previously, ganaxolone was being developed for infantile spasms (Gasior et al., 2000; Kerrigan et al., 2000; Kaminski et al., 2004). Standard treatment for status epilepticus is administration of benzodiazepines but in many cases these quickly become ineffective. $GABA_A$Rs are in a continuous cycle of insertion into the cell membrane and internalization (Goodkin et al., 2005). Internalization of $GABA_A$Rs occurs through clathrin-dependent endocytosis. This process is activated by calcium-phospholipid dependent protein kinase C (Chapell et al., 1998; Filippova et al., 2000) and brain-derived neurotrophic factor (Jovanovic et al., 2004). Internalization of the surface $GABA_A$Rs correlates with a reduced response to GABA, whereas inhibition of internalization results in increased amplitude of synaptic $GABA_A$R currents (Kittler et al., 2000).

The benzodiazepine-binding site within the $GABA_A$R complex is located within the α-subunit interface with the δ-subunit and after synaptic receptor internalization, epilepsy patients often become resistant to therapy. Neurosteroid binding sites are within the α-subunit or α/β interface and are not reliant on the δ-subunit composition. Extrasynaptic $GABA_A$Rs that contain a δ-subunit rather than a γ-subunit are sensitive Allo molecular targets that do not have drug resistance complications and improve treatment of seizures. Recent resurgence of interest in Allo and its analogs has prompted attention for this important disease application to dampen epileptic seizure activity and reduce neuroexcitotoxicity (Rogawski et al., 2013).

TRAUMATIC BRAIN INJURY

Disease target

Glutamate excitotoxicity systems of the brain.

Therapeutic response to Allo

Anti-inflammatory (VanLandingham et al., 2007), neuroprotective (Sayeed et al., 2009), anti-convulsant (Rogawski et al., 2013).

Dose and treatment regimen

Immediate and continuous exposure. Within 8 h after injury, a continuous IV Allo regimen for traumatic brain injury (TBI) is administered during a 4-day treatment period followed by a 1-day dose de-escalation period in humans (ClinicalTrials.gov identifier: NCT01673828) (Rogawski et al., 2013).

The adult brain has been shown to possess regenerative mechanisms after infarct and injury. In rodents, proliferation in the SVZ, migration of new neurons to peri-infarct site, and survival of these new neurons has been demonstrated following stroke and involves the inflammatory cytokines and chemokines systems to aid recovery (He et al., 2004b; Djebaili et al., 2005). It has been hypothesized that angiogenesis leads to functional recovery through its interaction with one or more aspects of tissue repair, including neurogenesis (Carmichael, 2010). There is currently no effective treatment available for TBI victims. TBI may induce coma or a minimally conscious state and may lead to neurobehavioral deficits including cognitive deficits. Long-term consequences of TBI include increased risk for epileptic seizures, PD, and AD (Annegers et al., 1998; Mueller et al., 2009; Hutson et al., 2011; Johnson et al., 2012). A challenge for TBI research is the extent of heterogeneity of these brain injuries and the delayed secondary injuries due to increases in intracranial pressure, hypoxia, and glutamate excitotoxicity (Mueller et al., 2009). A TBI case study of two patients who recovered after years in a state of minimal consciousness suggests that brain regeneration and specific axonal regrowth to improve quality of life is possible even after severe TBI (Voss et al., 2006).

Allo is currently in clinical trials to assess the safety and efficacy of Allo in improving neurobehavioral outcome and reducing mortality in adults with moderate and severe TBI (ClinicalTrials.gov identifier: NCT01673828). Within 8 h after injury, a continuous IV Allo regimen for TBI is administered during a 4-day treatment period followed by a 1-day dose de-escalation period (ClinicalTrials.gov identifier: NCT01673828). This continuous treatment regimen is very different from the regenerative regimen approach taken for Allo in chronic neurodegenerative diseases. This regimen takes advantage of the anticonvulsant mechanisms of Allo action to limit acute brain excitoxicity (**Figure 3**). Allo and its precursor progesterone acutely reduce inflammatory cytokines after brain injury (He et al., 2004a,b; Djebaili et al., 2005). Allo has been shown to upregulate CD55, a cell surface protein that inhibits convertase enzymes to reduce neuroinflammation (VanLandingham et al., 2007). Another possible mechanism of

Allo-induced neuroprotection is a direct inhibition of the mitochondrial permeability transition pore. By this mechanism, Allo was shown to inhibit calcium ion-triggered swelling in functionally intact rat liver and brain mitochondria (Sayeed et al., 2009).

INTEGRATION OF TRANSLATIONAL DETERMINANTS INTO THE CLINICAL TRIAL DESIGN FOR AD AND OTHER DISORDERS MATTERS

Fundamental translational determinants of success are well-defined mechanisms of action, dose-response relationships, and optimal therapeutic regimen. Mechanistically, a key to therapeutic success is an understanding of the intended activated pathways as well as those that are unintentionally activated. This is not always possible but always beneficial. A well-defined understanding of dose-response relationship includes doses that are sub-optimal and which induce toxicity or off-target effects. In the case of Allo, the dose-response relationship indicates that a sedative dose suppresses regeneration and thus establishes the maximally tolerated dose. Further, daily exposure to Allo at a non-sedative dose also suppresses regeneration. The dose and exposure relationships provide critical information for clinical trial design as well as providing key insights into the regenerative system of the brain.

The maximally tolerated dose will depend on the neurological condition, intended mechanism of action, and the therapeutic goals (**Figure 3**). For example, a low dose of Allo with intermittent exposure is optimal for activating regenerative responses. Whereas, TBI and status epilepticus require emergency suppression of glutamate excitotoxicity necessitating a high dose of Allo administered by continuous infusion.

Clinical trial design is inextricably linked to therapeutic success. Recently, the Food and Drug Administration (FDA) released a draft guidance document for developing drugs for early-stage AD trials (Food Drug Administration, Center for Drugs Evaluation Research, 2013) that aimed to improve the design of future AD clinical studies. The FDA guidance emphasized the use of the Clinical Dementia Rating Scale as a primary clinical scale to accelerate development of AD drugs. In addition to these cognitive and functional measures, exploratory outcomes for early stage AD trials can be incorporated into the design as secondary outcomes (Schneider, 2014). Exploratory outcomes are useful to detect responsiveness to new therapies such as correlations between neuroregenerative indicators and improvement in brain activity (Mullard, 2012, 2013; Schneider, 2014).

Adaptive clinical trials are designed to conduct interim analyses that are prospectively planned (Food Drug Administration, Center for Drugs Evaluation Research, 2010). An adaptive design affords the opportunity to modify one or more aspects of the study based on a hypothesis-driven analysis of the data. The benefit of an adaptive design is the opportunity to learn about the impact of the therapeutic agent early in the course of the study and to make course corrections earlier rather than later. These analyses would be hypothesis driven and based on predictive biomarkers of efficacy.

What defines biomarkers that align with stage of disease and are predictive of therapeutic efficacy? Potential biomarkers of efficacy relevant to regeneration could be evident as either decreased rate of degeneration or an increased rate of structural recovery. Thus we reasoned that if Allo was promoting regeneration in the brain, that regeneration could be evident in MRI-based measures of hippocampal volume, diffusion tensor imaging of white matter, and resting default mode network (Brinton, 2013). If structural integrity is related to function, then one would predict a delay in severity of dementia and or recovery of cognitive function. We anticipate, based on the temporal requirements for regeneration in the context of a degenerated brain, that the regeneration of the neural circuitry that underlies the resting default mode network has the greatest probability of being the first imaging biomarker to exhibit change over time.

Clinical trials of potential disease modifying agents require the ability to characterize a well-defined study cohort (Sperling et al., 2011a). The National Institutes of Aging and the Alzheimer's Association published disease-staging criteria that describe the clinical stages of AD (Albert et al., 2011). For clinical development of Allo, initial targeted populations are those with mild cognitive impairment or early AD (Brinton, 2013). These populations were targeted based on preclinical analyses indicating that Allo exerted a regenerative response in transgenic AD mice with burden of pathology relevant to early stage AD. These animals exhibited cognitive and neurogenic deficits that were reversed by Allo (Brinton, 2013). At later stages of the disease, the regenerative capacity was depleted and thus not appropriate for a therapeutic that promotes endogenous regeneration (Brinton, 2013).

Collectively, it is clear that clinical trial testing of Allo across multiple neurodegenerative diseases will require translational research specific to the disease, dosing to the intended target, treatment regimen specific to the respective system, and study population specific to stage of disease.

CONCLUDING REMARKS

The goal of this review was to integrate existing knowledge relevant to translational development of Allo as a therapeutic agent for multiple neurological disorders. There are multiple leverage points across programs of therapeutic development that could significantly accelerate time-to-clinical trial of Allo in each of these conditions (**Figure 5**). Allo activates multiple systems in multiple cell types in multiple anatomical regions with therapeutic implications for multiple diseases. Each neurological disease has specific Allo dose and therapeutic regimen requirements that must be tested preclinically and must be carefully translated to clinical study design. Leverage points—such as preclinical efficacy data, preclinical toxicology data, regulatory knowledge, access to clinical-grade material, and access to clinical data matter to development of expedited timelines to utilize Allo across these disorders (**Figure 5**). For Allo to reach its potential as a therapeutic option, the formulation, dosing regimen, and route of administration are critical determinants of success. Considerations of gender, genetics, age, and progression of disease for both preclinical translational analyses and clinical trials are critical (**Figure 4**).

The therapeutic potential of Allo for multiple neurological diseases is increasingly appreciated. Different etiologies, different courses of disease progression, different mechanisms involved in the disease, will impact the therapeutic efficacy of Allo. In

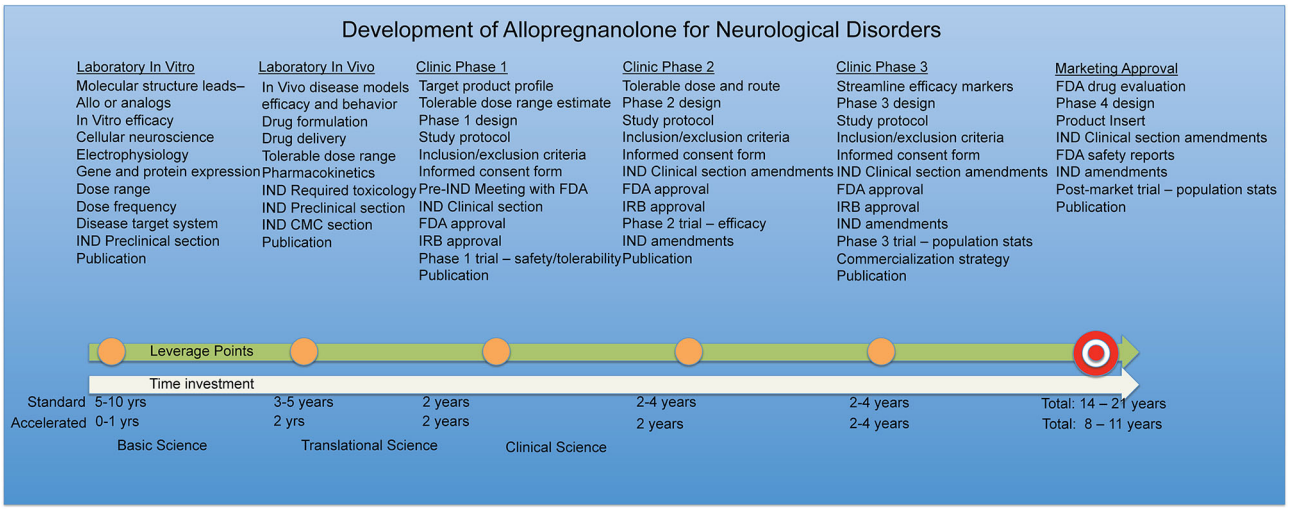


FIGURE 5 | Development of allopregnanolone for neurological disorders. Leverage points along the standard timeline of 12 years and up to 20+ years as drug development proceeds to the marketing approval target. By sharing resources and information, it is plausible to accelerate therapeutic development timelines to 8 years or less, completing clinical trials more rapidly for unmet medical needs.

turn, it is essential that translational research target each of the neurological disorders and take into consideration the similarities and differences in molecular targets and etiology of disease. To suppress neuronal excitability in TBI or epilepsy, it makes sense to inhibit excitability as early as possible following injury. In other cases, maximizing GABAergic suppression of the brain in the absence of glutamate excitotoxicity is unlikely to have therapeutic benefit and could induce harm. Pragmatically, this translates into dosage levels and treatment regimens tailored to the neurological disorder and therapeutic goals in the context of the relevant systems biology.

We have reviewed herein compelling preclinical discovery outcomes that strongly suggest the therapeutic potential of Allo. Translation of Allo to clinical studies requires systematic establishment of the optimal target engagement, dose, treatment regimen, duration of treatment, and safety. What remains is to leverage the discovery science into the path of translation. Fortunately, and remarkably, there are multiple opportunities to leverage existing translational knowledge and to systematically apply that knowledge in disease relevant models to accelerate determination of Allo efficacy across multiple disease states.

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Allopregnanolone and neuroinflammation: a focus on multiple sclerosis

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The progesterone derivative allopregnanolone (ALLO) is one of the most widely studied compounds among neurosteroids. Through interactions with GABA-A receptors expressed by neurons and glial cells, ALLO has been shown to affect diverse aspects of neural cell physiology, including cell proliferation and survival, migration, and gene expression. Recent data point to important roles for ALLO in different neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis (MS). Dysregulation in ALLO biosynthesis pathways has been reported in brain tissue from MS patients as well as in the central nervous system (CNS) tissue derived from MS animal models. Administration of ALLO has been shown to ameliorate neurobehavioral deficits together with neuropathology and inflammation in the CNS of animals with autoimmune demyelination. These findings are in line with previous reports indicating growth- and differentiation-promoting actions of ALLO on neurons and glial cells as well as its neuroprotective effects in the context of other CNS diseases. Nonetheless, these findings have also raised the possibility that ALLO might influence leukocyte biology and associated neuroinflammatory mechanisms independent of its neuroregenerative properties. Herein, we review the current knowledge regarding the role of ALLO in the pathogenesis of MS, and discuss the potential cellular and molecular pathways that might be influenced by ALLO in the context of disease.

Keywords: neurosteroid, allopregnanolone, neuroinflammation, multiple sclerosis, experimental autoimmune encephalomyelitis

INTRODUCTION

Steroids synthesized from precursors in central or peripheral nervous systems have attracted substantial attention in recent years (Mellon and Griffin, 2002; Brinton, 2013). While the blood-brain barrier (BBB) is permissive to steroids produced by gonads and adrenals, the precise neuroanatomical segregation of the locally-synthesized steroids, i.e., neurosteroids, gives them a distinct advantage over peripheral steroids in terms of exerting region-specific effects in the nervous system. In addition to binding to intracellular steroid receptors, some neurosteroids interact with neurotransmitter receptors, thus influencing the biology of select types of neural cells (Belelli and Lambert, 2005). Reduction of locally-synthesized or peripherally-derived progesterone to dehydroprogesterone (DHP) and tetrahydroprogesterone (THP) is one of the widely studied arms of the neurosteroid synthesis pathway. While progesterone and DHP can both bind to intracellular steroid receptors, the predominant THP form, allopregnanolone, can only interact with and signal through cell surface GABA-A receptors.

Multiple sclerosis (MS) is a complex disease of the CNS with both inflammatory and degenerative aspects (Sospedra and Martin, 2005; Trapp and Nave, 2008). Neuropathologically, MS is characterized by leukocyte infiltration of CNS followed by myelin damage, local gliosis, and axonal injury. It is generally believed that the pathogenic process of MS is initiated by a breakdown of immune tolerance to CNS antigens due to genetic or environmental factors, leading to activation and proliferation of neuroantigen-reactive T cells in the peripheral immune system in susceptible individuals (Hemmer et al., 2002). Activated T cells and macrophages then infiltrate the CNS and become reactivated, leading to local microglial activation and intraparenchymal generation of chemokines and inflammatory cytokines. Subsequent waves of lymphocytic and monocytic cell infiltration into the CNS give rise to widespread neuroinflammation with ensuing myelin damage and axonal injury (Sospedra and Martin, 2005). Some evidence gives more weight to innate immune events, compared with adaptive immune processes, in MS neuroinflammation (Tsutsui et al., 2005; Mayo et al., 2012). An alternative view holds that initial neurodegenerative events, including apoptosis of oligodendrocytes or structural alteration of myelin, might occur in early stages of disease and then trigger subsequent inflammatory phenomena (Moscarello et al., 2007; Prineas and Parratt, 2012; Stys et al., 2012). Regardless of whether the initiating event in the MS disease process is an innate or adaptive immune dysregulation or a neurodegenerative/cell death phenomenon, destruction of myelin and axonal damage are the final pathogenic outcomes which underlie signs and symptoms of the disease.

The localized and well-demarcated nature of pathology in MS begs the question of whether a focal dysregulation in the CNS microenvironment might contribute to the disease process in addition to the infiltration of myelin-reactive T cells and other leukocytes. Numerous studies have shown the link between MS with steroid hormones (El-Etr et al., 2005; Simpkins et al., 2005; Kipp and Beyer, 2009). Following the discovery of neurosteroids in the CNS, efforts were made to elucidate the role of these compounds and their potential dysregulation in the context of MS and neuroinflammation. Herein, we review the current knowledge regarding the role of ALLO in the MS disease process. The first part of this review concentrates on the effects of ALLO on major cellular players in MS pathogenesis, i.e., oligodendrocytes, monocytoid cells, and lymphocytes. While direct effects of ALLO on neuronal physiology might also be important in the context of MS and in particular on the degree of axonal injury which is correlated with MS symptoms, we have not included the neuronal effects of ALLO here and we refer the interested reader to other reviews on this subject (Charalampopoulos et al., 2006, 2008; Leskiewicz et al., 2006). In the second part of the review, we assess the evidence supporting the involvement of ALLO in MS pathogenesis, including studies in animal models of disease, as well as its potential for therapeutic interventions.

ALLO AND MS PATHOGENESIS; CELLULAR PLAYERS

ALLO, OLIGODENDROCYTES, AND MYELINATION

The effects of progesterone and its derivatives in promoting myelin formation in the peripheral nervous system (PNS) have been recognized for almost two decades (Baulieu and Schumacher, 1997). Schwann cells have been shown to express functional GABA-A receptors and respond to ALLO treatment by upregulating two major myelin proteins, i.e., myelin protein 22 and P0 (Melcangi et al., 1999). Progesterone and ALLO were later reported to induce myelin basic protein (MBP) gene expression in brain organotypic slice cultures (Ghoumari et al., 2003). While the effect of progesterone on MBP gene expression was shown to be largely mediated by intracellular progesterone receptors, effects of ALLO were mediated by GABA-A receptors expressed on oligodendrocytes (Ghoumari et al., 2003). In addition to promoting myelin protein gene expression in mature oligodendrocytes, progesterone has been illustrated to enhance the proliferation of oligodendrocyte precursors in cerebellar slice cultures through intracellular progesterone receptors, but a similar effect has not been reported for ALLO (Ghoumari et al., 2005). That said, ALLO has been shown to induce proliferation of rat hippocampal neuroprogenitor cells or human cortical neural stem cells, with the resulting cells showing neuronal phenotype (Wang et al., 2005). In addition to its promyelinating effects, ALLO has been reported to protect oligodendrocytes against cytotoxic stimuli. When treated with recombinant TNF-α, cultured rat oligodendrocytes display reduced viability as quantified by CNPase immunoreactivity. Pretreatment with ALLO (100 nM) reduced the toxicity of TNF-α (Noorbakhsh et al., 2011). Overall, it seems that ALLO exerts promyelinating as well as cytoprotective effects on oligodendrocytes against inflammatory stimuli, and both effects are relevant because of the beneficial outcomes in the context of autoimmune demyelination.

