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Neurosteroid enantiomers as potentially novel neurotherapeutics

Douglas F. Covey[a,b,c,d], **Alex S. Evers**[b,c,d], **Yukitoshi Izumi**[a,d], **Jamie L. Maguire**[e], **Steven J. Mennerick**[a,d], **Charles F. Zorumski**[a,d,*]

[a]Departments of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA

[b]Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA

[c]Anesthesiology Washington University School of Medicine, St. Louis, MO, USA

[d]The Taylor Family Institute for Innovative Psychiatric Research, Washington University School of Medicine, St. Louis, MO, USA

[e]Department of Neuroscience, Tufts University School of Medicine, Boston, MA, USA

Abstract

Endogenous neurosteroids and synthetic neuroactive steroids (NAS) are important targets for therapeutic development in neuropsychiatric disorders. These steroids modulate major signaling systems in the brain and intracellular processes including inflammation, cellular stress and autophagy. In this review, we describe studies performed using unnatural enantiomers of key neurosteroids, which are physiochemically identical to their natural counterparts except for rotation of polarized light. These studies led to insights in how NAS interact with receptors, ion channels and intracellular sites of action. Certain effects of NAS show high enantioselectivity, consistent with actions in chiral environments and likely direct interactions with signaling proteins. Other effects show no enantioselectivity and even reverse enantioselectivity. The spectrum of effects of NAS enantiomers raises the possibility that these agents, once considered only as tools for preclinical studies, have therapeutic potential that complements and in some cases may exceed their natural counterparts. Here we review studies of NAS enantiomers from the perspective of their potential development as novel neurotherapeutics.

Keywords

Allopregnanolone; Pregnanolone; Pregnenolone sulfate; Neuroprotection; Neuroinflammation; $GABA_A$ receptors; NMDA receptors; Calcium channels

*Correspondence to: Department of Psychiatry, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA. zorumskc@wustl.edu (C.F. Zorumski).

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1. Introduction

There is now considerable interest in developing neuroactive steroids (NAS) as novel treatments for a variety of neuropsychiatric illnesses. The past three years have witnessed the approval of two NAS by the US Food and Drug Administration – brexanolone in 2019 for treatment of postpartum depression (PPD), and ganaxolone in 2022 for treatment of seizures in the context of a neurodevelopmental disorder caused by cyclin-dependent kinase-like 5 deficiency (Meltzer-Brody and Kanes, 2020; Lamb, 2022). A third NAS, zuranolone, is in late phase clinical trials for use in major depressive disorder (MDD) and PPD (Gunduz-Bruce et al., 2019; Deligiannidis et al., 2021) (Fig. 1). NAS are also under development and in clinical trials for other indications including epilepsy, neurodegenerative illnesses, essential tremor and anesthesia, among further potential uses (Belelli et al., 2020; Goodchild et al., 2020; Johansson et al., 2016; Mitchell et al., 2008; Raikes et al., 2021; Reddy and Estes, 2016; Zorumski et al., 2019).

The approved NAS are potent and highly effective positive allosteric modulators (PAMs) of Δ-aminobutyric acid A receptors ($GABA_AR$s), the predominant class of receptors mediating both phasic (synaptic) and tonic (extrasynaptic) inhibition in the brain (Zorumski et al., 2013). The steroids presently approved and in development have similar, though not identical effects on $GABA_AR$s and differ in chemical structure. Brexanolone is a formulation of the endogenous neurosteroid, allopregnanolone (AlloP, 3α-hydroxy-5α-pregnan-20-one) in a cyclodextrin for intravenous infusion (Meltzer-Brody and Kanes, 2020). The other two steroids in advanced development are orally active agents. Ganaxolone is a 3α-hydroxyl, 5α-reduced steroid that also features a 3β-methyl group that improves oral bioavailability (Lamb, 2022). Similarly, zuranolone has 3α-hydroxyl and 3β-methyl substitutions but is also 5β-reduced without a C-19 methyl group, and features a more complex substitution at C-17 of the steroid D-ring that improves bioavailability (Martinez Botella et al., 2017; Althaus et al., 2020) (Fig. 1).

Detailed structure-activity studies of NAS indicate that there are two critical portions of the molecule for $GABA_AR$ PAM activity (Covey et al., 2001; Zorumski et al., 2013). These include a hydrogen bond donor in the 3α configuration (usually a hydroxyl group) and a C-17β hydrogen bond acceptor (a methyl ketone in the case of brexanolone and ganaxolone). Configuration at C-5 is less critical, and both 5α- (brex-anolone and ganaxolone) and 5β-reduced (zuranolone) steroids are effective at enhancing activity at $GABA_AR$s and have progressed in human clinical trials.

The endogenous neurosteroid, AlloP, has eight chiral centers and a key finding made over 25 years ago is that activity of AlloP and structural analogues are highly enantioselective for PAM activity at $GABA_AR$s, with EC_{50} values differing by over an order of magnitude between the natural and unnatural steroids (Wittmer et al., 1996). 5β-Reduced neurosteroids (e.g. pregnanolone) also show enantioselectivity at $GABA_AR$s, but potency separation between enantiomers is only about 3–4-fold (Covey et al., 2000, 2001; Evers et al., 2000). These enantiomers represented a major advance in understanding NAS as $GABA_AR$ modulators and indicated strongly that these neurosteroids act in chiral environments likely on the receptors. This concept was borne out by later site-directed mutagenesis,

photoaffinity labeling and structural studies (Hosie et al., 2006; Chen et al., 2019; Sun et al., 2023). In contrast, accumulation of NAS in membranes and intracellular compartments shows no enantioselectivity (Chisari et al., 2009, 2010; Jiang et al., 2016). However, the lack of activity at $GABA_A$Rs and further study of enantiomeric steroids has resulted in identification of unexpected actions of these agents – actions that suggest strongly that unnatural NAS analogues may have unique therapeutic potential that can both complement and differ from the natural steroids. In this paper, we review the evolution and current state of NAS enantiomers, and highlight important effects of these agents that have therapeutic potential in neuropsychiatric illnesses.

2. Literature search

To date and to our knowledge, almost all unnatural NAS enantiomers have been synthesized in the laboratory of the first author of this paper and have been disseminated to other laboratories for independent testing. Thus, this review emphasizes work done with these compounds in laboratories within and outside the United States over the past 30 years. We also conducted a literature search using PubMed and Chemical Abstracts with search terms that included neurosteroid enantiomers, allopregnanolone, pregnanolone, androgens, estrogens and sulfated steroids. We refined the search returns to emphasize work done with unnatural enantiomers. Broad effects of natural neurosteroids have been reviewed elsewhere (Belelli et al., 2020; Mitchell et al., 2008; Reddy et al., 2016; Zorumski et al., 2013, 2019).

3. Neurosteroids & neurosteroid enantiomers

3.1. Definitions and chemistry of enantiomers

The term “neurosteroid” was first used by Etienne Baulieu and colleagues in the early 1980′s to describe the finding that dehydroepiandrosterone sulfate is synthesized in situ in rat brain (Baulieu, 1981; Corpechot et al., 1981). The term is now widely used for steroids made in the brain that have rapid non-genomic effects on neuronal activity. This definition should not be interpreted to mean that neurosteroids made in the brain are not also made in other organs of the body. For example, the adrenal gland, testes and ovaries make steroid hormones that are also synthesized in brain; and progesterone, testosterone and 17β-estradiol, which are referred to as sex hormones in the endocrine literature, are widely referred to as neurosteroids in the neuroscience literature. Further, the periphery may serve as a major endogenous source of neurosteroids that are also synthesized in the brain. Analogues of neurosteroids are referred to as “neuroactive steroids” (NAS), a term coined by Steven Paul and Robert Purdy in the early 1990′s (Paul and Purdy, 1992). The term “NAS” has broader definition than “neurosteroid” and includes neurosteroids made in brain, endogenous steroids made in the body that impact brain function, and synthetic steroids that modulate brain activity.

The mirror images (enantiomers) of naturally occurring steroids are defined as *ent*-steroids. Enantiomeric steroids are sometimes mistaken for diastereomeric steroids. In molecules like steroids which have more than one chiral center inverting one or more, but not all, chiral centers produces a diastereomer, which is not a mirror image (i.e. not an enantiomer). For example, changing the configuration of the 3-hydroxyl group of AlloP from the alpha

to the beta configuration yields epiallopregnanolone (also named as isoallopregnanolone), a diasteromer, not an enantiomer of AlloP. This distinction is important because only enantiomers, and not diastereomers, have identical physiochemical properties (except for rotation of polarized light). Thus conclusions based on diastereomers have the major limitation that these molecules have different physical properties that could account for any differences in actions. Biosynthetic pathways for the conversion of squalene, the precursor to steroids, to *ent*-steroids are unknown as are biosynthetic pathways for the interconversion of a steroid to its corresponding *ent*-steroid. Accordingly, *ent*-steroids are made by chemical synthesis. A review by Biellmann (2003) describes synthetic methods for their preparation. More recent synthetic methods also exist (Jastrzebska and Covey, 2022), but are not reviewed here.

Because *ent*-neurosteroids are mirror images of the naturally occurring neurosteroids (Covey et al., 2001), all of the stereocenters, indicated by wedges (β configuration) and dashed wedges (α configuration) in Fig. 2, are the opposite of the stereocenters in their corresponding neurosteroids. Fig. 2 shows the structures of the enantiomers of cholesterol and of the most studied neurosteroids. Cholesterol fulfills part of the definition of “neurosteroid” because the brain makes its own cholesterol; peripheral cholesterol bound to lipoproteins such as LDL does not cross the blood brain barrier (Bjorkhem and Meaney, 2004). Although not reviewed here, *ent*-cholesterol has been used in many different studies (for early reviews see Westover and Covey, 2004; Covey, 2009). The role of cholesterol and its metabolites in the brain is also currently of great interest in Alzheimer’s and other neurodegenerative diseases (Kacher et al., 2022; Rudajev and Novotny, 2022).