ALLO AND MONOCYTOID CELLS

Macrophages are known to express functional GABA-A receptors, and the activation of the receptors leads to reduced production of inflammatory cytokines by these cells (Reyes-Garcia et al., 2007). Treatment with different GABA-A agonists have been shown to alter the behavior of macrophages and dendritic cells and the resulting T cell response following antigen presentation (Bhat et al., 2010). When treated with ALLO, murine peritoneal macrophages have been demonstrated to produce lower levels of TNFα after LPS stimulation (Ghezzi et al., 2000). In a study from our group, treatment of human monocyte-derived macrophages with ALLO (100 nM) reduced the production of IL-1β and TNF-α transcripts after PMA exposure. ALLO treatment also reduced macrophage expression of IDO, an enzyme involved in a variety of inflammatory processes (Noorbakhsh et al., 2011).

In addition to monocyte infiltration of the CNS, activation of microglia, the resident monocytoid cells of the brain, also plays an critical role during neuroinflammatory processes (Jack et al., 2005). Similar to macrophages, microglia have been illustrated to express both GABA-A and GABA-B receptors, and treatment with the GABA-A agonist muscimol reduces microglial production of inflammatory mediators following LPS stimulation (Lee et al., 2011). Nonetheless, studies investigating the effect of ALLO on microglial function are limited. A study by Muller et al. has shown decreased production of NO by LPS-stimulated microglial BV2 cell lines after treatment with either progesterone or ALLO (Muller and Kerschbaum, 2006). While still an underappreciated area, the consequences of microglial GABA-A signaling might not be limited to reduced proinflammatory activity of these cells. An interesting study by Mead et al. has shown that activation of GABA-A receptors on microglia can lead to enhanced activity of different isoforms of NADPH oxidase (Nox), the enzyme responsible for generation of superoxide ions (Mead et al., 2012). While Nox activation by glutamate signaling leads to a neurotoxic phenotype, GABA-A-receptor-mediated activation of the enzyme promotes a neuroprotective phenotype (Mead et al., 2012). It remains to be explored if ALLO-mediated activation of microglial GABA-A receptors might contribute to differentiation of the cells toward a neuroprotective anti-inflammatory phenotype.

ALLO AND LYMPHOCYTES

While altered activity of antigen presenting cells after exposure to ALLO or other GABA-A receptor agonists could translate into alterations in lymphocyte proliferation and differentiation, little is known about direct effects of ALLO on lymphocytes. Human and murine lymphocytes have been shown to express functional GABA-A receptors (Mendu et al., 2012). Treatment with the GABA-A agonist muscimol have been shown to inhibit antigen-specific T cell proliferation (Tian et al., 1999) and GABA-A activation leads to whole-cell transient and tonic currents in T lymphocytes (Mendu et al., 2012). Nonetheless, in a study by our group, ALLO treatment of splenocyte cultures derived from animals immunized with a myelin antigen did not affect cell proliferation after antigenic re-stimulation (Noorbakhsh et al., 2011). Moreover, ALLO treatment did not affect differentiation of antigen-stimulated lymphocytes to Th1 or Th17

pathogenic phenotypes, as measured by intracellular immunostaining of IFN-γ and IL-17, the prototypic Th1/Th17 cytokines (Noorbakhsh et al., 2011). While these findings do not rule out the possibility of ALLO acting directly on lymphocytes, they are more supportive of the role of ALLO as a modulator of innate immune function.

ALLO AND LEUKOCYTE MIGRATION THROUGH BLOOD BRAIN BARRIER (BBB)

A critical step in the process of autoimmune neuroinflammation is the traversing of the BBB by peripherally-activated leukocytes. These cells include neuroantigen-reactive lymphocytes or monocytes entering the CNS following the chemokine gradient generated by locally activated microglia or previously infiltrated lymphocytes. Both progesterone and ALLO have been demonstrated to reduce BBB dysfunction following focal ischemia (Ishrat et al., 2010). This effect has been partly attributed to suppressed expression of MMP-2 and MMP-9 in ischemic brain following ALLO treatment (Ishrat et al., 2010). Moreover, ALLO was shown to prevent degradation of the BBB tight junction proteins occludin 1 and claudin 5 (Ishrat et al., 2010). While it remains to be investigated, it is conceivable that ALLO treatment could also affect BBB permeability and leukocyte trafficking during autoimmune inflammation.

We have summarized the effects of ALLO on different cellular elements with known roles in MS pathogenesis (**Figure 1**).

ALLO AND MS PATHOGENESIS, HUMAN STUDIES AND ANIMAL MODELS

The role of progesterone and its potential derivatives in demyelinating diseases has been studied for many years (Shuster, 2008). Before the identification of neurosteroid synthesis pathways in the brain, it had been reported that treatment of mice with experimental autoimmune encephalomyelitis (EAE) with progestins ameliorated disease severity (Arnason and Richman, 1969a,b). Later studies further highlighted the protective and anti-inflammatory role of progesterone in EAE (Garay et al., 2007). Progesterone treatment of neuroantigen-reactive CD4+ T cells isolated from MS patients was shown to affect their cytokine production (Correale et al., 1998). Progesterone and its reduced form dehydroprogesterone (DHP) can bind to intracellular progesterone receptors and exert genomic effects via altered expression of progesterone-responsive genes. However, ALLO lacks this ability and can only exert its effects through interactions with GABA-A receptors (Paul and Purdy, 1992). Considering that exogenously-administered progesterone could be readily reduced to ALLO in different cells, the protective effects reported for progesterone have likely been the consequence of both genomic regulation and GABA-A receptor-mediated pathways.

Subsequent studies provided more specific evidence supporting the role of ALLO in MS pathogenesis. In a study performed on human autopsy brain tissues, our group showed that ALLO levels were significantly reduced in the brain white matter derived from

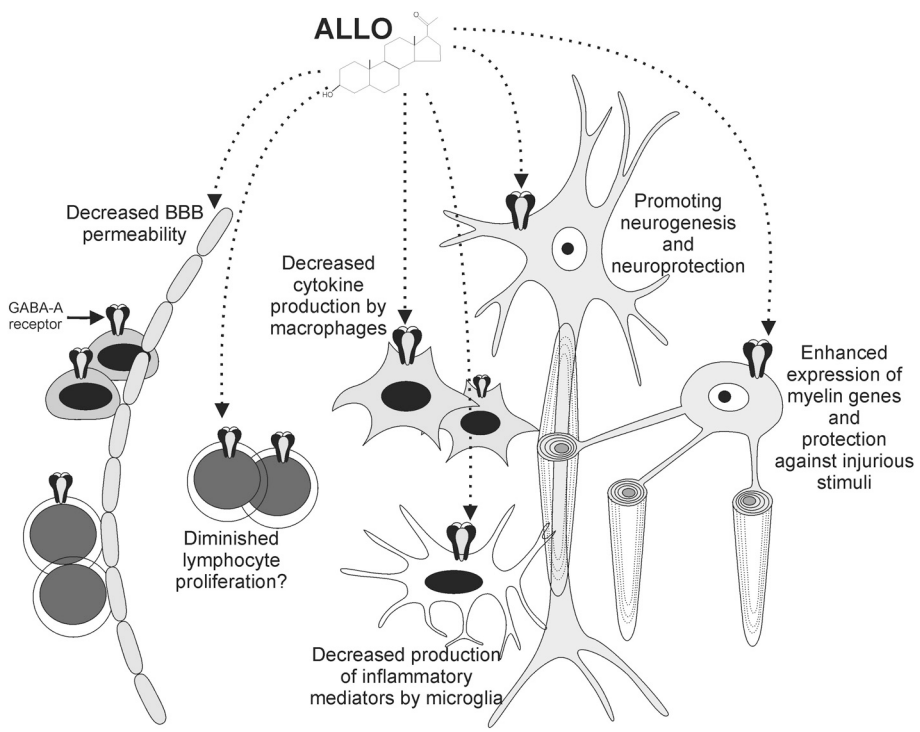

FIGURE 1 | ALLO exerts various effects on cells involved in MS pathogenesis. Functional GABA-A receptors are expressed by neurons, oligodendrocytes, monocytoid cells, and lymphocytes. ALLO promotes myelin gene expression by oligodendrocytes and protects them against injurious stimuli. Neuroprotective effects have also been reported for neurons. ALLO's binding to GABA-A receptors on monocytoid cells leads to diminished production of inflammatory mediators by these cells. These effects, together with diminished BBB permeability and potential influence on lymphocytes, contribute to the beneficial roles of ALLO in the context of autoimmune demyelination.

MS patients compared with control individuals (Noorbakhsh et al., 2011). The reduction was associated with diminished levels of two crucial upstream enzymes which are involved in conversion of progesterone to ALLO, i.e., 5-α-reductase and AKR1C1/C2, in brain white matter of MS patients. The levels of the further upstream enzyme, 3-α-HSD, which is involved in conversion of pregnenolone to progesterone, was not altered in MS brains. Interestingly, analysis of the levels of 3α,5β-THP, a minor isoform of THP, did not show any changes in the same brain tissues, indicating a specific downregulation in 3α,5α-ALLO isoform. Similar analysis of spinal cord tissues derived from EAE mice showed decreased levels of ALLO, but not the minor THP isoforms in EAE mice compared with control animals (Noorbakhsh et al., 2011). Again, ALLO suppression was associated with diminished RNA and protein levels of murine isoforms of the AKR1 enzymes, akr1c14, and akr1e1. Treatment of EAE mice with daily intraperitoneal injections of ALLO, which were started after the onset of neurological signs, reduced disease severity. Neuropathological analyses of the spinal cords from the same animals revealed diminished myelin damage and axonal injury in ALLO-treated animals, a finding that was consistent with the clinical features. These observations were also associated with lower levels of lymphocyte infiltration and monocyte/microglial activation in the CNS (Noorbakhsh et al., 2011).

Enhanced synthesis of endogenous ALLO has also been reported to be protective in the context of autoimmune demyelination. Translocator protein (TSPO) is a transfer protein located in the outer membrane of mitochondria (Papadopoulos et al., 2006; Gatliff and Campanella, 2012) previously termed the peripheral benzodiazepine receptor and a recognized marker for activated glial cells (Venneti et al., 2006). Through interactions with StAR (steroidogenic acute regulatory protein), TSPO exerts a rate-limiting step in the transfer of cholesterol into mitochondria, where it can be converted to pregnenolone (Rone et al., 2009). It has been shown that modulation of TSPO activity with etifoxine, a TSPO ligand, can enhance neurosteroid synthesis in brain (Girard et al., 2012; Nothdurfter et al., 2012). A recent study has shown that modulation of TSPO activity with etifoxine ameliorates disease symptoms and neuropathology in mice affected with EAE (Daugherty et al., 2013). The treatment was effective when the drug was administered at the presymptomatic stage of disease or at the peak of disease. The protective effect of TSPO activation was shown to be associated with specific upregulation of the murine enzyme responsible for ALLO synthesis, i.e., akr1c14 (Daugherty et al., 2013). Other TSPO ligands have also been reported to diminish microglial activation, an effect that is likely mediated through enhanced microglial neurosteroid synthesis (Zhao et al., 2011; Leaver et al., 2012). Of note, TSPO shows upregulation on astrocytes and microglia in a variety of neuroinflammatory disorders, which might be a part of an endogenously-regulated protective response in the context of neuroinflammation (Abourbeh et al., 2012; Lavisse et al., 2012).

FUTURE PERSPECTIVES: ALLO AS A THERAPY FOR MS

Since the discovery of ALLO dysregulation in several neurological disorders, ALLO and its synthetic analogs have been considered for their potential therapeutic or disease modifying effects in brain disease (Brinton, 2013). Currently, the majority of evidence supporting a beneficial role for ALLO and its analogs comes from animal models, with few studies having tested the effects of ALLO treatment on humans. In a pioneering study by Griffin et al., ALLO was shown to diminish neurological signs and neuropathology in a mouse model of Niemann-Pick disease (Griffin et al., 2004). ALLO treatment was later shown to decrease beta-amyloid burden and memory deficits in triple-transgenic mouse models of Alzheimer's disease (Wang et al., 2010; Singh et al., 2012), restore dopaminergic neurons and motor performance in the MPTP model of Parkinson's disease (Adeosun et al., 2012), exert anticonvulsant activity and decrease neuronal injury in models of epilepsy (Mares et al., 2006, 2010; Singh et al., 2010), reduce infarct volume and improve cognitive outcome in models of brain ischemia (Sayeed et al., 2006; Morali et al., 2011), and reduce neuronal death and gliosis after traumatic brain injury (TBI) (Djebaili et al., 2005). Of note, the protective effects of ALLO in TBI have been clearly linked with its anti-inflammatory functions (He et al., 2004; VanLandingham et al., 2007). Evidence for potential therapeutic roles for ALLO in MS also comes from animal studies showing reduced neuroinflammation and disease burden in the EAE animal model of disease after treatment with ALLO or TSO ligands, which lead to induction of ALLO-synthesizing enzymes (Noorbakhsh et al., 2011; Daugherty et al., 2013). While several reports have demonstrated the safety of ALLO administration to humans (Timby et al., 2006; van Broekhoven et al., 2007), ALLO trials on human disease are limited, in part because of costs and the quantity of drug needed for therapeutic purposes. Of interest, ganaxolone, an ALLO synthetic analog, has been successfully used to control epilepsy in human cases (Nohria and Giller, 2007; Pieribone et al., 2007), and recent studies from our group indicate that ganaxolone also exerts anti-inflammatory effects in EAE (Paul et al., 2014). Clinical trials to evaluate the therapeutic effects of ALLO in TBI are currently underway (Clinicaltrials.gov, NCT01673828), while ALLO trials for treating mild cognitive impairment and Alzheimer's disease are also expected (Irwin and Brinton, 2014). Overall, considering the wealth of knowledge derived from *in vitro* and *in vivo* analyses, together with low toxicity and good tolerance in human subjects, ALLO and its analogs are excellent candidates waiting to be tested in MS and other neuroinflammatory disorders.

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Biosynthesis and biological action of pineal allopregnanolone

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The pineal gland transduces photoperiodic changes to the neuroendocrine system by rhythmic secretion of melatonin. We recently provided new evidence that the pineal gland is a major neurosteroidogenic organ and actively produces a variety of neurosteroids *de novo* from cholesterol in birds. Notably, allopregnanolone is a major pineal neurosteroid that is far more actively produced in the pineal gland than the brain and secreted by the pineal gland in juvenile birds. Subsequently, we have demonstrated the biological action of pineal allopregnanolone on Purkinje cells in the cerebellum during development in juvenile birds. Pinealectomy (Px) induces apoptosis of Purkinje cells, whereas allopregnanolone administration to Px chicks prevents cell death. Furthermore, Px increases the number of Purkinje cells that express active caspase-3, a crucial mediator of apoptosis, and allopregnanolone administration to Px chicks decreases the number of Purkinje cells expressing active caspase-3. It thus appears that pineal allopregnanolone prevents cell death of Purkinje cells by suppressing the activity of caspase-3 during development. This paper highlights new aspects of the biosynthesis and biological action of pineal allopregnanolone.

Keywords: neurosteroids, allopregnanolone, caspase-3, apoptosis, cell survival, pineal gland, Purkinje cell

INTRODUCTION

De novo formation of neurosteroids in the brain was originally demonstrated in mammals (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Robel et al., 1987; Jo et al., 1989; Mathur et al., 1993; Mellon and Deschepper, 1993; Compagnone et al., 1995; Ukena et al., 1998, 1999; Sakamoto et al., 2001b, 2003a), and subsequently in non-mammalian vertebrates, such as birds, amphibians, and fish (Mensah-Nyagan et al., 1994, 1996a,b, 1999; Tsutsui and Yamazaki, 1995; Usui et al., 1995; Vanson et al., 1996; Tsutsui et al., 1997, 1999, 2003a, 2008; Beaujean et al., 1999; Schlinger et al., 1999; Takase et al., 1999, 2002, 2011; Freking et al., 2000; Matsunaga et al., 2001, 2002, 2004a; Sakamoto et al., 2001a; Tsutsui and Schlinger, 2001; Ukena et al., 2001; Inai et al., 2003; London et al., 2003, 2006, 2010; Soma et al., 2004; Menuet et al., 2005; Do-Rego et al., 2007; London and Schlinger, 2007; Tam and Schlinger, 2007; Bruzzone et al., 2010; Haraguchi et al., 2010, 2012a; Diotel et al., 2011; Brion et al., 2012). Therefore, *de novo* synthesis of neurosteroids from cholesterol is considered to be a conserved property in the brain across vertebrate species (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003a, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009).

Until recently, it was generally accepted that neurosteroids are produced in glial cells and neurons which are located in the brain and peripheral nervous systems (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003a, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009). However, we recently discovered that the pineal gland actively produces neurosteroids *de novo* from cholesterol in the juvenile chicken and quail (Hatori et al., 2011; Haraguchi et al., 2012b). Notably, allopregnanolone (3α,5α-tetrahydroprogesterone; 3α,5α-THP) is a major neurosteroid produced in the pineal gland (Haraguchi et al., 2012b). Importantly, allopregnanolone secreted by the pineal gland prevents cell death of Purkinje cells by suppressing the activity of caspase-3, a crucial mediator of apoptosis, in the cerebellum during development (Haraguchi et al., 2012b).

NEUROSTEROIDOGENIC CELLS IN THE BRAIN

Past studies demonstrated that oligodendrocytes are the primary site for neurosteroid formation in the brain (for reviews, see Baulieu, 1997; Compagnone and Mellon, 2000). Subsequently, astrocytes were shown to express steroidogenic enzymes (Mellon and Deschepper, 1993). Based on extensive studies, it was generally accepted that glial cells are the site for neurosteroid formation in the brain. However, whether neurons located in the brain produce neurosteroids was unknown in vertebrates until the middle 1990s. We discovered that Purkinje cells, a major neuronal population actively produce a variety of neurosteroids *de novo* from cholesterol in the brain of various vertebrates (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Ukena et al., 1998, 1999; Takase et al., 1999; Matsunaga et al., 2001; Sakamoto et al., 2001a,b, 2003a; Agís-Balboa et al., 2006, 2007). The Purkinje cell expresses several kinds of key steroidogenic enzymes in rat (Furukawa et al., 1998; Ukena et al., 1998, 1999; Sakamoto et al., 2003a). In the rat hippocampus, the expression of steroidogenic enzymes has also been found in pyramidal neurons in the CA1-CA3 regions as well as granule cells in the dentate gyrus (Kimoto et al., 2001; Hojo et al., 2004; Okamoto et al., 2012). In addition to these

brain neurons, the expression of steroidogenic enzymes has been reported in neurons in the retinal ganglion, sensory neurons in the dorsal root ganglia and motor neurons in the spinal cord of rat (Guarneri et al., 1994; Compagnone et al., 1995). Based on these findings, not only glial cells but also neurons have been demonstrated as the sites of neurosteroid formation in the central and peripheral nervous systems (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003a, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009).

BIOSYNTHESIS OF NEUROSTEROIDS IN THE PINEAL GLAND

NEUROSTEROIDS FORMED IN THE PINEAL GLAND

The pineal gland that is an endocrine organ located close to the parietal region of the brain is known to transduce photoperiodic changes to the neuroendocrine system by rhythmic secretion of melatonin. However, the biosynthesis of neurosteroids in this endocrine organ was, until recently, unknown. We recently provided new evidence that the pineal gland is a major neurosteroidogenic organ actively producing a variety of neurosteroids *de novo* from cholesterol (Hatori et al., 2011; Haraguchi et al., 2012b) (**Figure 1**). This is a paradigm shift of neurosteroid formation, because it was accepted that neurosteroids are synthesized only in glial cells and neurons which are located in the brain and peripheral nervous systems for the past 30 years (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003a, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009).

Pregnenolone is a main precursor of steroid hormones and the production of pregnenolone is initiated by cleavage of the cholesterol side-chain by cytochrome P450scc (P450scc; gene name *Cyp11a*), a mitochondrial enzyme, in vertebrates. We first showed that the pineal gland expresses P450scc in juvenile chickens and quail by reverse transcription polymerase chain reaction (RT-PCR) analysis (Hatori et al., 2011; Haraguchi et al., 2012b) (**Figure 1**). P450scc antibodies stained the cells forming follicular structures in the pineal gland of juvenile birds (Haraguchi et al., 2012b). Incubation of pineal glands from juvenile birds with ^{3}H-cholesterol led to the formation of radioactive pregnenolone as revealed by high-performance liquid chromatography (HPLC) analysis (Haraguchi et al., 2012b) (**Figure 1**). Gas chromatography-mass spectrometry (GC-MS) analysis further demonstrated the occurrence of pregnenolone in the pineal gland (Haraguchi et al., 2012b).

Subsequently, RT-PCR analyses demonstrated the expressions of key steroidogenic enzymes, such as cytochrome P450 7α-hydroxylase ($P450_{7\alpha}$; gene name *Cyp7b*), 3α-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3α-HSD; gene name *Hsd3a*), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD; gene name *Hsd3b*), 5α-reductase (gene name *Srd5a*), 5β-reductase (gene name *Srd5b*), cytochrome P450 17α-hydroxylase/c17,20-lyase ($P450_{17\alpha,lyase}$; gene name *Cyp17*), 17β-hydroxysteroid dehydrogenase (17β-HSD; gene name *Hsd17b*) and cytochrome P450 aromatase (P450arom; gene name *Cyp19*) in the pineal gland of juvenile birds (Hatori et al., 2011; Haraguchi et al., 2012b) (**Figure 1**).

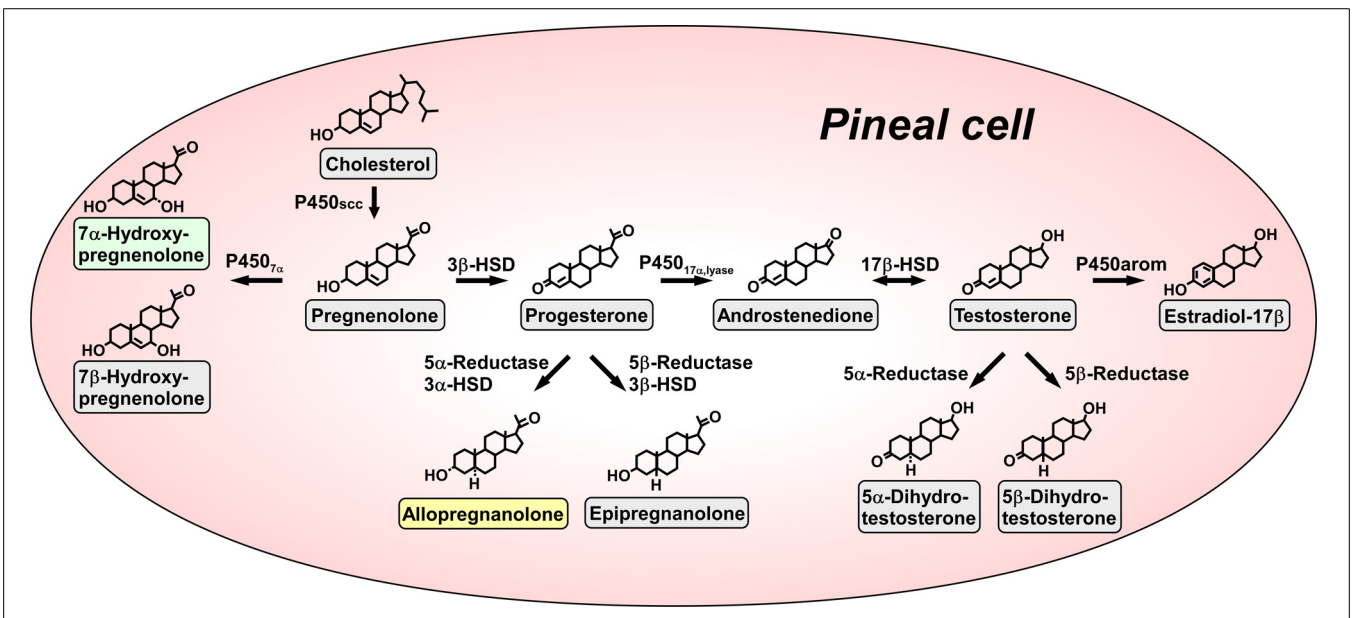

FIGURE 1 | Biosynthetic pathways for neurosteroids in the pineal gland. The arrows indicate the biosynthetic pathways of neurosteroids identified in the pineal glands of juvenile quail. The pineal gland actively produces a variety of neurosteroids *de novo* from cholesterol. Allopregnanolone and 7α-hydroxypregnenolone are major products secreted by the pineal gland. P450scc, cytochrome P450 side-chain cleavage enzyme (gene name *Cyp11a*); $P450_{7\alpha}$, cytochrome P450 7α-hydroxylase (gene name *Cyp7b*); 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (gene name *Hsd3b*); 3α-HSD, 3α-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (gene name *Hsd3a*); 5α-reductase (gene name *Srd5a*); 5β-reductase (gene name *Srd5b*); $P450_{17\alpha,lyase}$, cytochrome P450 17α-hydroxylase/c17,20-lyase (gene name *Cyp17*); 17β-HSD, 17β-hydroxysteroid dehydrogenase (gene name *Hsd17b*); P450arom, cytochrome P450 aromatase (gene name *Cyp19*). See Haraguchi et al. (2012b) and the text for details.

Biochemical studies combined with HPLC and GC-MS analyses were further conducted to demonstrate the biosynthetic pathways of neurosteroids in the pineal gland. Incubation of pineal glands from juvenile birds with ^{3}H-pregnenolone as a precursor led to the formation of 7α- and/or 7β-hydroxypregnenolone as revealed by HPLC analysis (Haraguchi et al., 2012b) (**Figure 1**). In addition to these neurosteroids, progesterone, allopregnanolone (3α,5α-THP) and/or epipregnanolone (3β,5β-THP), androstenedione, testosterone, 5α- and/or 5β-dihydrotestosterone and estradiol-17β were produced from the precursor pregnenolone (Haraguchi et al., 2012b) (**Figure 1**). Isomers, such as 7α- and 7β-hydroxypregnenolone; allopregnanolone and epipregnanolone; and 5α- and 5β-dihydrotestosterone, were not separated by HPLC analysis, but GC-MS analysis was capable of separating several pairs of isomers (Haraguchi et al., 2012b). As summarized in **Figure 1**, pregnenolone, 7α- and 7β-hydroxypregnenolone, progesterone, allopregnanolone, epipregnanolone, androstenedione, testosterone, 5α- and 5β-dihydrotestosterone, and estradiol-17β were identified as the neurosteroids produced in the pineal gland (Haraguchi et al., 2012b). In sum, molecular and biochemical techniques have demonstrated that the pineal gland produces a variety of neurosteroids from cholesterol *via* pregnenolone in juvenile birds. This is the first observation of *de novo* neurosteroidogenesis in the pineal gland in any vertebrate.

MAJOR PINEAL NEUROSTEROIDS

We further investigated major neurosteroids formed and released in the pineal gland. Incubation of the pineal glands from juvenile birds with ^{3}H-pregnenolone led primarily to the formation of 7α- and/or 7β-hydroxypregnenolone and allopregnanolone and/or epipregnanolone as revealed by HPLC analysis (Haraguchi et al., 2012b). The formation of 7α- and/or 7β-hydroxypregnenolone and the expression $P450_{7\alpha}$ mRNA in the pineal gland of juveniles were higher than those of adults (Haraguchi et al., 2012b). The formation of allopregnanolone and/or epipregnanolone and the expression of 5α-reductase mRNA in the pineal gland of juveniles were also higher than those of adults (Haraguchi et al., 2012b). Surprisingly, in juvenile birds, the formation of 7α- and/or 7β-hydroxypregnenolone and the expression of $P450_{7\alpha}$ mRNA in the pineal gland were higher than those in brain regions, such as the diencephalon and cerebellum (Haraguchi et al., 2012b). The formation of allopregnanolone and/or epipregnanolone and the expression of 5α-reductase mRNA in the pineal gland were also higher than those in the diencephalon and cerebellum in juvenile birds (Haraguchi et al., 2012b). Thus, the pineal gland of juvenile birds produces 7α- and/or 7β-hydroxypregnenolone and allopregnanolone and/or epipregnanolone far more abundantly than brain tissue.

Subsequently, to clarify the release of neurosteroids from the pineal gland, the pineal glands of juvenile birds were cultured in medium 199. The released neurosteroids were measured by GC-MS. Unlike 7β-hydroxypregnenolone and epipregnanolone, significant amounts of 7α-hydroxypregnenolone and allopregnanolone were released from the pineal gland into the culture medium (Haraguchi et al., 2012b). Thus, it appears that 7α-hydroxypregnenolone and allopregnanolone are the major neurosteroids secreted from the pineal gland (Haraguchi et al., 2012b) (**Figure 1**).

BIOLOGICAL ACTION OF PINEAL ALLOPREGNANOLONE ON PURKINJE CELL SURVIVAL DURING DEVELOPMENT

The two major pineal neurosteroids, 7α-hydroxypregnenolone and allopregnanolone, are abundantly released from the pineal gland during development (Haraguchi et al., 2012b). Therefore, these major pineal neurosteroids may play important roles in the avian brain during development. In birds, the pineal gland is located near the cerebellum (**Figure 2**). The Purkinje cell integrates the process of memory and learning. It has been reported that, in birds and mammals, pinealectomy (Px) induces cell loss in the brain including Purkinje cells during development (Kilic et al., 2002; Tunç et al., 2006). Based on these findings, we hypothesized that allopregnanolone and/or 7α-hydroxypregnenolone secreted by the pineal gland may play a role in preventing the death of developing Purkinje cells. To test this hypothesis, we conducted a series of experiments in the male juvenile birds. Px decreased the concentration of allopregnanolone in the cerebellum and induced apoptosis of Purkinje cells, whereas administration of allopregnanolone to Px birds increased allopregnanolone concentration in the cerebellum and prevented apoptosis of Purkinje cells (Haraguchi et al., 2012b). We further indicated that pineal allopregnanolone reaches Purkinje cells in the cerebellum by diffusion shown by injection of ^{3}H-allopregnanolone close to the pineal lumen (Haraguchi et al., 2012b). Thus, allopregnanolone secreted by the pineal gland is considered to be a key factor for Purkinje cell survival during development (**Figure 2**).

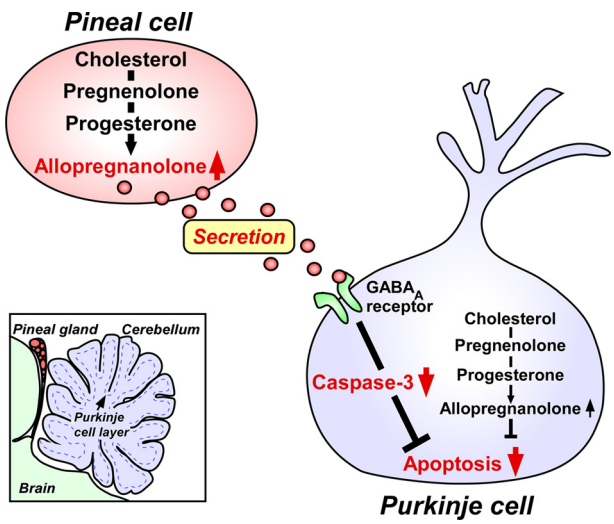

FIGURE 2 | Neuroprotective action of pineal allopregnanolone on Purkinje cell survival during cerebellar development. The square in the left bottom indicates the location of the pineal gland in the quail chick brain. The pineal gland is located adjacent to the cerebellum. Allopregnanolone is exceedingly produced in the pineal gland compared with brain regions, and may affect the adjacent cerebellar Purkinje cells by diffusion, and saves Purkinje cells from apoptosis in the juvenile quail. Secreted pineal allopregnanolone inhibits the expression of active caspase-3 that facilitates apoptosis of Purkinje cells in the cerebellum during development. See Haraguchi et al. (2012b) and the text for details.

In contrast to allopregnanolone, administration of 7α-hydroxypregnenolone to Px birds did not increase Purkinje cell survival (Haraguchi et al., 2012b). Although 7α-hydroxypregnenolone did not facilitate Purkinje cell survival, recent studies have demonstrated that this neurosteroid is involved in the regulation of locomotor rhythms of birds (Tsutsui et al., 2008; Hatori et al., 2011).

The induction of cell death of Purkinje cells in the cerebellum by Px suggests that certain other component(s) in the pineal gland may contribute to Purkinje cell survival during development. However, pineal melatonin did not facilitate Purkinje cell survival during development in juvenile birds (Haraguchi et al., 2012b). It thus appears that allopregnanolone but not melatonin acts as an important component of the pineal gland for Purkinje cell survival during development. Allopregnanolone produced in the pineal gland is considered to reach the target site within the cerebellum by diffusion, because allopregnanolone was abundantly released from cultured pineal gland of juvenile birds (Haraguchi et al., 2012b).

MODE OF ACTION OF PINEAL ALLOPREGNANOLONE ON PURKINJE CELL SURVIVAL DURING DEVELOPMENT

Finally, we investigated the mode of action of pineal allopregnanolone on Purkinje cell survival. Caspase-3, a crucial mediator of apoptosis, is known to play an important role in Purkinje cell death in vertebrates (Puig and Ferrer, 2001; Matsunaga et al., 2004b; Olkowski et al., 2008). Interestingly, Px increased the number of Purkinje cells that expressed active caspase-3 in juvenile birds and administration of allopregnanolone to Px birds decreased the number of Purkinje cells expressing active caspase-3 (Haraguchi et al., 2012b). Accordingly, the neuroprotective effect of pineal allopregnanolone on Purkinje cells is accompanied with the decrease in caspase-3 activity during development. We thus provide new evidence that pineal allopregnanolone exerts anti-apoptotic effects in Purkinje cells by suppressing the activity of caspase-3 during development (**Figure 2**).

It is unclear whether the action of pineal allopregnanolone on caspase-3 activity in the Purkinje cell is rapid (i.e., mediated through a membrane receptor) or slow (i.e., involving transcriptional activation). On the other hand, the action of allopregnanolone produced in the brain is likely mediated through interaction with the pathway of γ-aminobutyric acid type A ($GABA_A$) receptor, since allopregnanolone is a potent allosteric modulator of $GABA_A$ receptor (Paul and Purdy, 1992; Lambert et al., 1995). However, the mode of action of pineal allopregnanolone suppressing the activity of caspase-3 in the Purkinje cell remains unclear. We need to clarify the mode of action exerting neuroprotective effect of pineal allopregnanolone in the Purkinje cell.

INVOLVEMENT OF PINEAL AND BRAIN ALLOPREGNANOLONE IN PURKINJE CELL SURVIVAL DURING DEVELOPMENT

The Purkinje cell is known as a major site of neurosteroid formation in the brain of various vertebrates (for reviews, see Tsutsui, 2008a,b). In mammals, the Purkinje cell possesses several kinds of steroidogenic enzymes, such as P450scc and 3β-HSD, and actively produces progesterone during neonatal life (Furukawa et al., 1998; Ukena et al., 1998, 1999) (**Figure 2**). Allopregnanolone is also synthesized in the neonatal cerebellum (Tsutsui and Ukena, 1999; Tsutsui et al., 2003b,c, 2004; Agís-Balboa et al., 2006, 2007) (**Figure 2**). Subsequently, biological actions of progesterone (Sakamoto et al., 2001b, 2002, 2003b; Ghoumari et al., 2003) and allopregnanolone (Griffin et al., 2004; Langmade et al., 2006) have been demonstrated by the studies on mammals using the Purkinje cell. In addition, this neuron expresses P450arom, a key enzyme of estrogen formation, and actively produces estradiol-17β in the neonate (Sakamoto et al., 2003a; Tsutsui et al., 2003b). Estradiol-17β also contributes to important events in the developing Purkinje cell (Sakamoto et al., 2003a; Sasahara et al., 2007). Purkinje cells express the receptors for progesterone and estradiol-17β and these neurosteroids promote dendritic growth, spinogenesis, and synaptogenesis of Purkinje cells via each cognate nuclear receptor during cerebellar development (Sakamoto et al., 2001b, 2002, 2003a,b; Sasahara et al., 2007).

It has been shown that allopregnanolone produced in the cerebellum is involved in Purkinje and granule cell survival (Griffin et al., 2004; Langmade et al., 2006) (**Figure 2**), although allopregnanolone failed to promote dendritic growth, spinogenesis, and synaptogenesis of Purkinje cells (Sakamoto et al., 2001b, 2002). The Niemann–Pick type C (NP-C) mouse has been used as an excellent animal model for understanding the action of allopregnanolone. NP-C is an autosomal recessive, childhood neurodegenerative disease characterized by defective intracellular cholesterol trafficking, resulting in Purkinje cell degeneration as well as neuronal degeneration in other regions. Brains from adult NP-C mice contained less allopregnanolone than wild-type (WT) brain (Griffin et al., 2004). Administration of allopregnanolone to neonatal NP-C mice increased Purkinje cell survival and delayed neurodegeneration (Griffin et al., 2004). According to Langmade et al. (2006), Purkinje cell number was reduced in *npc1*$^{-/-}$ mice, a model of NP-C disease, compared with WT mice. Thus, allopregnanolone produced in the cerebellum acts as a survival factor of Purkinje cells in the neonate (Griffin et al., 2004; Langmade et al., 2006) (**Figure 2**).

In addition to these findings, our recent studies on juvenile birds have demonstrated that the pineal gland is a major site of production of neurosteroids *de novo* from cholesterol (Hatori et al., 2011; Haraguchi et al., 2012b; Tsutsui et al., 2013a). Notably, allopregnanolone is exceedingly produced in the pineal gland compared with the brain and this major pineal neurosteroid is abundantly released from the pineal gland (Haraguchi et al., 2012b; Tsutsui et al., 2013b,c). Importantly, allopregnanolone secreted by the pineal gland prevents cell death of Purkinje cells by suppressing the activity of caspase-3, a crucial mediator of apoptosis, in the cerebellum during development (Haraguchi et al., 2012b; Tsutsui et al., 2013b,c). Taken together, it appears that both pineal allopregnanolone and cerebellar allopregnanolone are involved in Purkinje cell survival during development (**Figure 2**).