3.2. Uses of enantiomers

Neurosteroid enantiomers were initially prepared to investigate mechanisms underlying the anesthetic actions of AlloP. In 1986, it was established that AlloP potentiated the actions of GABA at $GABA_A$ receptors (Majewska et al., 1986). Potentiation of this receptor class contributes strongly to the mechanism of action for many general anesthetics in clinical use. Anesthetics are hydrophobic molecules and the long-standing Meyer-Overton hypothesis correlated anesthetic potency with their accumulation in membranes such that changes in membrane properties indirectly affected ion channel function. Specific binding sites for anesthetics on ion channels are not a premise of this hypothesis. Although it is now clear that there are specific binding sites for AlloP on $GABA_A$ receptors, this was not the case prior to 2006 when site-directed mutagenesis studies first unambiguously identified these sites (Hosie et al., 2006). Subsequent studies using x-ray crystallography and photoaffinity labeling methods have highlighted the complexity of NAS interactions with $GABA_A$Rs including multiple likely sites of receptor interaction (Chen et al., 2012; 2019; Laverty et al., 2017; Miller et al., 2017; Sugasawa et al., 2020, Sun et al., 2023). Earlier in the mid-1990 s, a medicinal chemical approach was taken to provide evidence that AlloP’s anesthetic actions resulted from its direct binding to $GABA_A$ receptors, not indirect actions caused by changes in membrane properties (Wittmer et al., 1996). Thus, *ent*-AlloP was synthesized and studied with the rationale being that a binding site on a receptor would likely distinguish AlloP from its enantiomer, whereas the membrane perturbation effects of AlloP and its enantiomer, which have identical physiochemical properties, would be equivalent leading to

no difference in their actions. Because *ent*-AlloP was not a potent modulator of $GABA_A$ receptors, this provided the first evidence for steroid binding sites on this class of receptors. Furthermore, these early studies provided evidence that anesthetic effects of AlloP and analogues in tadpoles and mice also show strong enantioselectivity (Wittmer et al., 1996). These initial results also provided the impetus to study other *ent*-neurosteroids and propelled investigations into the reasons for the observed enantioselectivity at $GABA_A$ receptors.

Since the initial report of enantioselective actions at $GABA_A$Rs, work with stereoisomers and enantiomers has progressed and provided important insights into how NAS likely align with $GABA_A$Rs to modulate function (Covey et al., 2000). In this regard, the substituent at the 17-position on the steroid D-ring is a major determinant of the stereoselectivity found for both AlloP and pregnanolone, the 5β-reduced analogue of AlloP (Krishnan et al., 2012). Changing the acetyl group at C-17 to a ketone group produces the androgen class steroids, androsterone and etiocholanolone, respectively. Interestingly, this structural change leads to a reversal of enantioselective action. Both *ent*-androsterone and *ent*-ethiocholanolone (Fig. 2) are more active $GABA_A$R PAMs than their corresponding natural NAS (Katona et al., 2008). Additional studies of structural modifications of substituents on the steroid D-ring have led to the discovery of *ent*-steroids with potent anesthetic and anticonvulsant actions (Krishnan et al., 2012; Qian et al., 2014; Zolkowski et al., 2014). Finally, *ent*-steroids that are weak or inactive modulators of what is generally considered to be the primary targets for their mechanism of action are powerful tools for identifying unexpected targets and pathways affected by neuroactive steroids. The potential clinical uses for *ent*-neuroactive steroids are discussed in the remaining sections of the review under the headings of their corresponding natural neurosteroids.

4. Progesterone

4.1. Effects of progesterone in the nervous system

Progesterone (PROG) is an important neurosteroid that is synthesized in brain from cholesterol, where it serves as both a modulator of certain brain functions and as a precursor to multiple other neurosteroids including AlloP (Belelli and Lambert, 2005; Belelli et al., 2020; Liang and Rasmussen, 2018). PROG itself has several well-known actions in the brain including direct effects on intracellular PROG receptors (PRs) that regulate gene transcription (Ghoumari et al., 2020; Gonzalez et al., 2020). Additionally, PROG can act at several other receptors including receptors expressed on the cell surface of neurons and other cells (Wendler and Wehling, 2022). These receptors include a class of G-protein coupled receptors (GPCRs) called membrane progesterone receptors (mPRs) that activate intracellular signaling pathways akin to other GPCRs and have neuroprotective effects (Thomas and Pang, 2020), and PGRMC1 (PROG receptor membrane component 1), receptors that activate cytochrome P450 enzymes, affect lipid metabolism and promote cell survival (Bassani et al., 2022; Ghoumari et al., 2020; Gonzalez et al., 2020). PROG was recently shown to be a PAM of the voltage-gated potassium channel Kir7.1 in epithelial cells of the choroid plexus (Bjorkgren et al., 2022). While it is unclear whether PROG directly modulates $GABA_A$Rs, it is certain that some of its metabolites (e. g. AlloP and others) are highly effective and direct acting PAMs. Also, activation of mPRs can modulate expression

and phosphorylation of $GABA_AR$ subunits (Parakala et al., 2019; Vien et al., 2022) to alter GABAergic inhibition. Furthermore, PROG (and AlloP) withdrawal upregulates expression of certain $GABA_AR$ subunits (α4 in particular) and this contributes to altered inhibition in the hippocampus and an anxiety-like behavioral phenotype (Gulinello et al., 2002; Smith et al., 2013). PROG can also modulate neuronal excitability via changes in expression of glutamate receptors and other signaling proteins (Kapur and Joshi, 2021).