CONCLUSIONS AND FUTURE DIRECTIONS

The pineal gland actively produces neurosteroids *de novo* from cholesterol in juvenile birds. This is a new aspect of the biosynthesis of neurosteroids, because it was accepted that

neurosteroids are produced only in glial cells and neurons which are located in the brain and peripheral nervous systems. The major pineal neurosteroid allopregnanolone prevents cell death of Purkinje cells by suppressing the activity of caspase-3 during development. P450scc is expressed in the cells forming follicular structures in the pineal gland (Haraguchi et al., 2012b). Further study is needed to determine which cell types within the pineal gland, such as epithelial cells and/or neuronal cells express steroidogenic enzymes, P450scc, 3α- and 3β-HSD, 5α-reductase, *etc*. The coordinated action of steroidogenic enzymes is essential for neurosteroidogenesis. As for the production of allopregnanolone in the pineal gland, the coordinated action of P450scc, 3α- and 3β-HSD and 5α-reductase is required. Therefore, future study is also needed to determine whether all these enzymes are expressed in the same cell. Interaction of pineal and brain allopregnanolone in the regulation of brain development deserves further investigations. Px not only induces cell loss in the brain including Purkinje cells during development (Kilic et al., 2002; Tunç et al., 2006) but also abolishes circadian rhythm of locomotor activity (Gaston and Menaker, 1968; Tsutsui et al., 2008). In addition, allopregnanolone administration increases locomotion (Darbra and Pallarès, 2009). These observations indicate that pineal allopregnanolone may play an important role in the regulation of circadian locomotor activity.

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Analgesic strategies aimed at stimulating the endogenous production of allopregnanolone

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A growing number of studies indicate that 3-alpha reduced neurosteroids are remarkable analgesics in various pain states. This is the case for allopregnanolone (AP), one of the most potent endogenous positive allosteric modulators of $GABA_A$ receptor function. From the pioneering work of Hans Selye, who described the sedative properties of steroids, synthetic compounds resembling the progesterone metabolite AP have been developed. If some of them have been used as anesthetics, it seems difficult to propose them as a therapeutic option for pain since they display several adverse side effects such as sedation, amnesia and functional tolerance. An alternative strategy, chosen by few laboratories around the world, is aimed at stimulating the local production of 3-alpha reduced neurosteroids in order to limit these well-known side effects. This pharmacological approach has the advantage of targeting specific structures, fully equipped with the necessary biosynthetic enzymatic machinery, where neurosteroids already act as endogenous pain modulators. The various pharmacological trials which attempted to treat pain symptoms by stimulating the production of 3-alpha reduced neurosteroids are reviewed here, as well as novel neurotransmitter systems possibly regulating their endogenous production.

Keywords: allodynia, hyperalgesia, nociception, pain, neurosteroids, etifoxine

INTRODUCTION

In 1941, Selye reported that intraperitoneal injections of high doses of progesterone produce anesthesia in the rat (Selye, 1941). Several years later, this led to the development of steroidal anesthetics (Child et al., 1971). Of particular interest are the 3α-reduced steroid compounds, such as alphaxalone, which display potent anesthetic properties. Indeed, they were found to selectively act as positive allosteric modulators of the inhibitory functions of $GABA_A$ receptors ($GABA_A$Rs), expressed either at extrasynaptic (Harrison and Simmonds, 1984) or synaptic sites (Poisbeau et al., 1997; Cooper et al., 1999). After two decades of research, two steroid binding sites on $GABA_A$Rs have been identified (Hosie et al., 2006). Modulation of $GABA_A$R function is observed after binding of a 3α-reduced steroid in a cavity formed by the α-subunit transmembrane domains. A direct activation of the receptor-channel is also observed at a higher concentration if the binding is effective at interfacial residues between α and β subunits. Interestingly, recent experiments strongly suggest that these binding sites are reached by steroids via lateral mobility in the cell membrane, and that they only affect $GABA_A$R function when accessing the intracellular part of the channel (Akk et al., 2005). This observation may help understanding the possible occlusion of steroid action when the receptor is submitted to intracellular phosphorylation by protein kinase C (Harney et al., 2003; Vergnano et al., 2007). Many other receptor-channels were found to be modulated by steroids (Schlichter et al., 2006) but, in most cases, this physiological action was only observed at micromolar concentrations. Without fully excluding elevated levels of steroids within specific neuronal microdomains, such concentrations are unlikely to occur in the central nervous system (CNS; Schumacher et al., 2003).

It is interesting to note at this stage that neuroactive steroids can be synthesized at the periphery (i.e., by gonads and adrenal glands), but also by neural cells in the nervous system. This discovery led Baulieu and collaborators to propose the term "neurosteroids" for neuroactive steroids produced by neural cells independently of the endocrine steroidogenic glands (Baulieu and Robel, 1990). In the brain, similarly to any steroid-synthesizing tissues, a mitochondrial protein complex called TSPO is necessary to initiate the synthesis of neuroactive steroids (Rupprecht et al., 2010). TSPO facilitates the translocation of cholesterol from the outer to the inner mitochondrial membrane, where the P450 side-chain cleavage enzyme is located. The intramitochondrial transport of cholesterol is considered as a rate-limiting step for neurosteroidogenesis. Of course, this complex metabolic step can

be bypassed if peripheral steroid hormones access the brain, as they easily cross the blood-brain barrier. In this case, circulating steroid hormones (i.e., progesterone, deoxycorticosterone, testosterone) may serve as precursors for neurosteroidogenic enzymes synthesizing $GABA_AR$ active steroids (**Figure 1A**).

The purpose of this topical review is to illustrate the current analgesic strategies aimed at stimulating the production of endogenous neurosteroid analgesics in animal models. Two major aspects will be covered: (i) the recent attempts to use TSPO agonists for the production of steroid analgesics; and (ii) the identification of endogenous signaling pathways which may regulate neurosteroidogenesis and could be targeted in the near future for therapeutic purposes.

WHAT ARE THE ENDOGENOUS STEROID ANALGESICS? LESSONS FROM EXOGENOUSLY-ADMINISTERED STEROIDS AND CURRENT HYPOTHESIS FOR SEX-SPECIFIC PAIN ISSUES

Since the early reports of Selye, several groups have characterized the analgesic efficacy of steroid hormones, when administered systemically, into cavities of the CNS (intracerebroventricular, i.c.v. or intrathecally) or directly into rodent brain structures. These observations also raised critical issues linked to the well-known sex-specific differences in pain responses (Greenspan et al., 2007). From many experiments, it is clear that the administration of testosterone or progesterone to rodents induces antinociception and analgesia (Kavaliers and Wiebe, 1987; Frye and Duncan, 1994; Pednekar and Mulgaonker, 1995). Conversely, low levels of theses hormones, after gonadectomy for example, are associated with low nociceptive thresholds and pain hypersensitivity. Experimental evidences have also shown that the effects of testosterone and progesterone are mediated by their neuroactive metabolites, since analgesia is never observed in mice deficient for 5α-reductase (Frye et al., 2002, 2004). We recently contributed to this field by showing that the analgesic action of AP (3α-hydroxy-5α-pregnan-20-one or 3α,5α-tetrahydroprogesterone) is mediated by a direct allosteric positive modulation of $GABA_ARs$ in the spinal cord of rats displaying mechanical or thermal pain symptoms (Charlet et al., 2008). Coming back to the endocrine control of nociception by steroid hormones, it is interesting to note that the duration of miniature $GABA_AR$-mediated synaptic currents in the spinal cord is submitted to large variations during the female reproductive cycle (**Figure 1B**). We found these currents to be significantly longer in duration while recording from layer II spinal neurons of adult females during proestrus (i.e., during the progesterone surge) compared to those recorded in late estrus when circulating progesterone is low (mean decay time constant in proestrus: 31.9 ± 3.3 ms, $n = 10$ vs. estrus: 16.8 ± 2.9, $n = 14$; two-tailed unpaired Student's t-test, $p < 0.001$). Interestingly, glycinergic synapses during proestrus are also converted into mixed glycine/GABAergic synapses. Together, this led to an increased inhibitory control of layer II neurons processing pain informations and in a reduction in the intensity of pain symptoms, as previously demonstrated (Keller et al., 2001, 2004; Inquimbert et al., 2008). Since layer II neurons are fully equipped to synthesize AP from progesterone, it is likely that progesterone reaching the spinal cord is converted to AP, which potentiates the affinity of $GABA_ARs$ for GABA at individual synapses. This is fully in agreement with previous studies indicating that female pain thresholds are controlled by the levels of circulating gonadal steroids and, among them, progesterone (Greenspan et al., 2007).

Based on these observations, several groups around the world, including ours, have demonstrated the efficacy of administering AP for alleviating pain symptoms (Kavaliers and Wiebe, 1987; Frye and Duncan, 1994; Charlet et al., 2008; Meyer et al., 2008, 2011). This strategy, although very efficient in the short-term, has major disadvantages since a systemic/oral administration of AP-like steroids may generates negative side effects such as sedation, fatigue, nausea and functional tolerance. If the treatment is stopped or not properly controlled, it may also give rise to severe withdrawal symptoms (Smith, 2001, 2002; Gulinello and Smith, 2003). It remains that AP has an interesting therapeutic potential as pain killer in pathological pain states. Interestingly, endogenous concentrations of AP are affected in various neuropathologies including peripheral neuropathies (Melcangi et al., 2011). For example, AP concentrations were found particularly low in the distal portion of an injured sciatic nerve and correlated with low expression levels of 5α-reductase (Roglio et al., 2008). In apparent contradiction, 5α-reductase activity is high in the spinal cord of rats exhibiting inflammatory pain symptoms (Poisbeau et al., 2005). Since pain responses are exacerbated after inhibition of 5α-reductase activity, this demonstrates that the synthesis of AP-like steroids may limit pain symptoms. More recently, 3α-HSOR expression and activity were also found to be elevated in the spinal cord dorsal horns of neuropathic rats (Meyer et al., 2008).

Taken together, these results show that AP-like compounds, exogenously administered, are particularly efficient for limiting pain symptoms. Several reports also indicate that they may exert a general neuroprotective action. In peripheral nerves, in the spinal cord and in various supraspinal structures, there is a significant production of these putative pain killers (Caruso et al., 2013). A straightforward strategy would be to stimulate this widespread endogenous analgesic system. This has, at least, one major advantage: to limit the possible unwanted side effects seen when AP is given *per os* or via the general circulation.

STIMULATING THE SYNTHESIS OF ALLOPREGNANOLONE FOR PRODUCING ANALGESIA

The mitochondrial TSPO complex is the main molecular target exploited so far to efficiently stimulate neurosteroidogenesis (Rupprecht et al., 2009). Many TSPO ligands have been developed and tested successfully as neurotherapeutics in experimental models of painful diabetic and chemotherapy-induced neuropathy (Bordet et al., 2008; Aouad et al., 2009), mononeuropathy after chronic nerve constriction (Aouad et al., 2014a) and monoarthritis induced by persistent knee inflammation (Aouad et al., 2014b).

When we first attempted to stimulate the production of 3α-reduced neurosteroids in the spinal cord, we incubated the slices in the presence of the classical benzodiazepine diazepam and of flumazenil, a silent antagonist of the benzodiazepine site on $GABA_ARs$ (Keller et al., 2004). This favored the binding of

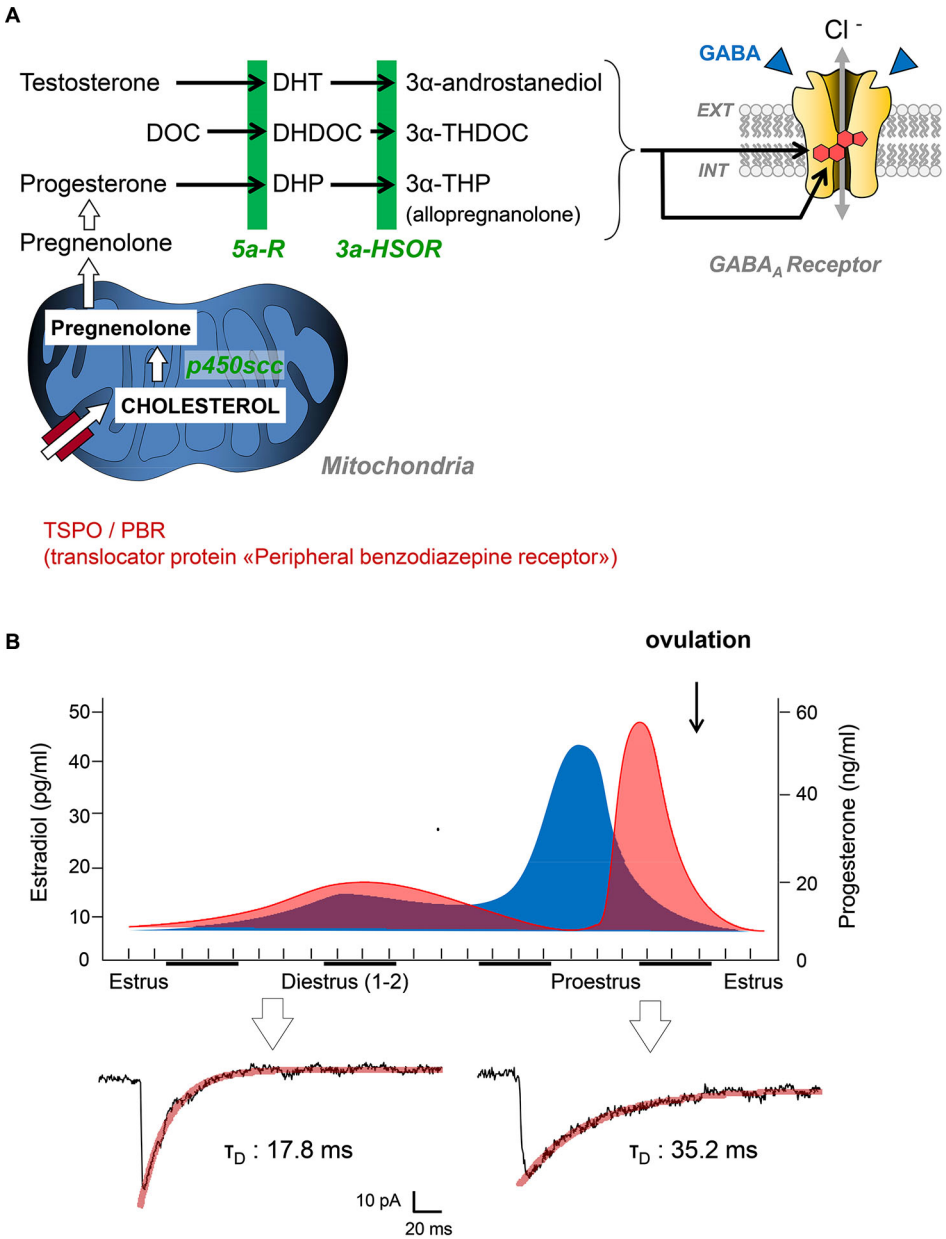

FIGURE 1 | (A) Simplified diagram summarizing the key steps of neurosteroidogenesis from cholesterol precursors to 3α-reduced neurosteroids (3α-androstanediol, 3α-THDOC, 3α-THP). These end-chain metabolites are potent positive allosteric modulators of $GABA_A$Rs after binding to specific binding sites. Abbreviations: DOC, deoxycorticosterone; DHDOC, 5α-dihydrodeoxycorticosterone; THDOC, 3α,5α-tetrahydrodeoxycorticosterone; DHP, 5alpha-dihydroprogesterone; THP, 3α,5α-tetrahydroprogesterone (= AP); 5α-R, 5α-reductase; 5α-HSOR, 3α-hydroxysteroid oxydoreductase; P450scc, P450 side-chain cleavage enzyme. **(B)** Top graphs summarize the global changes in blood estradiol (blue) and progesterone (red) during different phases of the reproductive cycle in female rats. Patch clamp traces below are representative of inhibitory postsynaptic currents mediated by $GABA_A$Rs in the spinal cord of female Sprague-Dawley rats during estrus and proestrus (i.e., when progesterone levels are low and high, respectively). Adapted from Keller (2002).

diazepam on the mitochondrial TSPO and promoted the local synthesis of AP-like neurosteroids. Indeed, $GABA_A$R-mediated synaptic currents were longer in duration, suggesting an increased affinity of the receptor-channel for GABA. We further confirmed 3α-reduced neurosteroids to be responsible for this increased spinal inhibition by blocking this effect with the TSPO inhibitor PK11195 or the 3α-reductase inhibitor finasteride (Keller et al., 2004). Apart from characterizing the key role of neurosteroids in the developmental maturation of inhibitory spinal synapses, the increased spinal inhibition was found to be responsible for the limitation of thermal hyperalgesia in a rodent model of inflammatory pain (Poisbeau et al., 2005). Beside $GABA_A$Rs,

note here that 3α-reduced neurosteroids may also inhibit T-type calcium channels to produce analgesia as previously published (Pathirathna et al., 2005). As mentioned earlier, it is difficult to use exogenous administration of AP-like steroids or benzodiazepines *in vivo* (see however Brinton, 2013). We thus tried to use a TSPO ligand to achieve similar goals. We choose etifoxine (EFX) because its properties had been already characterized on primary culture of hypothalamic neurons and on freshly-dissociated neonatal spinal neurons (Schlichter et al., 2000).

EFX (2-ethylamino-6-chloro-4-méthyl-4-phényl-4H-3,1-benzoxazine chlorhydrate) is prescribed in several countries as a non-benzodiazepine anxiolytic (Servant et al., 1998; Nguyen et al., 2006). At an efficient anxiolytic dose in human, it can be used safely (e.g., no functional tolerance and no physical dependence after treatment cessation) and displays limited adverse side effects on cognitive functions and vigilance (Micallef et al., 2001). EFX preferentially binds and modulates $GABA_A$Rs containing β2/3 subunits, at a site close to the chloride channel and distinct from that of benzodiazepines (Verleye et al., 1999, 2002; Hamon et al., 2003). Beside this direct effect on $GABA_A$Rs, EFX also binds to TSPO with an apparent affinity of about 20 μM (Verleye et al., 2005). If rats are sacrificed 30 min after a single injection of EFX of the reference anxiolytic dose of 50 mg/kg i.p., the plasmatic and brain concentrations of AP are increased by 2–4 times (Verleye et al., 2005). An increase in AP was also seen in EFX-treated male rats in the absence of gonads and adrenal glands. Because AP is a potent allosteric positive modulator of $GABA_A$Rs, this molecular mechanisms is referred to as "indirect" on $GABA_A$R function. While studying the functional consequence of EFX on hypothalamic primary cultures, we found that it strongly affected the tonic inhibition mediated by extrasynaptic receptors (Schlichter et al., 2000). More recently, we also found that the indirect neurosteroid-mediated effect of EFX prolonged $GABA_A$R-mediated synaptic currents in layer II neurons of the spinal cord (Aouad et al., 2014b).

Spinal inhibition, when reduced, gives rise to pathological pain symptoms and it is then crucial to maintain or to increase inhibitory controls to limit pain states. Because EFX may theoretically increase inhibitory controls mediated by $GABA_A$Rs, directly or indirectly, we choose to administer EFX (50 mg/kg, five daily injections i.p.) to animals exhibiting generalized neuropathic pain symptoms after chemotherapy with vincristine (VCR) sulphate, oxaliplatin or paclitaxel (**Figure 2**). The very low values for mechanical thresholds after chemotherapy were consistent with the presence of mechanical allodynia in all rat groups and models. Normal mechanical thresholds were, however, restored after EFX treatment, except if rats were pre-treated with an inhibitor of 3α-HSOR (depo-provera = medroxyprogesterone acetate) as shown in **Figure 2** (orange bar). Interestingly, EFX analgesia persisted even if the gonads and adrenals were removed (**Figure 2**, right graph). This strongly suggests that EFX analgesia is almost exclusively carried by the production of AP-like neurosteroids.