Among the major effects of PROG in brain is its ability to serve as a neuroprotectant in a wide range of neurodegenerative conditions (Gonzalez et al., 2020), including animal models of traumatic brain injury (TBI) where PROG (and its downstream product AlloP) dampen neuronal death and improve learning and memory (Galani et al., 2001; Shear et al., 2002). Additionally PROG (and AlloP) dampen production of proinflammatory cytokines and decrease cerebral swelling along with promoting functional recovery from injury (VanLandingham et al., 2006). Mechanisms involved in neuroprotection by PROG are not completely understood but all three PROG receptors described above have been implicated in the beneficial effects (Bassani et al., 2022; Cao et al., 2021; Ghoumari et al., 2020; Gonzalez et al., 2020) and PROG can upregulate antioxidant signaling and the cellular process of autophagy (Ghandiri et al., 2019; Kim et al., 2012, 2013). One of the challenges in understanding neuroprotective mechanisms of PROG is its metabolism to other active neurosteroids, including AlloP, a powerful $GABA_AR$ positive allosteric modulator (PAM) and known neuroprotectant (Zorumski et al., 2019).

4.2. Effects of ent-progesterone

The enantiomer of PROG (*ent*-PROG) has not been studied extensively but has several important properties (Auchus et al., 2003). *ent*-PROG binds nuclear PRs with moderate affinity and appears to inhibit binding of PROG competitively (VanLandingham et al., 2006). In COS cells, however, *ent*-PROG does not activate PR-mediated gene transcription (VanLandingham et al., 2006). Early studies showed that *ent*-PROG competitively inhibits CYP450c17 and CYP450c21, enzymes that metabolize PROG in the pathway leading to corticosteroids; these two P450 enzymes do not hydroxylate *ent*-PROG (Auchus et al., 2003). These results indicate that there is enantioselectivity in at least some of PROG's actions, although it is presently unknown whether other PROG receptors including mPRs and PGRMC1 show similar enantioselectivity.

Intriguingly, in a well-characterized in vivo rat model of TBI, *ent*-PROG showed substantial neuroprotection at the same dose (16 mg/kg) at which PROG was effective. Effects of both enantiomers resulted in marked decreases in brain edema, reactive gliosis and production of proinflammatory cytokines, along with increases in generation of endogenous antioxidants (VanLandingham et al., 2006). Mechanisms and pathways underlying these effects of *ent*-PROG are unlikely to involve classical intracellular PRs, but remain unknown.

Results in preclinical studies of PROG led to human clinical trials in TBI, but, to date, those trials have not been successful (Lu et al., 2016; Zeng et al., 2015). Numerous factors could contribute to the failure of these TBI trials, not the least of which would be metabolism of PROG and uncertainties about concentrations of the agent achieved in critical brain regions. Given protective effects of *ent*-PROG observed in rodent studies, it is possible that this agent

could be developed for therapeutic purposes in TBI and perhaps other conditions associated with neurodegeneration and cognitive impairment (VanLandingham et al., 2013). At present little is known about pharmacological properties of *ent*-PROG.

5. 5α- and 5β-reduced pregnanes

5.1. Effects of pregnane steroids in the nervous system

The prototype for this class of neurosteroids is AlloP, a 3α-hydroxy, 5α-reduced pregnane that is a metabolite of PROG and a potent and effective PAM at $GABA_A$Rs. This steroid is synthesized endogenously in brain in multiple cell types including excitatory (glutamatergic) neurons in response to certain drugs and acute cellular stressors (Agis-Balboa et al., 2006; Tokuda et al., 2010, 2011; Zorumski and Izumi, 2012). In addition to powerfully modulating neuronal excitability and brain network function via GABA-enhancing effects, AlloP also has sedative/anesthetic, analgesic, anti-inflammatory and neuroprotective effects that make it and its derivatives attractive candidates as possible therapeutics for multiple neuropsychiatric illnesses (Kim et al., 2012; Belelli et al., 2020; Zorumski et al., 2019). Some of these effects (sedation and anticonvulsant effects) are consistent with GABAergic actions, while other effects are less clearly GABA-related.

5.2. Effects of ent-pregnanes

As described above, the enantiomer of AlloP (*ent*-AlloP) was synthesized in the mid-1990′s (Wittmer et al., 1996; Hu et al., 1997; Covey et al., 2000; Covey, 2009), and its effects and those of structural analogues have been examined in multiple pre-clinical studies. Initial efforts centered on understanding whether effects of AlloP on $GABA_A$Rs show enantioselectivity as could be expected if this neurosteroid acts in the defined chiral environment of a receptor binding pocket. These early studies showing high degrees of enantioselectivity provided strong support for 3α-hydroxyl, 5α-reduced NAS potentiation and direct gating of $GABA_A$Rs, with enantiomers differing by at least an order of magnitude in effects on GABA currents and displacement of the non-competitive antagonist, TBPS, in binding assays (Table 1). Similar high enantioselectivity was observed in anesthetic assays in tadpoles and mice (Wittmer et al., 1996). Enantioselectivity also extended to a class of tricyclic AlloP analogues called benz[*e*]indenes, which have an open A-ring (Rodgers-Neame et al., 1992; Zorumski et al., 1996, 1998).

In contrast to AlloP, the enantiomer of pregnanolone (a 3α-hydroxy, 5β-reduced analogue of AlloP) showed much lower (about 3-fold) enantioselectivity in assays of $GABA_A$R activity, TBPS binding and anesthesia (Table 1) (Covey et al., 2000, 2001). The reason for the decreased enantioselectivity of pregnanolone versus AlloP initially suggested in binding sites for these steroids on $GABA_A$Rs. Possibly consistent with this interpretation, a novel NAS, 3α,5α-17-phenyl-androst-16-en-3-ol (17PA), has no intrinsic effect on $GABA_A$Rs alone, but acts as an antagonist and effectively inhibits potentiation and direct channel gating by 5α-reduced NAS PAMS while only weakly altering effects of 5β-reduced PAMs. In contrast to 5α-NAS, 17βA has no effect on barbiturate-induced $GABA_A$R potentiation (Mennerick et al., 2004). 17PA and similar agents are referred to as $GABA_A$R modulating steroid antagonists (GAMSA) (Johansson et al., 2016). Interestingly, pregnanolone, in

contrast to AlloP, inhibits rather than potentiates a unique homopentameric subtype of GABA receptors expressing rho-1 subunits that are sometimes called GABAc receptors. However, both the inhibitory effects of pregnanolone and the potentiating effects of AlloP on rho-1 receptors are enantioselective, although at higher concentrations, *ent*-pregnanolone appears to have the opposite effect of pregnanolone, potentiating rho-1 receptors (Li et al., 2006).

Recent photoaffinity labeling studies have examined the interactions of AlloP, pregnanolone and their enantiomers with recombinant α1β3 $GABA_A$Rs. These studies found that *ent*-AlloP binds with low affinity to the β3-α1 intersubunit site and in a pose that is inverted 180° from the binding poses of AlloP, pregnanolone, and *ent*-pregnanolone (Tateiwa et al., 2023). Interestingly, *ent*-AlloP interacts similarly to AlloP with intrasubunit binding sites on the α1 and β3 subunits. The markedly reduced ability of *ent*-AlloP to potentiate GABA-elicited currents was attributed to its interaction with the canonical β3-α1 intersubunit NAS binding site.

AlloP also inhibits T-type (low voltage activated) calcium channels in rodent dorsal root ganglion neurons and in recombinant channels. Akin to what was observed with $GABA_A$Rs, inhibition of T-channels by AlloP and a 5α-reduced structural analogue also exhibited significant enantioselectivity with an order of magnitude separation between enantiomers (Pathirathna et al., 2005). The 5α-reduced steroids also exhibited analgesic effects in rodent models of peripheral pain, and this effect showed strong enantioselectivity. Importantly, T-channel activity is critical for peripheral anti-nociception, but activation of $GABA_A$ channels alone can enhance analgesia without altering baseline pain processing. Enantioselectivity for effects on T-channels extended to a steroid analogue that lacks significant effects on $GABA_A$Rs (Todorovic et al., 1998).

The high degree of enantioselectivity of AlloP and other 3α-hydroxyl, 5α-reduced pregnanes on $GABA_A$Rs and T-channels stands in distinct contrast to a lack of difference between the effects of enantiomers on lipid bilayers. In early studies, the enantiomers of both AlloP and pregnanolone produced indistinguishable effects on the physical properties of lipid bilayers of different composition (Alakoskela et al., 2007). This important set of results effectively excludes changes in membranes as being critical or relevant for anesthetic effects or effects on $GABA_A$Rs and T-channels.

5.3. Intracellular accumulation and effects of ent-pregnanes

Subsequent studies with novel enantiomeric pairs of NAS provided additional insights into membrane and intracellular accumulation. In one set of studies, cellular and membrane accumulation and effects on $GABA_A$Rs were studied using a 7-nitrobenz-2-oxa-1,3-diazole (NBD)-tagged fluorescent analogue of AlloP and its enantiomer. The steroid with the configuration of natural AlloP is a potent and effective modulator of $GABA_A$Rs, while its unnatural enantiomer is ineffective at these receptors (Chisari et al., 2009). However, these two steroids demonstrated identical fluorescence accumulation in membranes and neurons. An NBD-tagged fluorescent analogue of the anesthetic steroid, alphaxalone, which has lower lipophilicity compared to AlloP, showed faster accumulation and departitioning from cell membranes and intracellular compartments. Using analysis of NAS with different

degrees of lipophilicity, other studies demonstrated a relationship between hydrophobicity and potency for effects on $GABA_A$Rs, indicating that aqueous concentration is not an accurate indicator of NAS binding affinity for the receptors and supporting the hypothesis that membrane accumulation contributes significantly to potency and duration of actions, likely by enhancing access to the receptors (Chisari et al., 2009, 2010; Shu et al., 2004). Importantly, these studies provided strong support for the hypothesis that both lipophilicity and pharmacophore considerations are important in the effects of NAS $GABA_A$R PAMs.

Other studies used a series of NAS analogues with less complex chemical substitutions that allowed use of click chemistry to label steroids bio-orthogonally following administration to track cellular accumulation and effects on $GABA_A$Rs. A clickable analogue based on AlloP effectively modulated $GABA_A$Rs and selectively labelled intracellular Golgi in cultured rat hippocampal neurons (Jiang et al., 2016). This distribution of the steroid was mimicked by its enantiomer and did not require cellular energy, although the enantiomer was inactive at $GABA_A$Rs. Furthermore, the distribution of the GABA-active steroid differed from cholesterol and was not mimicked completely by other non-enantiomeric structural analogues that were inactive at $GABA_A$Rs (Jiang et al., 2016). These initial studies also provided evidence that accumulation of NAS in neuronal cell bodies can act as either a sink or a source of NAS on somatic $GABA_A$Rs. The GABAergic clickable NAS was subsequently used in photoaffinity labeling studies to determine amino acid residues on $GABA_A$R subunits that interact with NAS and other cellular targets of NAS (see below) (Chen et al., 2019; Cheng et al., 2019).