TSPO EXPRESSION, POSSIBLE CELL COOPERATION AND SIGNALING MECHANISMS

The above results, combined with recently published data (Aouad et al., 2010, 2014a,b), raise several critical questions regarding the TSPO-mediated mode of action of EFX. First, EFX clearly displays anti-allodynic/hyperalgesic properties but does not modify basal nociceptive thresholds of symptom-free animals (or body parts; e.g., sham-operated paw or contralateral territories to the lesion site). The second important observation is related to the efficacy of the effect, which not only fully alleviates pain symptoms, but also prevents their re-appearance after cessation of the treatment. In the model of rat sciatic nerve constriction, we failed to observe any pain symptoms for about 90 days after the treatment (Aouad et al., 2014a). As highlighted in a Pain editorial, there is thus an urgent need for a clinical trial testing the translational interest of this molecule in human pain pathologies (Zeilhofer, 2009). In addition, the molecular and cellular mechanisms recruited by EFX need to be identified and will require complementary experiments. Beside the sensori-discriminative component of pain responses, the real action of EFX on the affectivo-emotional component need, for example, to be clarified. This will require studying the supraspinal action of EFX in brain structures processing nociceptive informations and setting the emotional pain responses.

Of particular interest are the neuroregenerative and neuroprotective effects of the molecule described in a model of sciatic nerve lesion (Girard et al., 2008) or of diabetic neuropathy (Giatti et al., 2009). After cryolesion of the rat sciatic nerve, EFX therapy not only accelerated but also improved the quality of axonal regeneration and functional recovery (Girard et al., 2008). This was an important observation, as poor regeneration of axons may result in chronic neuropathic pain. Once again, little is known regarding the molecular mechanisms recruited to protect and promote peripheral nerve recovery, and there is so far little information concerning the cellular targets of EFX. Within the peripheral nervous system, TSPO is upregulated in macrophages, Schwann cells and dorsal root ganglia (DRG) sensory neurons in response to injury and disease (Karchewski et al., 2004; Rupprecht et al., 2010). A synergistic cooperation between these different cell types could be of utmost importance for neuroregenerative processes as well as pain-related neuropathologies.

Indeed, TSPO protein levels are upregulated in the ipsilateral spinal cord in rats displaying inflammatory pain symptoms after knee injection of complete Freund's adjuvant (Hernstadt et al., 2009). A recent study also reports an increase in the number of TSPO binding sites (e.g., using [^{3}H] PK11195, a well known TSPO ligand) in the spinal cord of rats, exhibiting neuropathic and osteoarthritic pain symptoms (Miller et al., 2013). According to this study, the increase in [^{3}H] PK11195 binding in the spinal cord seems to occur in microglial cells. TSPO is strongly expressed in activated microglia (Benavides et al., 1983; Moynagh et al., 1991; Itzhak et al., 1993; Park et al., 1996; Karchewski et al., 2004; Hernstadt et al., 2009; Varga et al., 2009). Microglial TSPO requires a particular attention due the key role of this cell type in the initiation and maintenance of chronic pain states (Tsuda et al., 2005). The recruitment of TSPO signaling is also of interest while dealing with pain models associated with lesions during the process of recovery. For example, regulation of the expression of TSPO and of their endogenous ligands have been well studied during rat sciatic nerve degeneration and regeneration (Rupprecht et al., 2010). After nerve freezing lesion or chronic

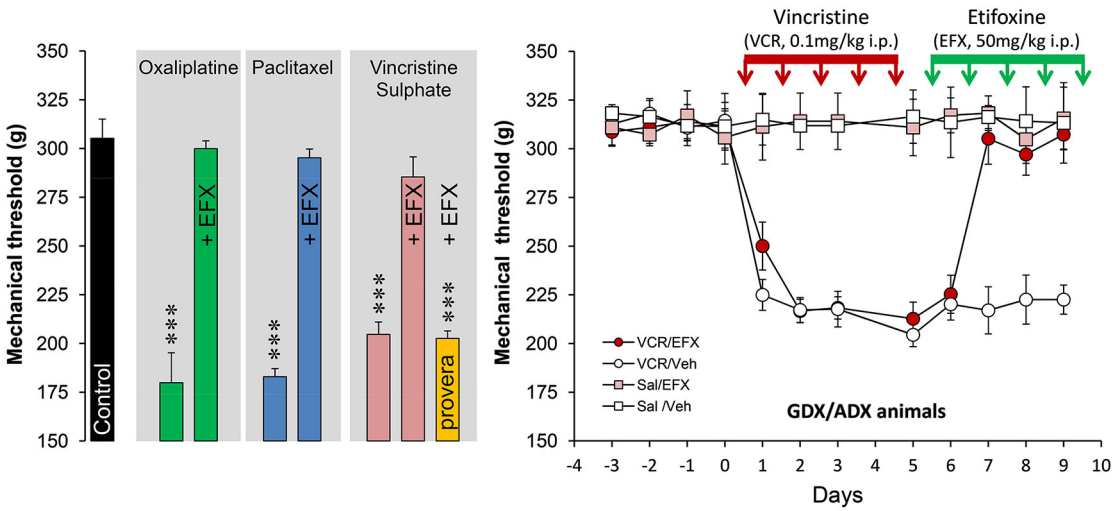

FIGURE 2 | Histogram on the left illustrates the mechanical nociceptive threshold measured with a calibrated forceps in control (black bar, treated with the vehicles) and after treatment with oxaliplatin (green bars; 2 mg/kg, injection i.p. twice-weekly for four-and-a-half consecutive weeks), paclitaxel (blue bars; 2 mg/kg i.p. on four alternate days: day 1,3,5,7), vincristine sulphate (pink bars; 0.1 mg/kg, five daily injections i.p.). Note that all rats exhibited low mechanical thresholds after chemotherapy (Tukey, $p < 0.001$; $n = 6$–8 rats per groups) but normal thresholds were restored after five daily injections with etifoxine (EFX, 50 mg/kg i.p.). EFX analgesia was abolished in animals pre-treated for 1 week before VCR with depo-provera (orange bar; inhibitor of 3α-HSOR, five daily injections of 5 mg/kg s.c.). Graph on the right illustrates the time course of mechanical threshold in gonadectomized/adrenalectomized (GDX/ADX) rats, submitted to VCR chemotherapy (or saline) and treated for their mechanical allodynia with EFX (or vehicle). Note that EFX analgesia persisted even in the absence of peripheral source of steroids. Abbreviations: EFX: etifoxine, Sal: saline, VCR: vincristine, Veh: vehicle. Adapted from Aouad et al. (2010).

denervation, a clear over-expression of TSPO and octadecaneuropeptide (ODN) is observed in Schwann cells and macrophages, suggesting their crucial role during regenerative processes (Lacor et al., 1996). Axonal injury-dependent induction of TSPO has been also observed in small-diameter adult rat primary sensory neurons (Karchewski et al., 2004).

ENDOGENOUS CONTROL OF ALLOPREGNANOLONE SYNTHESIS AND ANALGESIC FUNCTION

Many endogenous ligands of TSPO have been identified and are described in detail in a recent review (Rupprecht et al., 2010). If their binding characteristics and neurosteroidogenic activities are well understood, very little is known regarding their role in brain functions and pathologies (Rupprecht et al., 2010). Cholesterol and porphyrins are important endogenous ligands of TSPO and display nanomolar to micromolar affinities, respectively. Endozepines, discovered at the end of the 80 s, are peptidergic ligands capable of displacing the binding of classical benzodiazepines at their $GABA_AR$ binding sites (Costa and Guidotti, 1991). The endozepine diazepam-binding inhibitor (DBI) and its metabolites were later shown to stimulate neurosteroidogenesis after binding to TSPO (Papadopoulos et al., 1991; Do-Rego et al., 1998). Few reports are available suggesting their implication in pain. An antinociceptive effect of DBI on thermal and mechanical thresholds has been reported after intrathecal or i.c.v. infusion of DBI (Wang et al., 2002). In line, i.c.v. injection of a bovine endozepine potentiated morphine analgesia in mice (Chen et al., 1991). In apparent contradiction, the DBI-derived ODN was shown to increase aggressive interactions in mice and, surprisingly, to reduce defeat-induced analgesia (Kavaliers and Hirst, 1986). In summary, these rare reports suggest a role for endogenous TSPO ligands in the control of nociception. The real expression levels of endogenous TSPO ligands, their cellular localization and changes in pain pathologies are, however, still largely unknown. This is in sharp contrast with TSPO expression which has been found to be increased in several neuropathologies including in pain models.

Beside TSPO ligands, we showed that oxytocin, a neuropeptide released by hypothalamic neurons projecting onto spinal nociceptive neurons, is exerting its long-lasting analgesic effect by stimulating the production of AP (Juif et al., 2013). Apart from its fast antinociceptive action in the spinal cord which increases GABAergic inhibitory transmission and reduces neuronal excitability (Breton et al., 2008, 2009), the tonic activation of spinal oxytocin receptors in inflammatory pain states also stimulates AP synthesis via ERK (for extracellular signal-regulated kinase) signaling pathways, resulting in the potentiation of GABAergic inhibition and limitation of pain symptoms (Juif et al., 2013). It is not excluded at this stage that other descending inhibitory controls may also exert their antinociceptive action by modulating neurosteroid synthesis, but this remains to be demonstrated. Another example is related to substance P secretion by nociceptive primary afferents which may inhibit the production of AP through its NK1 receptor, as previously demonstrated (Patte-Mensah et al., 2005). Taken into account this result, we may speculate the diffuse AP-based analgesic system to be under the control of

a balance of stimulating and inhibiting neuropeptides in the spinal cord. This hypothesis requires to be tested in order to identify the most promising peptides for future therapeutic interventions.

CONCLUSIVE REMARKS

In conclusion, there are now several convergent experimental evidences demonstrating that the local production of AP-like steroids in the nociceptive system constitutes a diffuse analgesic system. The development of numerous TSPO ligands capable of stimulating this system is on the way, but little is known regarding other possible physiological stimulators. In line with this idea, neuropeptides could play a key role.

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PKCε and allopregnanolone: functional cross-talk at the $GABA_A$ receptor level

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Changes in GABAergic inhibition occur during physiological processes, during response to drugs and in various pathologies. These changes can be achieved through direct allosteric modifications at the γ-amino butyric acid (GABA) type A ($GABA_A$) receptor protein level, or by altering the synthesis, trafficking and stability of the receptor. Neurosteroids (NSs) and protein kinase C (PKC) are potent modulators of $GABA_A$ receptors and their effects are presumably intermingled, even though evidence for this hypothesis is only partially explored. However, several PKC isoforms are able to phosphorylate the $GABA_A$ receptor, producing different functional effects. We focused on the ε isoform, that has been correlated to the sensitivity of the $GABA_A$ receptor to allosteric modulators and whose expression may be regulated in peripheral sensory neurons by NSs. The cross-talk between PKC-ε and NSs, leading to changes in $GABA_A$ receptor functionality, is considered and discussed in this perspective.

Keywords: GABAA receptor, phosphorylation site, neurosteroids, receptor traffiking, PKCepsilon

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Introduction

$GABA_A$ receptors are fundamental for fast synaptic inhibition in the brain. Given the role of these receptors in synaptic transmission, all the mechanisms that regulate their activity are of primary importance. One that is particularly significant is receptor phosphorylation. Kinases represent a superfamily of isoenzymes that, through protein phosphorylation, regulate several cellular processes, including proliferation, differentiation, tumorigenesis, cytoskeletal remodeling, receptor function and synaptic transmission modulation (Battaini, 2001). This superfamily comprises the protein kinase (PK) A, G and C, which are serine/threonine phosphotransferases.

Protein kinase C (PKC) is one of the most significant kinases for $GABA_A$ receptor modulation. Phosphorylation of the receptor can produce different effects, ranging from enhancement to inhibition of protein function, depending on the subtype of subunit targeted and on the location of the sites being phosphorylated (Moss and Smart, 1996).

In particular, among PKC enzymes there are conventional (α, βI, βII and ϒ) and new (δ, ε, η and θ) isoforms (Nishizuka, 1995), that are differently expressed in the nervous system. The differences among PKC isoforms contribute to the large range of functions they may perform.

Some PKCs directly bind to the intracellular domain of specific $GABA_A$ receptor subunits, providing in this way a rapid regulation of the receptor activity by all the intracellular pathways that activate this kinase (Brandon et al., 1999).

PKC-Epsilon (PKC-ε)

PKC-ε is classified as a novel isoform present in the brain, found mainly in the cerebral cortex, cerebellum, hippocampus and, in small amount, in non-nervous peripheral tissues (Saito et al., 1993; Chen et al., 2000).

The characterization of PKC-ε null mice has provided insights into the role of PKC-ε in the central nervous system (CNS; Hodge et al., 1999). Animals lacking PKC-ε show hypersensitivity to the behavioral effects of allosteric modulators of $GABA_A$ receptors, such as ethanol and neurosteroids (NSs; Hodge et al., 2002). Among NSs, the progesterone metabolite Allopregnanolone (Allo) is one of the most important endogenous steroid in the CNS (Baulieu and Robel, 1990), as changes in its concentration correlate with physiological and pathological conditions(Maguire and Mody, 2007; Luchetti et al., 2011).

In vitro studies on cortical synaptosomes demonstrate that a peptide able to inhibit PKC-ε translocation (Khasar et al., 1999) produces an increase in Allo sensitivity (Hodge et al., 1999). Similarly, in primary cultures of cortical neurons Allo is more effective in potentiating GABA-evoked current when the cells are intracellularly perfused with this same inhibitory peptide (**Figure 1A**). From analysis of the dose-response curves of this Allo effect, it is evident that the potency of NSs remains unchanged after blocking PKC-ε translocation, whereas the efficacy is increased (**Figure 1B**). We cannot determine whether the receptors mediating the increased response to Allo were at synaptic or extrasynaptic sites. However, it is possible that a selective phosphorylation by PKC-ε occurs, in this case the kinase activity determines selective changes only in synaptic or in tonic currents. The use of a heterologous system, expressing different $GABA_A$ receptor subunits, or specific agonists for "tonic receptors" could provide the answer to this issue.

Recent studies showed that PKC-ε action depends on the phosphorylation of the $GABA_A$ receptor at the level of Ser327 of the γ2 subunit (Qi et al., 2007), in turn regulating the response of the receptors to allosteric modulators. Furthermore, PKC-ε kinase controls $GABA_A$ receptor trafficking through the N-Ethylmaleimide-Sensitive Factor (NESF)-signaling pathway. Indeed, as suggested by the changes in Allo efficacy from *in vitro* studies (**Figure 1B**), the activation of PKC-ε is able to decrease cell surface expression of these receptors (Chou et al., 2010).

NSs and Phosphorylation

Allo, and other NSs that are positive modulators of $GABA_A$ receptors, potentiate GABA-evoked chloride current through an increase in the channel opening probability (Puia et al., 1990; Twyman and Macdonald, 1992; Zhu and Vicini, 1997). This results in a prolongation in the decay time of inhibitory post-synaptic currents (IPSCs; Harrison et al., 1987; Fáncsik et al., 2000). However, NSs also act on extrasynaptic $GABA_A$ receptors, causing large effects on δ-containing receptors that mediate the tonic current in certain brain regions (Belelli et al., 2002; Stell et al., 2003).

Interestingly, the desensitization of $GABA_A$ receptors plays an important role in NSs' modulation (Zhu and Vicini, 1997), suggesting that the receptor needs to be in a "specific" state to be responsive to these endogenous NSs.

Furthermore, NSs produce long-lasting changes in the efficacy of GABAergic neurotransmission by modulating the phosphorylation of synaptic and extrasynaptic receptors. Indeed, in a recent paper, Abramian et al. showed a new molecular mechanism by which NSs change the efficacy of GABAergic inhibition by increasing surface expression of specific $GABA_A$ receptors (mainly containing α4 subunits), responsible for the tonic current in hippocampus (Abramian et al., 2014). They suggested that this component of GABAergic neurotransmission may be a key regulator of excitability. In this way the phosphorylation process may alter the function and/or trafficking of $GABA_A$ receptors, thus changing the efficacy of $GABA_A$-mediated inhibition.

Interestingly other allosteric modulators of $GABA_A$ receptors, i.e., benzodiazepine, may also change GABA signaling, influencing the diffusion and clustering of receptors at synapses (Lévi et al., 2015). Conversely, it was shown that the potentiating effect of NSs can be decreased after stimulation of the PKC signaling pathway, either in physiological (Brussaard et al., 2000; Brussaard and Koksma, 2003; Maguire et al., 2005; Oberlander et al., 2012) or pathological conditions (Mtchedlishvili et al., 2001; Kia et al., 2011). For example, the phosphorylation state of the $GABA_A$ receptor changes during pregnancy or over the estrous cycle, a phenomenon that can compromise $GABA_A$ modulation by NS action.

The modulatory efficacy of NSs is decreased in dentate granule cells from epileptic rats (Mtchedlishvili et al., 2001). This agrees with the observation that brain and cell specific changes in $GABA_A$ receptors may occur in several epileptic models (Schwarzer et al., 1997; Fritschy et al., 1999; Peng et al., 2004) and in the temporal lobe of human epileptic subjects (Loup et al., 2000; Ferando and Mody, 2012). Recent studies show changes in the phosphorylation of $GABA_A$ receptors after kindling; these modifications together with changes in $GABA_A$ subunit composition could account for a decreased responsiveness to NSs (Kia et al., 2011; Carver et al., 2014). The diminished sensitivity to endogenous positive allosteric modulators, such as Allo, increases susceptibility to seizures, similarly to what happens in women with catamenial epilepsy, where seizures occur more frequently before the onset of menses and NS levels fall due to progesterone crash (Reddy, 2009).

Also important is the cross-talk among PKC-ε, $GABA_A$ receptors and NSs in pain perception. NS production is stimulated as a result of inflammatory pain (Poisbeau et al., 2005), and accordingly changes in PKC-ε expression were

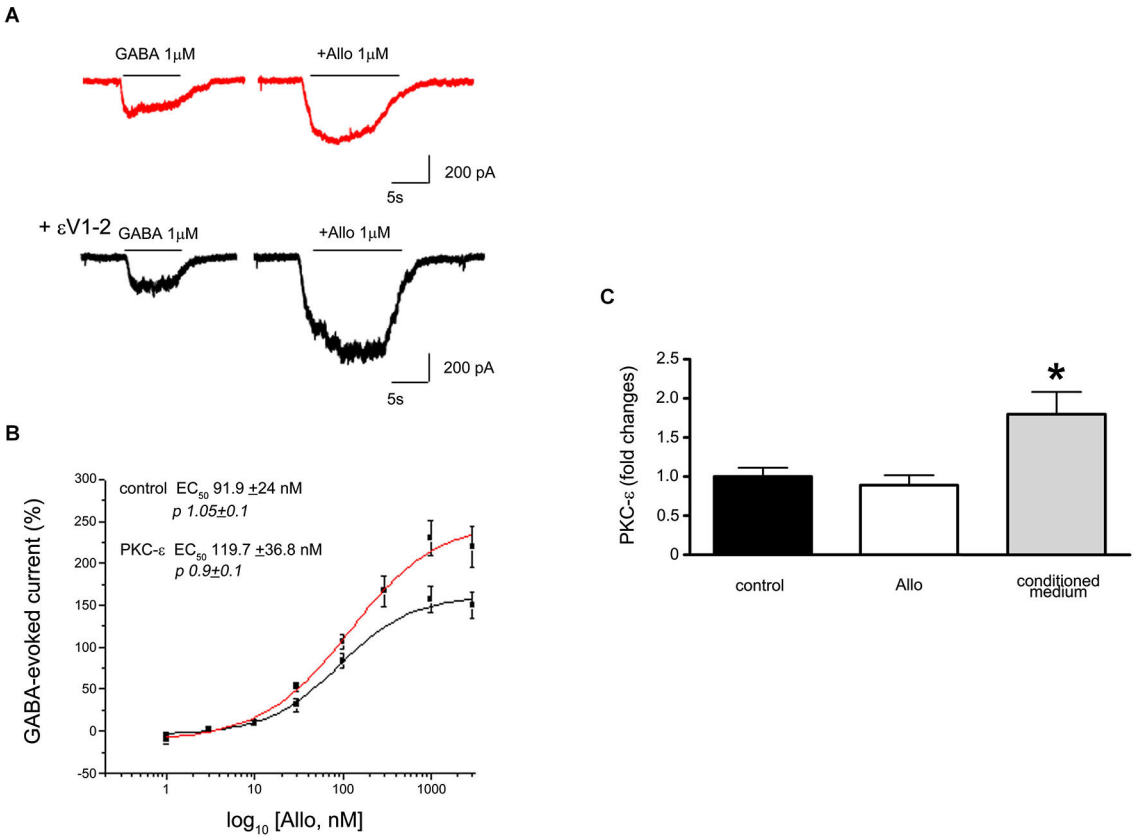

FIGURE 1 | (A) Electrophysiological traces showing the effect of Allo on GABA-evoked currents recorded from cortical neurons in culture (7DIV). The upper trace shows a recording from a control cell (red) and the lower trace is from a cell intracellularly perfused with the peptide εV1–2, a PKC-ε inhibitor. **(B)** Dose response curves of Allo effect in control and in the presence of the peptide eV1–2. Each point is the mean+/−SE of 8–15 cells. PKC-ε blockade did not change the potency of Allo but affected its efficacy. **(C)** Assessment of PKC-ε gene expression changes (as fold changes) in primary culture of dorsal root ganglia (DRG) neuron after 24 h exposure to 10^{-6} M Allo (white column), or to the culture conditioned medium obtained from Schwann cell cultures treated with 10^{-6} M Allo (gray column). The relative quantification of mRNA was obtained by quantitative real time PCR. Data were normalized to the housekeeping genes α-tubulin and β2-microglobulin and expressed as difference ($^{\Delta\Delta}$Ct) vs. controls, then averaged for each experimental group. The black column represents the controls (DRG neurons treated with vehicle, ethanol). Experiments were repeated at least three times (*$p < 0.05$ Anova Test).

observed during pathological pain (Parada et al., 2003). Vergnano et al. proposed that the 3α-5α-NSs (such as Allo) could be part of an endogenous compensatory mechanism, in response to sustained activation of the spinal nociceptive system that occurs during pathological conditions (Vergnano et al., 2007).