5.4. Neuroprotective effects of ent-pregnanes

The studies outlined above indicate that enantiomers of AlloP analogues serve as useful monitors of non-specific effects of NAS (particularly intramembranous and intracellular accumulation), but other work with these steroids indicates that the unnatural AlloP enantiomer has important effects, independent of $GABA_A$Rs that could have relevance for neurotherapeutics. An intriguing, but incompletely understood property of AlloP is its ability to protect neurons in models of neurodegeneration. These protective effects include a murine model of Niemann-Pick C (NPC) disease that results in developmental neurodegeneration and involves constitutive knockout of the *npc* gene (*npc−/− mice*) (Griffin et al., 2004). In both humans and mice, the NPC phenotype results in changes in neuronal lipid storage, defective neurosteroidogenesis, Purkinje cell loss in the cerebellum and premature death. In the mouse model, all knockout animals die by 12–14 weeks of age. Intriguingly, a single injection of AlloP (25 mg/kg sc) at postnatal day 7 (PND 7), during the period of developmental synaptogenesis, extends lifespan by over 40% (with death occurring by about 18 weeks of age) (Griffin et al., 2004; Langmade et al., 2006). This dose of AlloP causes acute sedation lasting about 45 min. The knockout mice also exhibit loss of Purkinje cells and neuroinflammatory changes with microglia invasion and production of proinflammatory cytokines including tumor necrosis factor-α (TNFα) and interleukin 1β (IL1 β). All of these cellular changes are dampened significantly by the single AlloP injection on PND 7. Remarkably, the identical dose of *ent*-AlloP at PND 7 produced similar neuroprotection, survival and effects on inflammatory changes without causing sedation following acute injection (the latter being a GABAergic effect). Furthermore, both enantiomers activated

pregnane X receptors, nuclear receptors involved in regulating genes that help detoxify cells and promote expression of pro-survival factors, suggesting a possible contributing mechanism (Lamba et al., 2004; Frye et al., 2014; Wnuk and Kajta, 2017). AlloP, unlike certain oxysterols, did not activate liver X receptors, a related class of nuclear receptors (Langmade et al., 2006). In cell culture studies, the AlloP enantiomers were equally protective against oxidative stress induced by NPC mutation or knockdown (Zampieri et al., 2009).

ent-AlloP is also neuroprotective in *ex vivo* and in vivo rodent models of glaucoma. In the *ex vivo* model, dissected rat eye cups are acutely incubated under varying degrees of pressure for 24 h. At 10 mm Hg of ambient pressure (akin to normal conditions in the eye) retinas have normal histological appearance; high pressure (75 mm Hg) results in degenerative changes in retinal ganglion cells (RGCs) and swelling of their axons. Treatment with 1 μM AlloP during the 24 h incubation results in complete preservation of retinal histology (Ishikawa et al.,2021). Remarkably, 1 μM *ent*-AlloP produces identical protection (Ishikawa et al., 2022). Effects of natural AlloP involve both activation of $GABA_A$Rs and stimulation of the cellular process of autophagy. $GABA_A$Rs do not contribute to the effects of *ent*-AlloP, yet *ent*-AlloP effectively induces autophagic flux leading to retinoprotection (Ishikawa et al., 2022). These effects of AlloP enantiomers extend to an in vivo glaucoma model. The in vivo model involves a 21 day experiment that begins with intravitreal injection of microbeads into the anterior eye chamber. Over the first three days after injection intraocular pressure increases from about 10 mm Hg to 30 mm Hg and remains elevated at this level over the remainder of the experiment. The increased intraocular pressure damages ganglion cells and their axons with diminished retinal function as measured by the scotopic threshold response. A single intravitreal injection of either AlloP or *ent*-AlloP on day 7 of the experiment produces complete histological protection of the retina and preservation of retinal function. Both AlloP and *ent*-AlloP promote autophagic flux in the in vivo model contributing substantially to neuroprotection with the unnatural enantiomer showing greater effectiveness in autophagy activation (Covey et al., 2022; Ishikawa et al., 2021, 2022).

The protective mechanisms of AlloP enantiomers are incompletely understood. Photoaffinity labeling studies using some of the NAS outlined above have identified several potential targets that could be important. These include voltage dependent anion channels (VDACs) that are labelled at Glu-73, a site that also binds cholesterol (Cheng et al., 2019). VDACs are important contributors to the mitochondrial permeability transition pore that helps to regulate cell death programs. A second site previously identified is on β-tubulin at Cys-354, the same site also labelled by colchicine (Chen et al., 2012a, 2012b). It is unknown whether effects on this key microtubule protein contributes to anti-inflammatory or other effects of AlloP. Presently it is also unknown whether effects on VDAC or β-tubulin are enantioselective.

Taken together studies of enantiomeric pairs of pregnane steroids have been highly informative, indicating certain effects that are unequivocally enantioselective (potentiation of $GABA_A$Rs and sedation, and inhibition of T-channels and peripheral analgesia) and effects that show no enantioselectivity (membranous and intracellular accumulation, and neuroprotection in two models studied to date). A summary of enantioselective effects of

AlloP and derivatives is shown in Fig. 3. These findings raise important opportunities in the potential development of enantiomers as therapeutic agents.

6. Androstanes

6.1. Effects of androstanes in the nervous system

Regarding the naming of steroids, pregnane and androstane classes are distinguished by the presence of a two-carbon side chain at the C-17 position on the steroid D-ring of the former. While this nomenclature rule is salient for indicating the different hormonal activities of these two classes of steroids, it does not predict differences in their non-hormonal activities. Thus, many analogues of AlloP that lack the pregnane side chain will be named as androstanes even though their actions on ion channels are similar to the pregnanes. For this reason, we discuss steroids and their enantiomers in both classes sequentially because they have qualitatively similar actions on ion-channels, particularly $GABA_AR$s, and potentially similar clinical utility. In general, the enantiomers of steroid hormones lack hormonal activities and are not substrates for enzymes involved in hormone biosynthesis. The literature on the enzymology and physiology of *ent*-steroid hormones was reviewed in detail by Biellmann in 2003.

6.2. Effects of ent-androstanes

Two androstane steroids are of particular interest based on effects of their enantiomers. The natural steroids, androsterone and etiocholanolone differ from AlloP and its derivatives in being weak potentiators of $GABA_AR$s. These weaker effects likely result from differences in substitutions at the C-17 position of the steroid D-ring (Katona et al., 2008). In contrast, the unnatural enantiomers of these androstanes were found to be surprisingly potent and effective modulators of GABA receptors (Table 1) (Katona et al., 2008). Single channel recordings and site directed mutagenesis studies indicate that the enantiomers of etiocholanolone may each act at distinct sites on $GABA_AR$s (Li et al., 2007).

Other structure-activity studies pursuing features of androstane enantiomers that contribute to effectiveness at GABA receptors made several important observations. These include the presence of a ketone group at position C-16, a C-18 methyl group and an axial methoxyl substitution at the 4α-position (Qian et al., 2014). Additionally, enantiomers with these substitutions were more potent and effective in producing anesthetic effects in tadpoles and mice. In other studies, the enantiomers of androsterone and etiocholanolone were found to be anticonvulsants, consistent with their enhanced GABAergic activity and with more potent and longer durations of actions than the natural steroids in vivo (Zolkowski et al., 2014). These results strongly suggest that certain enantiomeric androstanes could be developed for therapeutic purposes based on GABAergic activity, including possible antidepressant, anxiolytic, anesthetic, anticonvulsant, analgesic, neurogenic and neuroprotective effects (Melcangi et al., 2021; Kelava et al., 2022).

7. Estrogens

7.1. Effects of estrogens in the nervous system

Neuroactive estrogens, particularly 17β-estradiol (17β-E2), represent another important class of neurosteroids that modulate multiple brain functions including behavior, neuronal structure, neural excitability and survival (Woolley et al., 2007; Simpkins et al., 2013; Brann et al., 2022). These steroids can be synthesized locally in brain from cholesterol via pregnenolone in both neurons and glia (Belelli and Lambert, 2005; Fester and Rune, 2021) and act at classical estrogen receptors (ERα and ERβ) that are expressed both intracellularly and on the surface of cells. Classical intracellular ERs play key roles in mediating hormonal actions in the periphery (Farkas et al., 2022). Acutely, estrogens can modify intrinsic neuronal physiology and synaptic function of both excitatory (glutamatergic) and inhibitory (GABAergic) transmission by presynaptic and postsynaptic effects acting on specific receptors expressed on the surface of neurons and other cells (Huang and Woolley, 2012; Oberlander and Woolley, 2016). Furthermore, 17β-E2 and derivatives have powerful neuroprotective effects in several preclinical models in vitro and in vivo, including models of oxidative stress, cerebral trauma, ischemia and neurodegenerative illnesses, among others (Brann et al., 2022; Farkas et al., 2022; Engler-Chiurazzi et al., 2017; Saeed et al., 2021).

There is considerable information about structure-activity relationships of 17β-E2 and derivatives at classical estrogen receptors, likely because of the importance of these steroids in reproductive biology, behavior and pharmacology. Less is known about structure-activity requirements for estrogens on neuronal function and excitability. In contrast, detailed structure-activity studies have explored how 17β-E2 produces neuroprotection. These studies have shown that estrogenmediated neuroprotection in preclinical models can occur independently of effects on classical ERs. The steroid A-ring, particularly its phenolic structure, is a minimal requirement for neuroprotection (Engler-Chiurazzi et al., 2017); methylation of the phenolic A-ring hydroxyl group eliminates protective effects. However, neuroprotection is enhanced by substitution of non-polar groups at the C2 and C4 carbons of the A-ring (Qian et al., 2016). Mechanistically, 17β-E2 neuroprotection involves synergistic modulation of the antioxidant effects of glutathione via generation of NADPH from the hexose monophosphate shunt, resulting in increased levels of reduced forms of glutathione (Dykens et al., 2004). Importantly, these detailed structure-activity studies provided strong support for the hypothesis that neuroprotective effects can be separated from classical feminizing actions of estrogens.