The persistence of incoming pain messages, and the associated increases in intracellular Ca^{2+} concentration, could induce a strong stimulation of Ca^{2+}-dependent PKC. This leads to a functional block of $GABA_A$ receptors in their current state. Therefore, PKC prevents further 3α-5α-NS dependent potentiation, without decreasing the basal modulatory effect of 3α-5α-NSs (Vergnano et al., 2007).

PKC-ε exerts a modulatory action on the pain pathways acting on CNS neurons, but also at the peripheral nervous system (PNS) level, for instance on dorsal root ganglia (DRG) neurons. Indeed, PKC-ε may alter the permeability of Na-type Ca^{2+} channels in DRG, and it has been shown to enhance nociception (Van Kolen et al., 2008). The capability of endogenous mediators to regulate PKC-ε gene expression has already been demonstrated for several molecules, including the neuropeptide ghrelin, thyroid hormones, the apolipoprotein E3 and some miRNAs (Rybin and Steinberg, 1996; Alipour et al., 2011; Sen et al., 2012). However, no data reported on the possible modulation exerted by NSs on PKC-ε gene expression. Recent studies by qRT-PCR analysis evaluated the possible modulation of PKC-ε expression in DRG neuronal cultures following 24-h treatment with Allo (10^{-6} M). Allo did not change PKC-ε expression under basal conditions, but was significantly upregulated in DRG neurons exposed to the culture medium from Allo-treated Schwann cells (**Figure 1C**). These findings suggest that Allo-treated Schwann cells can release one or more factors able to modulate PKC-ε expression in DRG neurons. However, Schwann cells also express basal levels of PKC-ε (Borghini et al., 1994). Overall, we speculate that these mechanisms identify novel putative circuits involved

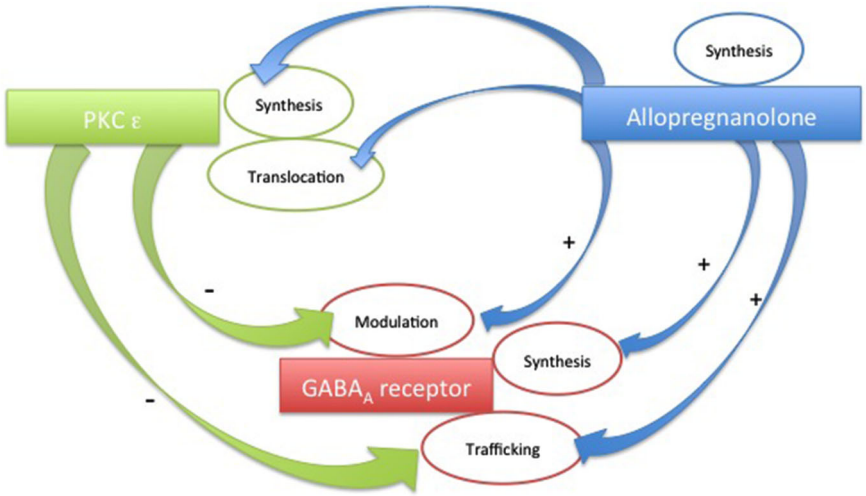

FIGURE 2 | Schematic plot of the possible cross-talks among PKC-ε, Allopregnanolone and GABA$_A$ receptors.

in the regulation of pain processes at PNS and spinal cord levels.

Given that Allo is considered one key factor in the modulation of peripheral pain pathways, its capability to regulate PKC-ε is promising and opens new perspectives for the identification of the basic mechanisms regulating chronic pain onset.

Conclusions

The activity of GABA$_A$ receptors must be finely regulated in the CNS. For this reason when NSs increase GABA$_A$ receptor activity, the "system" tries to re-equilibrate by activating different PKs. The increase in neuronal activity that occurs by activating L-type Ca^{2+} channels leads to a Ca^{2+}/calmodulin-dependent PK type II phosphorylation of the GABA$_A$ receptor β3 subunit. This in turn produces a rapid insertion of receptors in the membrane, with a consequent increase in tonic current (Saliba et al., 2012). Similarly, NSs promote GABA$_A$ receptor phosphorylation, leading to an increase in extrasynaptic receptor expression (Abramian et al., 2014).

These findings shed light on a new type of modulatory activity played by NSs. Indeed, NSs not only allosterically modulate GABA$_A$ receptor function and synthesis, but can also regulate membrane trafficking of the receptor protein, which is particularly important in determining synapse efficacy. Another NS, pregnenolone sulphate, uses the same "strategy" to modulate N-Methyl-D-aspartate (NMDA) receptor-mediated neurotransmission. Indeed, its effects are determined by direct modulation of the NMDA receptor, but also by increasing receptor expression on the cell surface (Kostakis et al., 2013).

Interestingly, in physio-pathological situations, or after pharmacological treatments, NSs (endogenous and exogenous) and PKC activities may vary a lot. The precise role of functional cross-talk between these "modulators" (Adams et al., 2015) and how these interactions can affect GABA$_A$ receptor function are still a matter of investigation.

In **Figure 2** we summarize some of the interrelationships among these three players. We believe that it is important to keep in mind these pathways. Eventually, shedding light on other presently unknown cross-talks will help to better understand the mechanisms underlying some neuropathologies, as well as unraveling the mechanisms of action of novel GABA$_A$ modulating drugs. However, a complex picture emerges from the recent findings. The pharmacological response to endogenous molecules or to exogenous drugs results from the dynamic interrelationship between modulators and receptor proteins. This cross-talk can produce different responses, depending on factors such as cellular distribution or subtype and phosphorylation state of the receptor involved.

As a general perspective, the importance of GABA$_A$ receptor phosphorylation is particularly relevant when a pharmacological treatment involving allosteric GABA$_A$ receptor modulators, such as NSs, is started. However, it should be emphasized that GABA$_A$ receptor rearrangement at the synapse level, and/or changes in receptor subunit composition, can lead to different pharmacological effects. Altogether, these hypotheses should be taken into account to better understand the complex behavior of NSs at the level of neuronal circuitries (Puia et al., 2012) and in *in vivo* studies.

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REVIEW ARTICLE
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Novel receptor targets for production and action of allopregnanolone in the central nervous system: a focus on pregnane xenobiotic receptor

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Neurosteroids are cholesterol-based hormones that can be produced in the brain, independent of secretion from peripheral endocrine glands, such as the gonads and adrenals. A focus in our laboratory for over 25 years has been how production of the pregnane neurosteroid, allopregnanolone, is regulated and the novel (i.e., non steroid receptor) targets for steroid action for behavior. One endpoint of interest has been lordosis, the mating posture of female rodents. Allopregnanolone is necessary and sufficient for lordosis, and the brain circuitry underlying it, such as actions in the midbrain ventral tegmental area (VTA), has been well-characterized. Published and recent findings supporting a dynamic role of allopregnanolone are included in this review. First, contributions of ovarian and adrenal sources of precursors of allopregnanolone, and the requisite enzymatic actions for *de novo* production in the central nervous system will be discussed. Second, how allopregnanolone produced in the brain has actions on behavioral processes that are independent of binding to steroid receptors, but instead involve rapid modulatory actions via neurotransmitter targets (e.g., γ-amino butyric acid-GABA, N-methyl-D-aspartate- NMDA) will be reviewed. Third, a recent focus on characterizing the role of a promiscuous nuclear receptor, pregnane xenobiotic receptor (PXR), involved in cholesterol metabolism and expressed in the VTA, as a target for allopregnanolone and how this relates to both actions and production of allopregnanolone will be addressed. For example, allopregnanolone can bind PXR and knocking down expression of PXR in the midbrain VTA attenuates actions of allopregnanolone via NMDA and/or $GABA_A$ for lordosis. Our understanding of allopregnanolone's actions in the VTA for lordosis has been extended to reveal the role of allopregnanolone for broader, clinically-relevant questions, such as neurodevelopmental processes, neuropsychiatric disorders, epilepsy, and aging.

Keywords: midbrain, ventral tegmental area, allopregnanolone, neurosteroid, reproduction, pregnane xenobiotic receptor, non-genomic

INTRODUCTION

Now it is generally understood that cholesterol-based hormones ("steroids") can be produced in the brain and peripheral nerves and not only in traditional steroid organs, such as the ovaries, adrenals, and placenta. This notion is based on initial findings by Baulieu and colleagues in the early eighties, and further supported by decades of follow-up studies (Baulieu, 1980, 1991). These steroids, which are produced in the brain and the peripheral nerves, were given the name "neurosteroids" to differentiate them from the same steroids that are produced by peripheral glands. These initial discoveries demonstrated that precursors to the pregnane steroids, such as pregnenolone were greater in the brain and peripheral nerves, than in circulation. As well, the same steroidogenic enzymes in the peripheral steroid gland were found to be expressed in the nervous system and involved in production of these molecules (Compagnone and Mellon, 2000; Furukawa et al., 2002). These steroids measured in the brain may be also products of metabolism of peripheral organ derived precursors; these molecules are referred to as "neuroactive" steroids. The pregnane steroid, 5α-pregnan-3α-ol-20-one (a.k.a. allopregnanolone or 3α,5α-THP) will be the focus herein. Levels of allopregnanolone in the nervous system can be much greater than circulating levels, and even persist after removal of the glands that produce pregnane steroids in the body (i.e., following ovariectomy—OVX and/or adrenalectomy—ADX). Indeed, these and other studies substantiated the notion that allopregnanolone

is synthesized *de novo* in the brain and peripheral nerves, and that levels in the nervous system are not only a product of metabolism from peripheral gland-derived precursors and subsequent accumulation in neural tissues (Baulieu, 1980, 1991; Majewska, 1992; Paul and Purdy, 1992; Mellon, 1994). A central question in our laboratory has been in determining the extent to which allopregnanolone's functional effects are related to its synthesis in the brain, and/or metabolism of its precursors from the periphery (e.g., progesterone), in the brain. A brief summary of the key information supporting the role of allopregnanolone as a neurosteroid and neuroactive steroid is as follows.

There are highly coordinated actions of steroidogenic enzymes in neurons and glia in regions of the brain supporting production of allopregnanolone as a neuroactive steroid and neurosteroid. The brain and peripheral nerves express all of the enzymes required for metabolism or biosynthesis of allopregnanolone (Compagnone and Mellon, 2000). Regarding metabolism, circulating progesterone, secreted from peripheral glands, can be sequestered and accumulated in the brain, and then can be metabolized by enzymes to other neuroactive metabolites. Formation of allopregnanolone from progesterone is dependent upon sequential actions of 5α-reductase (which produces dihydroprogesterone), and then 3α-hydroxysteroid dehydrogenase (3α-HSD). Additionally, allopregnanolone can be formed from biosynthesis in the brain itself (Baulieu, 1991; Paul and Purdy, 1992; Mellon, 1994; King et al., 2002; Papadopoulos et al., 2006a,b; Batarseh and Papadopoulos, 2010). The requisite factors for allopregnanolone biosynthesis involves the 18kDA translocator protein (TSPO, formerly known as the mitochondrial benzodiazepine receptor or the peripheral-type benzodiazepine receptor), which binds cholesterol at high affinity. TSPO, with the steroidogenic acute regulatory (StAR) protein, have actions to transport cholesterol into mitochondria, which is considered a rate-limiting step for allopregnanolone biosynthesis (Mellon and Deschepper, 1993; King et al., 2004; Papadopoulos et al., 2006a,b). Cholesterol is then oxidized to pregnenolone by cytochrome P450-dependent C27 side chain cleavage enzymes (P450scc), which is converted to progesterone by 3β-hydroxysteroid dehydrogenase enzymes. Progesterone from this biosynthesis, can then be converted to allopregnanolone by actions of 5α-reductase and 3α-HSD. As such, production of allopregnanolone can be from metabolism of circulating progesterone, or *de novo* production of progesterone in the nervous system. All of these factors involved in metabolism to, or biosynthesis of, allopregnanolone, described above, are expressed in the spinal cord, cerebellum, hindbrain (e.g., pons, medulla), midbrain (e.g., tegmentum), and forebrain (e.g., corticolimbic regions, such as prefrontal cortex and hippocampus, as well as basal ganglia, hypothalamus, and thalamus); however, there are differences in expression based upon many factors, including age, sex, hormonal milieu, cell type, context (Mellon, 2007; Frye, 2009). Nevertheless, the vast distribution of these factors, and their conservation across species (see review Mellon, 2007), implies the importance of neuro(active) steroids, such as allopregnanolone, for brain function, and supports investigations to understand the functional significance of allopregnanolone from metabolism and/or biosynthesis (Melcangi et al., 2014).

A focus in our laboratory for over 25 years has been how production of allopregnanolone is regulated, and the novel targets for allopregnanolone's functional effects, including behavioral endpoints. This review will summarize early studies about challenge/stressor-induced biosynthesis of allopregnanolone and what is known about allopregnanolone synthesis and its actions from studies using mating as a manipulation and measure in our laboratory. Additionally, there will be a focus on recent studies, and inclusion of data in support, regarding the role of the pregnane xenobiotic receptor (PXR) as a novel factor for allopregnanolone synthesis and actions. Lastly, there will be a discussion of how these basic studies centered on allopregnanolone synthesis and action in the midbrain of rodents have been extended to clinically-relevant findings.

CHALLENGE-INDUCED ALLOPREGNANOLONE SYNTHESIS

Early studies investigating allopregnanolone as a neurosteroid identified that environmental challenge, or stressors, can induce allopregnanolone biosynthesis. In support, acute cold-water swimming, an experimental model of an acute physical stressor in rodents, increases brain production of allopregnanolone (Purdy et al., 1991; Barbaccia et al., 1996; Vallée et al., 2000). Similar effects are observed with other acute stressors, such as footshock, ether exposure, and/or carbon dioxide exposure have been demonstrated in intact, gonadectomized/ovariectomized (OVX), and/or adrenalectomized (ADX) rodents (Paul and Purdy, 1992; Barbaccia et al., 1996). Analogous effects in intact rodents and those with peripheral sources of progesterone removed support allopregnanolone biosynthesis as a response to these challenges. Alternatively, allopregnanolone levels can be reduced following exposure to chronic laboratory stressors in adult rodents, such as social isolation (Serra et al., 2004; Agís-Balboa et al., 2007; Pibiri et al., 2008; Nin et al., 2011; Pinna and Rasmusson, 2012). Exposure to stressors of rodents *in utero* (e.g., immune challenges, restraint stress, immune challenges, exposure to cold, swim stress during the last week of gestation), or in early development (e.g., maternal deprivation) produces long-lasting reductions in allopregnanolone (Kellogg and Frye, 1999; Kehoe et al., 2000; McCormick et al., 2002; Paris and Frye, 2011; Paris et al., 2011a,b). There are functional effects of reducing allopregnanolone synthesis related to these responses in that greater stress responding is associated with lower levels of allopregnanolone (Zimmerberg and Blaskey, 1998; Frye and Walf, 2004; Agís-Balboa et al., 2007; Brunton and Russell, 2011; Paris et al., 2011a,b). Thus, it has been recognized for some time that extreme situations and behavioral experiences can alter allopregnanolone; however, there is now a greater understanding of robust effects of ecologically-relevant behavior, such as mating, on allopregnanolone synthesis.

MATING AS A MANIPULATION AND MEASURE FOR INVESTIGATING ALLOPREGNANOLONE SYNTHESIS AND ACTION IN THE BRAIN

To facilitate further understanding of allopregnanolone's functions and targets, and the role of its metabolism or biosynthesis, it has proved useful to focus on a behavior that is reliant upon allopregnanolone synthesis and actions (and subsequently

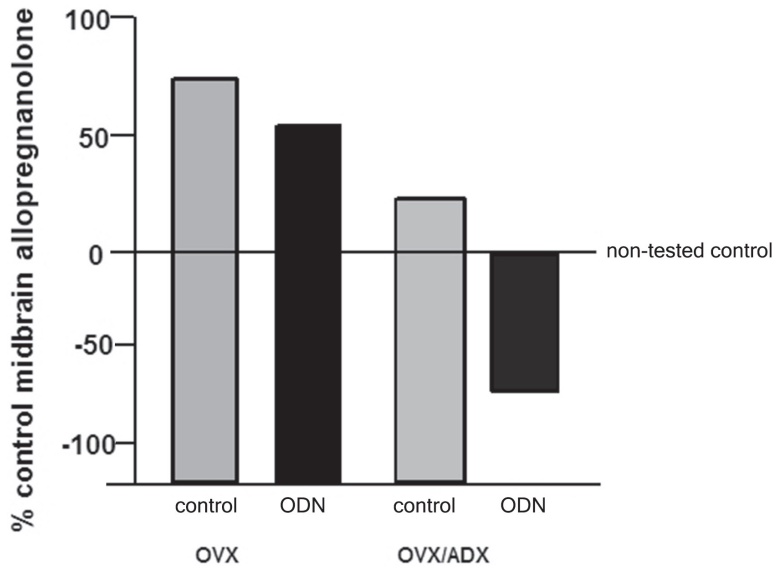

FIGURE 1 | Depicts the midbrain allopregnanolone levels of behaviorally tested animals compared to non-tested rats that were ovariectomized (OVX) or OVX and adrenalectomized (OVX/ADX), estradiol-primed and behaviorally tested in the paced mating task as a percent of the non-tested controls. Rats in these conditions were also administered saline vehicle or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotides (AS-ODNs) to the midbrain VTA. Paced mating increased midbrain allopregnanolone levels of rats compared to what was observed in the non-tested controls; this was attenuated with knockdown of PXR.

extend this approach to other functions, described later in this review). In our laboratory, mating behavior of female rodents is thus utilized as both a manipulation and measure to elucidate allopregnanolone's role. From studies using this approach, the importance of synthesis and actions of allopregnanolone in the midbrain ventral tegmental area (VTA) have been consistently revealed, and will be discussed in the following paragraphs.

MATING BEHAVIOR ASSESSMENT

The midbrain VTA is known for its actions for motivated responses, and mating can be considered such a motivated behavior. Mating responses of females are quantified with measures of lordosis, proceptivity, and aggression. Lordosis, the necessary posture of female rodents for mating, can be quantified in the laboratory as the number of such responses by the female as a ratio (or quotient; lordosis quotients) of the attempts by the male. Other behaviors, such as proceptivity (courtship behaviors; proceptivity quotients) or aggression (rejection of males' advances; aggression quotients) can be concurrently assessed with lordosis. As well, in our laboratory, we typically assess other behaviors beyond those directly related to mating, but those that may have consequences for successful reproduction, such as exploration, reductions in fear/anxiety, and social behavior with conspecifics (for review see Frye, 2009). Mating is a motivated behavior that is only observed under appropriate endocrine and environmental contexts, and one in which the brain circuitry necessary for it to occur (namely in the hypothalamus and midbrain for female rodents), and may modify its expression (e.g., corticolimbic structures), are becoming better characterized (DeBold and Malsbury, 1989; Frye and Walf, 2008; Pfaff et al., 2008; Frye, 2011).

ALLOPREGNANOLONE IN THE MIDBRAIN VTA IS NECESSARY AND SUFFICIENT FOR MATING

By utilizing this behavioral response of mating as a bioassay, we have been able to determine that allopregnanolone, from both metabolism of circulating progesterone, and biosynthesis in the midbrain, in the midbrain VTA is necessary and sufficient for mating (reviewed recently in Frye, 2011). Requisite enzymes and proteins for metabolism and biosynthesis of allopregnanolone are expressed in the midbrain VTA as well as in corticolimbic regions that may be involved (Cheng and Karavolas, 1975; Li et al., 1997; Furukawa et al., 2002; Frye, 2011; Frye et al., 2013a). Observations of age-related changes in reproductive behaviors and timing of reproductive senescence among female rats suggest that reductions in capacity to form allopregnanolone in the midbrain may be involved (Walf et al., 2011). Genetic knockout of 5α-reductase in female mice lowers allopregnanolone levels in the midbrain and attenuates lordosis during proestrous (when females typically have their highest levels of allopregnanolone, coincident with mating), and following ovariectomy and progesterone administration (Koonce and Frye, 2014). 5α-reductase knockout mice have normative responses to allopregnanolone administration. In addition to these findings that suggest the importance of progesterone metabolism in the midbrain for mating, there are data in support of the role of allopregnanolone synthesis in the VTA for mating. Antagonists of TSPO, P450scc, and 3β-HSD, delivered directly to the midbrain VTA, of receptive rats attenuates lordosis similarly as inhibitors of metabolism by 5α-reductase and 3α-HSD (reviewed in Frye, 2011; Frye et al., 2013a). As well, agonists of TSPO can have similar actions as allopregnanolone to OVX and ADX rats to increase midbrain levels of allopregnanolone and lordosis (reviewed

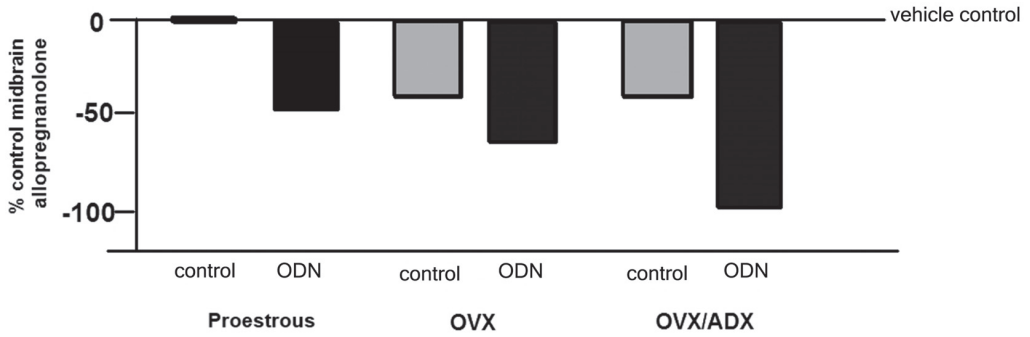

FIGURE 2 | Depicts the midbrain allopregnanolone levels of paced mated proestrous, ovariectomized (OVX) and estradiol-primed, or OVX and adrenalectomized (OVX/ADX) estradiol-primed rats infused with saline vehicle or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotides (AS-ODNs) to the midbrain VTA. Data are represented as a percent of the saline-infused proestrous control group. Administration of PXR AS-ODNs to the midbrain reduces allopregnanolone levels in the midbrain following mating across these hormone conditions.

in Frye, 2011; Frye et al., 2013a). Together, these approaches have suggested the importance of allopregnanolone, from both metabolism and biosynthesis, in the midbrain VTA for mating.

MATING-INDUCED ALLOPREGNANOLONE SYNTHESIS IN THE MIDBRAIN

In addition to being a measure of interest, mating can induce allopregnanolone formation in the nervous system, and, thereby, can be considered a manipulation as well. Among proestrous female rats that engage in mating with a male, there is a rapid increase in allopregnanolone levels in the midbrain; this same pattern of allopregnanolone synthesis is not observed with the smell, or site, of a sexually-experience male, or a female conspecific (Frye and Bayon, 1999; Frye et al., 2007). Notably, allopregnanolone levels are higher following "paced mating" compared to a standard mating task (Frye, 2001a,b, 2009, 2011; Frye et al., 2007, 2014b). Paced mating is considered a semi-naturalistic mating paradigm as compared to a standard mating paradigm, which is typically performed in a small chamber in a laboratory (e.g., a 10 gallon aquarium). Paced mating is considered closer to the natural experience because the chamber is larger and divided with an entry only a female can transverse to get to the other side of the chamber; as such, female rats can control the timing of (i.e., "pace") their mating contacts with males, which is a critical part of the natural response in the wild and to enhance fertility and fecundity (Frye and Erskine, 1990). Even in the situation that females are tested in a large, paced mating chamber in the laboratory, but do not spontaneously pace, or show a low pacing response (but the same number of mounts by the male), there are lower levels of allopregnanolone in the midbrain compared to females that do show the pacing response (Frye and Rhodes, 2006). These data support the notion that mating can induce allopregnanolone synthesis; albeit, a question is the role of other reproductively-relevant behaviors, which may precede or follow mating, for allopregnanolone synthesis.

PACED MATING, MORE SO THAN OTHER REPRODUCTIVELY-RELEVANT BEHAVIORS, INCREASES ALLOPREGNANOLONE SYNTHESIS

Reproductively-relevant behaviors are those that may improve reproductive success. For example, some of these reproductively-relevant behaviors are those that include increased exploration and reduced anxiety that would promote females leaving the natal nest and traversing a complex and novel environment to encounter other female conspecifics and potential mates for the first time. To address this in our laboratory, paced mating as well as measures of exploration (e.g., open field), anxiety (e.g., elevated plus maze), or social interaction with another female are assessed in a short battery of these tasks. Paced mating itself, or immediately following this battery of tasks, increases allopregnanolone synthesis in the midbrain, compared to testing in the battery without mating (Frye et al., 2007, 2014b). Together, these data support that mating can be utilized as a measure of allopregnanolone's actions as well as a way to manipulate allopregnanolone levels in the midbrain. This model has then been used to assess the mechanisms of allopregnanolone, with a focus on non-traditional actions for mating and reproduction-relevant behaviors.

NON-TRADITIONAL ACTIONS OF ALLOPREGNANOLONE IN THE MIDBRAIN VTA

Allopregnanolone has actions that are considered "non-traditional" when compared to actions peripherally secreted steroids have through binding to cognate steroid receptors in their distal target organs, including brain regions involved in reproductive and homeostatic processes, such as the hypothalamus, midbrain, and limbic system (Pfaff et al., 1976; Shughrue et al., 1997; Osterlund et al., 2000). These effects involve dimerization of the steroid bound receptor, DNA binding, mRNA transcription and translation, and, ultimately, protein expression that would alter the behavior of the cell/organism (often referred to as the "genomic" actions of steroids). It was believed that the shortest latency of when hormones are secreted and bind to receptors and initiate this intracellular process to ultimately alter behavior was on the order of tens of minutes (and even hours to days). This notion was challenged with the discovery of neurosteroidogenesis, by which steroids could be produced in the same tissue that they were having effects for behavior in, and that steroids could have such effects so rapidly that they cannot be explained by these genomic actions. To summarize decades

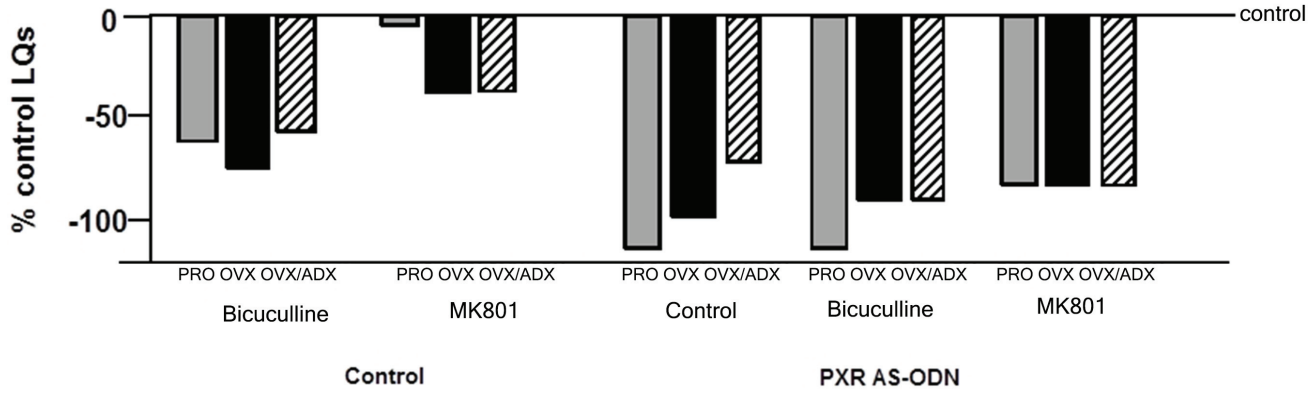

FIGURE 3 | Depicts the lordosis quotients of proestrous (PRO), ovariectomized (OVX) and estradiol-primed, or OVX and adrenalectomized (OVX/ADX) estradiol-primed rats infused with saline vehicle or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotides (AS-ODNs) and then a $GABA_A$ receptor antagonist (bicuculline), or a NMDAR antagonist (MK-801) to the midbrain VTA before behavioral testing. Data are represented as a percent of the saline-infused proestrous control group. PXR AS-ODNs to the VTA potentiated these effects of $GABA_A$ and NMDAR manipulations.

of work by many laboratories, neuro(active)steroids, such as allopregnanolone, are known to have rapid effects, including those on neuronal excitability and synaptic function (Majewska et al., 1986; Morrow et al., 1987; Gee et al., 1995; Brot et al., 1997; Qiu and Lange, 2003; Weir et al., 2004; Lange, 2004; Belelli and Lambert, 2005; Skildum et al., 2005). These rapid effects are understood to involve direct or indirect modulation of ion-gated or other metabotropic neurotransmitter receptors, rather than traditional actions via cognate nuclear steroid hormone receptors; these actions are referred to as a novel or non-traditional actions of steroids. Indeed, many decades ago Hans Selye reported rapid anesthetic and anti-convulsive properties of allopregnanolone and other progestogens (Selye, 1941). In the decades following these observations, GABAergic mechanisms have been described for these anesthetic and anticonvulsant effects of allopregnanolone as well as some of the anxiolytic effects of allopregnanolone (Harrison and Simmonds, 1984; Majewska et al., 1986; Belelli and Lambert, 2005). For over two decades, our laboratory has been focused on GABA, dopamine, and glutamate as targets of allopregnanolone in the midbrain VTA for mating and reproductively-relevant responses.

ALLOPREGNANOLONE HAS ACTIONS VIA GABA, DOPAMINE, AND GLUTAMATE FOR MATING

The VTA has rich innervation of dopamine targets and some of allopregnanolone's actions in the VTA for mating may involve these targets, as well as GABA and glutamate. Progestogens can increase release of GABA, dopamine and glutamate (Lévesque and Di Paolo, 1990; Frye et al., 2000; Frye, 2001a,b). High levels of progestogens enhance number, density, and affinity of $GABA_A$ receptors, coincident with enhancing lordosis (Mascó et al., 1986; Wilson, 1992; Frye and Vongher, 1999). There are D_1 receptors on dopaminergic cell bodies and GABAergic terminals as well as NMDARs (Stoof and Kebabian, 1984; Bayer and Pickel, 1991; Willick and Kokkinidis, 1995). Greater GABA input onto $GABA_A$ receptors that are located on GABAergic interneurons in the VTA mitigate inhibitory actions of these cells on dopamine cell bodies, thereby increasing dopamine release (from cell body and dendrites; Churchill et al., 1992). Excitation of D_1 receptors on GABAergic afferents in the VTA increases GABA release (Kalivas and Duffy, 1995). Antagonists of D_1 or $GABA_A$ reduce allopregnanolone-facilitated lordosis when administered to the VTA and the opposite pattern is observed with agonists of D_1 or $GABA_A$ (Frye et al., 2004; Sumida et al., 2005; Frye and Paris, 2009). Furthermore, antagonists of $GABA_A$ receptors to the VTA reduce allopregnanolone-facilitated lordosis, and the potentiation of this response by a D_1 agonist co-administered to the VTA (Frye et al., 2006c). A role of N-methyl-D-aspartate receptor (NMDARs) is also suggested in this pathway. In support, D_1 expressing GABAergic terminals, synapse on dopaminergic cell bodies that express both $GABA_A$ receptors and NMDARs (Bayer and Pickel, 1991; Willick and Kokkinidis, 1995). Infusions of a NMDAR antagonist to the VTA increases allopregnanolone-facilitated reproductive responding of female rodents (Petralia et al., 2007; Frye and Paris, 2011). Together these findings suggest that allopregnanolone's actions for reproductive responding in the VTA may be related to reductions in tonic inhibition of dopamine neurons in this region, involving actions of $GABA_A$, D_1, and NMDARs here. Additional studies have suggested down-stream pathways for these receptors, including, including activity of G-proteins, adenylyl cyclase, phospholipase C and protein (a discussion of which is beyond the scope of this review, but can be found in Frye and Walf, 2008). Moreover, the functional role of membrane targets of the progestogens, such as the membrane progestin receptors, for reproductive indices have been shown across aquatic species and terrestrial mammals (Tokumoto, 2012; Tokumoto et al., 2012; Frye et al., 2013b, 2014c; Pang et al., 2013; Petersen et al., 2013; Schumacher et al., 2014); the extent to which there are interactions between these ionotropic and metabotropic targets in the VTA is of continued interest. A microarray analysis of gene expression changes in the midbrain of proestrous rats that had been paced mated or not confirmed the role of the targets

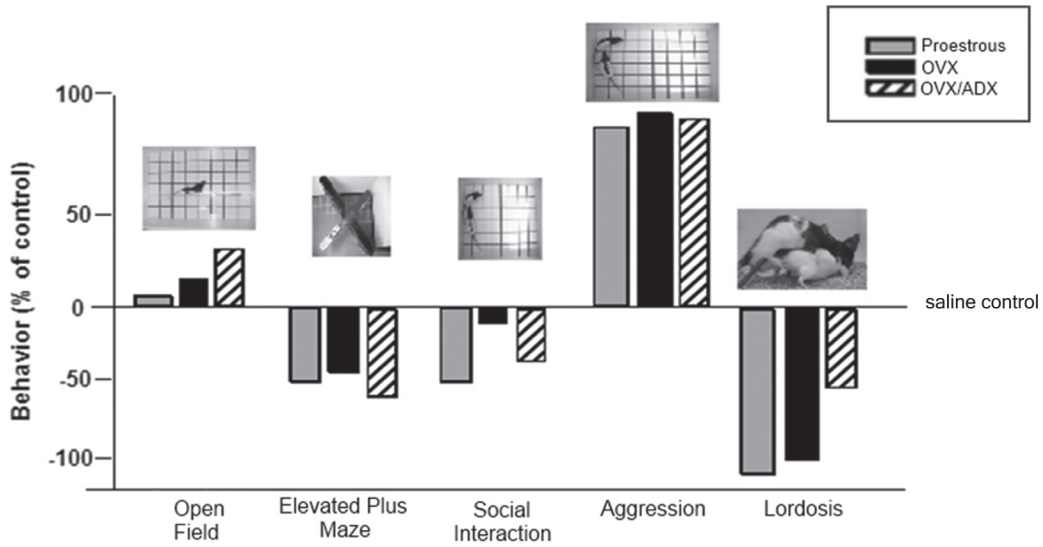

FIGURE 4 | Depicts the pattern of results for all behavioral measures in the testing battery (open field, elevated plus maze, social interaction and paced mating) of rats proestrous (PRO), ovariectomized (OVX) and estradiol-primed, or OVX and adrenalectomized (OVX/ADX) estradiol-primed rats infused with saline vehicle or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotides (AS-ODNs) to the midbrain VTA. Data are represented as a percent of the saline-infused control group for each respective hormone condition. Pictures of the tasks are included about the data bars. The most robust reduction in responding following PXR AS-ODNs infusions to the VTA were observed in the social interaction and paced mating task.

involved in allopregnanolone metabolism and biosynthesis, as well as these neurotransmitter targets, but also revealed a novel target of interest, the pregnane xenobiotic receptor (PXR; Frye and Walf, 2008; Frye, 2009). The findings to date about this novel target in the midbrain are described as follows.

BRIDGING SOURCES AND ACTIONS-ROLE OF PXR IN THE MIDBRAIN VTA

PXR IS EXPRESSED IN THE BRAIN AND MAY HAVE HORMONE-RELEVANT ACTIONS

A recent focus has been on characterizing the role of a promiscuous nuclear receptor, PXR, involved in cholesterol metabolism and expressed in the VTA, as a target for allopregnanolone and how this relates to both actions and production of allopregnanolone. PXR has well-known metabolic and clearance actions in the traditional organs for metabolism and excretion, such as the liver, kidneys, intestines, and the blood-brain barrier (Geick et al., 2001; Dussault and Forman, 2002; Kliewer et al., 2002; Francis et al., 2003; Bauer et al., 2004, 2006; Xu et al., 2005; Harmsen et al., 2007; Ma et al., 2008; Zhang et al., 2008; Ott et al., 2009). It is considered a promiscuous nuclear receptor with a long list of molecules that it positively modulates (including several steroids, and allopregnanolone) and much fewer molecules that are negatively modulated. Although early work on understanding the role of PXR outside of the liver and other excretory organs in the body was focused on the blood-brain-barrier, several laboratories, including our own, have demonstrated its expression in the brain proper (e.g., in rodents, rabbits, pigs, and humans; Bauer et al., 2004; Lamba et al., 2004; Marini et al., 2007; Mellon et al., 2008; Frye, 2011). In considering its role for metabolism and xenobiotic (including steroid) clearance in the liver, we sought to determine PXR's functional effects related to allopregnanolone production and/or action. PXR protein and mRNA was expressed in the midbrain of proestrous rats, with higher expression (determined by western blots) in female rats in proestrous versus those in diestrous or male rats (Frye et al., 2012, 2013b), suggesting a possible role of ovarian steroids (estradiol, progesterone) and/or pregnane neurosteroids. These studies were correlational in nature and demonstrated a relationship between hormonal milieu and sex differences for expression of PXR.

MANIPULATIONS OF PXR IN THE MIDBRAIN FOR MATING

Next, studies investigated manipulations of PXR in the midbrain for functional effects, including lordosis. Positive modulators of PXR, such as allopregnanolone, other pregnane steroids (3β,5α-THP, 3α,5β-THP), and RU486, when infused to the VTA, enhanced lordosis of OVX, estradiol-primed rats (Frye, 2011). However, these findings are tempered by the known promiscuity of PXR. Follow-up studies utilized a pharmacogenetic tool (antisense oligodeoxynucleotides, AS-ODNS) to reduce expression of PXR in the midbrain VTA to further understand functional outcomes (Frye, 2011; Frye et al., 2012, 2013a, 2014a,b). Investigations of the role of PXR, by using this PXR knockdown approach, for mating-induced neurosteroidogenesis and functional effects are ongoing, and some key findings are described as follows.

MANIPULATIONS OF PXR FOR ALLOPREGNANOLONE SYNTHESIS

The role of the PXR for biosynthesis of allopregnanolone in the brain has been investigated. An approach that was utilized to investigate this was to compare the capacity of rats with

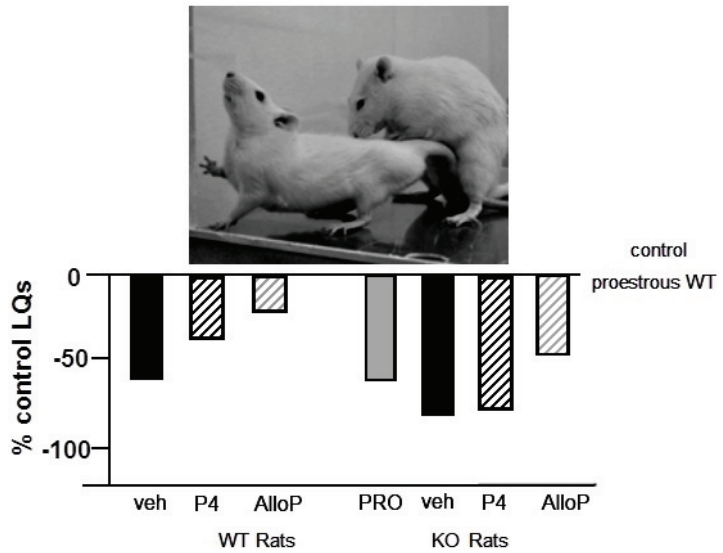

FIGURE 5 | Depicts the lordosis quotients of proestrous (PRO; positive control) and ovariectomized (OVX) rats administered vehicle, progesterone (P_4) and/or allopregnanolone (AlloP) via subcutaneous injections. Comparisons were made in Sprague-Dawley wildtype (WT) and pregnane xenobiotic receptor (PXR) knockout (KO) rats on a Sprague-Dawley background. This strain of rats is commercially available from SAGE Labs. Data are represented as a percent of the WT proestrous control group. A picture of lordosis is included above the data bars. PXRKO rats have lower lordosis than do WT rats across hormone conditions, with similar improvements in lordosis with replacement back of AlloP.

peripheral glands removed (e.g., the ovaries and/or the adrenal glands) to produce allopregnanolone in the midbrain following mating. In comparing rats that were OVX or OVX/ADX, estradiol-primed and behaviorally tested in the paced mating task to non-tested controls, there was a robust increase in midbrain levels of allopregnanolone, particularly among the OVX rats, with paced mating (**Figure 1**). This effect was attenuated when rats were administered PXR AS-ODNs to the midbrain VTA (**Figure 1**). Moreover, comparisons of rats that are paced mated and in different hormonal states (proestrous, OVX, OVX/ADX) and administered saline vehicle or PXR AS-ODN infusions to the midbrain VTA corroborate these findings. Administration of PXR AS-ODNs to the midbrain reduces allopregnanolone levels in the midbrain following mating across these hormone conditions (**Figure 2**). These data suggest a role of PXR for mating-induced allopregnanolone secretion in the midbrain VTA.

PXR IS UPSTREAM OF TSPO FOR ALLOPREGNANOLONE SYNTHESIS

A question is how PXR may interact with other downstream factors recognized to be involved in neurosteroidogenesis. Investigation of this question has begun by assessing the role of TSPO, given that this is one rate-limiting factor for allopregnanolone synthesis in the brain. Inhibiting TSPO with PK11195 reduced allopregnanolone in the midbrain and lordosis, an effect that could be reversed with allopregnanolone replacement, but not when AS-ODNs and allopregnanolone were co-administered. AS-ODNs blocked actions of FGIN 1-27 for lordosis and allopregnanolone levels among proestrous > OVX > OVX/ADX rats. Together, these data support the notion that PXR may be upstream of TSPO. Investigations of the regulation of other related factors are underway.

INTERACTIONS OF PXR, GLUTAMATE AND GABA RECEPTORS

Although these data suggest that PXR is important for the synthesis of allopregnanolone in the midbrain, a related research question is the downstream factors for allopregnanolone's actions. PXR AS-ODNs to the VTA, but not nearby midbrain sites, blocks reproductive responding among receptive rats associated with estrous cycle increases or following estradiol- and progestogen-administration to OVX rats (Frye et al., 2012, 2013a, 2014a). Moreover, knocking down expression of PXR in the midbrain VTA attenuates actions of allopregnanolone via NMDA and/or $GABA_A$ receptors for lordosis (**Figure 3**). That there were some differences noted across hormonal milieu in this study, suggestive of a role of allopregnanolone biosynthesis, follow-up questions would include capacity for allopregnanolone biosynthesis in the brain as well as responses to allopregnanolone administration across these groups.

BEYOND SYNTHESIS AND ACTIONS OF ALLOPREGNANOLONE IN THE MIDBRAIN VTA

MANIPULATIONS OF PXR ARE MOST SALIENT FOR SOCIALLY-RELEVANT BEHAVIORS

Another area of interest is the role of PXR for other socially-relevant behaviors. We have traditionally utilized studies such as those described above to ascertain mechanisms of allopregnanolone using lordosis in a mating task as one endpoint. Mating is typically assessed after other measures of behaviors that may support reproduction (i.e., reproductively-relevant behaviors, such as exploration, anxiety, and pro-social behavior) that allopregnanolone mediates (Frye, 2011; Frye et al., 2012, 2013a,b), perhaps through its actions at $GABA_A$ receptors and NMDARs

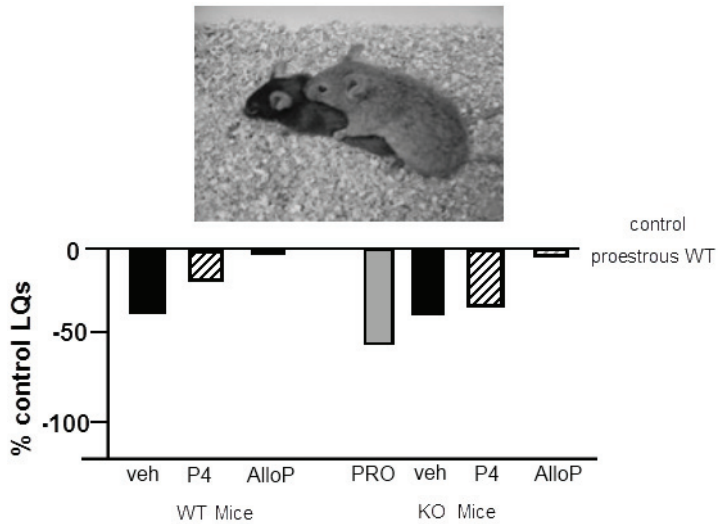

FIGURE 6 | Depicts the lordosis quotients of proestrous (PRO; positive control) and ovariectomized (OVX) mice administered vehicle, progesterone (P_4) and/or allopregnanolone (AlloP) via subcutaneous injections. Comparisons were made to C57BL/6Tac wildtype (WT) and pregnane xenobiotic receptor (PXR) knockout (KO) mice on a C57BL/6Tac background. This strain of mice is commercially available from Taconic. Data are represented as a percent of the WT proestrous control group. A picture of lordosis is included above the data bars. PXRKO mice have lower lordosis than do WT mice across hormone conditions, except for with replacement back of AlloP.

(Frye and Paris, 2009, 2011). In comparing the extent to which PXR AS-ODNs reduce such behaviors, we have consistently noted that the most robust effects are for lordosis quotients, followed by other socially-relevant measures (aggression/rejection during the mating task, and social investigation of a female conspecific), and then affective measures (open arm exploration in the plus maze) and then exploratory/ambulatory behavior (open field entries made; **Figure 4**). These data suggest overall that manipulations of PXR are most salient for socially-relevant behaviors, and that the midbrain infusions of such drug manipulations are not associated with non-specific effects of ambulatory behavior.

BRAIN TARGETS BEYOND THE MIDBRAIN VTA

These comparisons suggest the specificity of the response as well as brain targets outside of the midbrain VTA. Among receptive rats, mating-induced allopregnanolone synthesis is observed in the midbrain as well as corticolimbic structures (hippocampus, prefrontal cortex) and the hypothalamus (Frye et al., 2006a, 2007). PXR AS-ODNs to the midbrain VTA of receptive rats have the most salient effects to reduce allopregnanolone in the midbrain, but reductions are also observed in the hippocampus (Frye et al., 2013a, 2014a). Interestingly, PXR AS-ODNs to the midbrain VTA also reduce levels of the growth factor, brain-derived neurotrophic factor (BDNF), in the hippocampus coincident with differences in behavior (Frye et al., 2014b). Allopregnanolone has actions on BDNF as well as cognitive performance of rodents (Nin et al., 2011; Frye et al., 2013a; Bali and Jaggi, 2014). As such, the extent to which PXR is a target of allopregnanolone beyond the midbrain to corticolimbic structures is of great interest. Indeed, genetic knockout of a related nuclear receptor known for its actions in the liver, the liver X receptor, increases anxiety-like behavior of mice and alters GABAergic function in the hypothalamus, as well as may play a protective role in a Parkinson's disease mouse model (Dai et al., 2012; Tan et al., 2012). Thus, we consider that allopregnanolone may have a role via PXR in the midbrain and beyond for neural and behavioral plasticity.

MATING BEHAVIOR OF PXR KNOCKOUT RATS AND MICE

We have begun characterizing the role of lifelong knock down of PXR as well as species similarities/differences, using PXR knockout (KO) rats and mice. Progesterone administration produced similar rates of lordosis as observed among proestrous wildtype (WT), but not PXRKO, rats; both WT and PXRKO rats responded to allopregnanolone administration with increased lordosis (**Figure 5**). The same pattern was observed among WT and PXRKO mice, suggesting species similarities in this mechanism (**Figure 6**). These data corroborate what has been observed with PXR knockdown in the VTA of rats to reduce lordosis of receptive rats, but show that PXRKO rats can respond to allopregnanolone administration (unlike what has been observed with allopregnanolone infusions to the VTA following PXR AS-ODN infusions; Frye et al., 2014a). These results are promising in that they suggest a specific deficit in synthesis of allopregnanolone, rather than binding of allopregnanolone as just one of many, many positive modulators of this promiscuous nuclear receptor. However, it is not known what the capacity for mating-induced allopregnanolone, and whether there are similar brain targets, is in these rodent models at this time. As well, a typical concern with models of whole body and brain knockout of a gene throughout development is the potential for compensatory mechanisms. Studies are ongoing to characterize these animal model resources further.

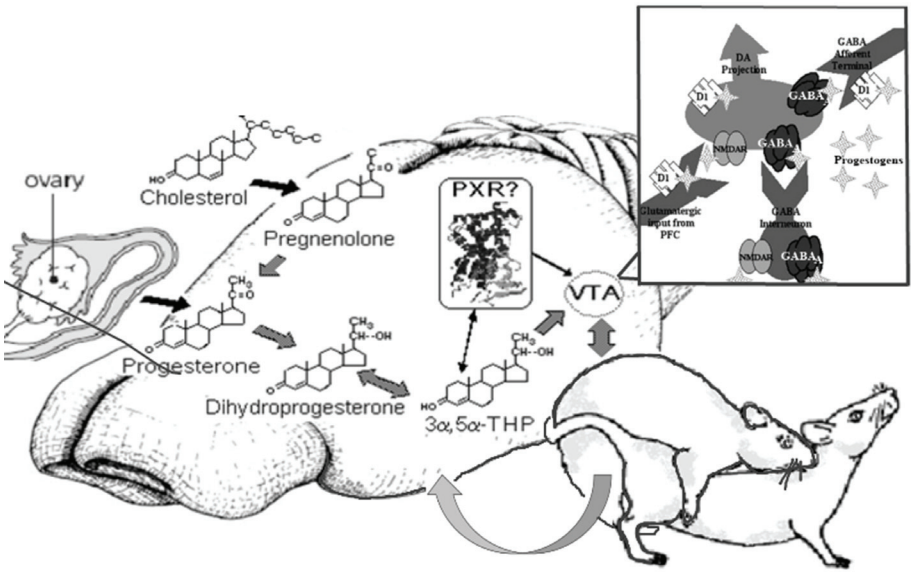

FIGURE 7 | Model of proposed actions of allopregnanolone produced in the brain via PXR and other novel targets, important for reproductive responding. In summary, our lab has been focused on elucidating the novel targets of allopregnanolone, such as PXR, for its functional effects. The data to date suggest that PXR is a novel target of allopregnanolone in the midbrain VTA, as are neurotransmitter targets (e.g., GABA, NMDA), for functional, reproductively-relevant responses, as well as biosynthesis of allopregnanolone in the brain (i.e., acting upstream of TSPO).

OTHER BEHAVIORAL PHENOTYPES IN THE PXR KNOCKOUT RATS AND MICE—THE MIRROR MAZE

Of interest is whether there are other behavioral phenotypes in the PXRKO rats and mice to consider. There are no apparent differences in their homecage behavior, and systematic analyses are underway to assess other behavioral endpoints. A prediction, based upon the data with PXR AS-ODNs, is that the most salient effects of PXR knockout may be for reproductive measures (as supported by data in **Figures 5** and **6**) or social behaviors. Indeed, a pilot assessment of WT and PXRKO mice in the mirror maze supports this notion. The mirror maze is a behavioral assessment of acute changes in rodents' responses to observations of their own image in a mirror (Houri, 1986; Lamberty, 1998). This task, like several others considered to be an index of affective responsing (e.g., elevated plus maze), is considered a free-choice conflict task in which the time spent by the rodent in the mirrored section of a cubed chamber is compared to the time spent away from the mirrors in an adjoined alleyway without mirrors (Henderson et al., 2004; Frye et al., 2006b). We have utilized this task to assess the role of allopregnanolone and other steroid targets, including the androstane equivalent of allopregnanolone (3α-androstanediol; Frye et al., 2006b, 2008; Walf et al., 2009). In initial assessments of female WT and PXRKO mice during the proestrus phase of the estrous cycle, we noted an approximately 15% increase in time spent in the mirror chamber among the PXRKO mice (197 s) than in the WT mice (172 s). This pattern is opposite to what has been noted with PXR knockdown or knockout for interaction with a mate or conspecific (as described above). As well, female mice with knockout of estrogen receptor beta, which may be another important factor in allopregnanolone synthesis, but not progestin receptor, respond poorly in this task. These data in the mirror maze are interesting as they suggest a role of PXR for mediating responses to another socially-relevant stimuli, the rodents' own image in a mirror, beyond a mate (as in the paced mating task) or another female conspecific (as in the social interaction task). Although a focus has been on actions of allopregnanolone in the midbrain VTA, how the understanding of these novel targets in this region can be extended elsewhere in the CNS relevant for clinical conditions is of continued interest.

BEYOND HOMEOSTASIS—ALLOPREGNANOLONE'S ROLE IN TRANSLATION

Diverse functions have been ascribed to the actions of allopregnanolone, including many of the actions described above for reproduction and other reproductively-relevant behaviors. Our understanding of allopregnanolone's actions in the VTA for lordosis has been extended to reveal the role of allopregnanolone for broader, clinically-relevant questions, such as neurodevelopmental processes, neuropsychiatric disorders, epilepsy, and aging (reviewed in Frye, 2009). Some examples about the role of allopregnanolone for seizure and affective processes in clinical populations are as follows. Large clinical trials and a case study support that allopregnanolone may be involved in seizure control (Herzog and Frye, 2003; Herzog et al., 2006, 2014). There are mediating effects of allopregnanolone for anxiety and depressive symptoms among women with premenstrual dysphoric disorder (Endicott et al., 1999; Freeman et al., 2002; Gracia et al., 2009) as well as self-reported anxiety in men with post-traumatic stress disorder following exposure to trauma cue (Casada et al., 1998; Frye, 2009). Furthermore, allopregnanolone may underlie some of the effects of therapeutics. Fluoxetine can enhance dihydroprogesterone (DHP)'s affinity for 3α-HSD, thereby

increasing allopregnanolone formation (Griffin and Mellon, 1999). Reductions in depressive symptoms of men or women diagnosed with major depression are correlated with higher cerebrospinal fluid levels of allopregnanolone (Romeo et al., 1998; Uzunova et al., 1998). Thus, biosynthesis and subsequent rapid effects of allopregnanolone at its non-traditional targets ($GABA_A$, glutamate, dopamine, and PXR) are mechanisms of continued interest with respect to these clinical conditions.

SUMMARY AND CONCLUSIONS

In summary, investigations of allopregnanolone's production and function in the midbrain VTA have focused on mating as a measure and manipulation of allopregnanolone. First, there are traditional (metabolism from peripheral steroids) and nontraditional (biosynthesis, or production in the brain from cholesterol, following challenges such as mating) means for production of allopregnanolone in the central nervous system. Second, the non-traditional mechanisms in the brain that allopregnanolone has for behavioral processes, including mating and reproduction-relevant behaviors, depends upon rapid modulation of neurotransmitters (GABA, glutamate, dopamine), instead of binding to steroid receptors. Third, PXR is a target bridging the synthesis of allopregnanolone with its functions in brain and may be upstream of TSPO and modulate actions of allopregnanolone via neurotransmitter targets (**Figure 7**). Fourth, the significance of studying the functions and mechanism of allopregnanolone in VTA can be extended to clinically-relevant findings for neuropsychiatric, neurodevelopmental, neurodegenerative, and/or age-related disorders. In conclusion, neurosteroids have novel actions, which are now well-accepted, related to their production in the brain and their actions through non-steroid receptor targets. Future considerations include further understanding another characteristic of neurosteroids, which their capacity to induce steroidogenic enzymes in the brain, and thus be involved in clearance (as is PXR). As such, the role of PXR as a factor involved in steroid production, action, *and* clearance in the brain is of continued study in our laboratory.

AUTHOR CONTRIBUTIONS

All authors on this paper substantially contributed to the work reviewed herein and the composition of this manuscript. Carolyn J. Koonce was involved in acquisition, analysis, and interpretation of data represented in figures, and drafting of figures for paper, the reference list and editing this entire work. Alicia A.Walf was involved in acquisition, analysis, and interpretation of data represented in figures, and drafting, editing, and revising of all sections of the paper. Cheryl A. Frye was involved in the conception and study design, acquisition, analysis, and interpretation of data of all studies in the lab described, reviewing, editing, and drafting versions of the work, and giving final approval of the paper to be submitted.

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