7.2. Effects of ent-estrogens

The enantiomer of 17β-E2 has been studied in a variety of models since the 1960 s and 1970 s (Terenius, 1968; Chernayaev et al., 1975; Payne et al., 1979). This enantiomer interacts weakly with classical ERs and lacks estrogen-like effects on reproductive tissues in animals. Only limited information is available about brain effects of *ent*-17β-E2. 17β-E2 potentiates certain neuronal nicotinic receptors, and this effect is enantioselective (Paradiso et al., 2001). These same nicotinic receptors are enantioselectively inhibited by a 3α-hydroxyl, 5α-reduced androstane with about three-fold separation between enantiomers (Paradiso et al., 2000).

Importantly, *ent*-17β-E2 shows highly effective neuroprotection in models of neurodegeneration including toxicity produced by hydrogen peroxide and oxidative stress in a hippocampal cell line model and in a neuroblastoma cell line in vitro. The E2 enantiomer is also effective in an in vivo rodent model of focal cerebral ischemia involving occlusion of the middle cerebral artery (Green et al., 2001). In both the in vitro and in vivo models, the enantiomer of 17β-E2 was equally effective and as potent as natural 17β-E2, but exhibited no stimulation of uterine growth or effects on vaginal opening in juvenile female rats, indicating a lack of effect on classical estrogen receptors. Furthermore, in cultured rat cortical neurons, both 17β-E2 and its enantiomer protect against glutamate toxicity via a non-ER-dependent mechanism by preserving serine phosphatase activity and dampening pathological kinase activation and resultant oxidative stress. Neither enantiomer was effective against toxicity in the presence of specific phosphatase inhibitors (YI et al., 2008, 2009). Taken together, these findings indicate that *ent*-17β-E2 has therapeutic potential as a neuroprotectant without the feminizing effects of the natural steroid. At present there is no information about *ent*-17β-E2 on other brain effects of estrogens including changes in excitability.

8. Sulfated steroids

8.1. Effects of sulfated neurosteroids in the nervous system

Most neurosteroids are unconjugated, but are subject to a variety of enzymatic modifications that affect their activities and targets. Certain neurosteroids can be sulfated by endogenous sulfotransferases and this generates molecules with interesting effects in brain. Sulfated steroids, most notably pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS), modulate certain ion channels to alter neuronal excitability and synaptic transmission, and different sulfated steroids can be positive or negative allosteric modulators. Known targets of sulfated steroids include ligand gated ion channels such as $GABA_A$Rs, glycine receptors, N-methyl-D-aspartate receptors (NMDARs), kainic acid receptors and AMPA receptors, mediators of the majority of fast (phasic) and tonic excitation and inhibition in the nervous system (Kudova, 2021; Vitku et al., 2022). Sulfated neurosteroids can also regulate the activity of certain GPCRs, transient receptor potential (TRP) channels and sigma 1 receptors (S1Rs). These steroids, in turn modulate learning and memory, neuronal survival, neurite outgrowth and neurogenesis. Sulfation increases water solubility of these agents and alters their half-lives, making sulfated steroids potential targets for drug development, although delivery of these charged molecules across the blood-brain barrier is a significant challenge.

Presently, most information about sulfated neurosteroids involves the effects of PREGS (and to a lesser extent DHEAS). PREGS and DHEAS are sometimes referred to as “excitatory neuroactive steroids” based on their effects on ion channels (Vitku et al., 2022). PREGS is an effective PAM at NMDARs, but is a negative allosteric modulator (NAM) of $GABA_A$Rs, giving it unique actions by which it can modulate the balance of brain excitation and inhibition. Notably, effects on NMDA receptors are generally less potent than effects on $GABA_A$Rs. Certain subtypes of NMDARs are more sensitive to potentiation by PREGS, including NMDARs that express GluN2A and GluN2B subunits, with lower potency and

effectiveness at receptors with GluN2C and GluN2D subunits (Horak et al., 2006). PREGS can also inhibit NMDARs under certain conditions, complicating interpretation of effects (Horak et al., 2006). A 5β-reduced analogue of PREGS (pregnanolone sulfate, PAS) is a negative modulator of NMDARs (Kapras et al., 2018). At $GABA_A$Rs, PREGS is a potent, activation-dependent, non-competitive inhibitor (Park-Chung et al., 1999; Seljeset et al., 2018) and appears to act, at least in part, by enhancing receptor desensitization (Shen et al., 2000; Eisenman et al., 2003). PREGS also shows little $GABA_A$R subtype selectivity except for rho-1 receptors that are less sensitive to inhibition (Seljeset et al., 2018).

8.2. Effects of enantiomers of sulfated neurosteroids

Enantiomers of sulfated steroids were synthesized in the late 1990′s (Nilsson et al., 1998) and most studies have examined the effects of *ent*-PREGS, an agent that has clear but complex effects on brain function, with some actions showing high degrees of enantioselectivity of the natural steroid, other effects showing no difference between enantiomers and some effects showing reverse enantioselectivity. Early studies found that the ability of PREGS to inhibit $GABA_A$RS (Nilsson et al., 1998) and to potentiate NMDARs (Vallee et al., 2001) showed no enantioselectivity. Additionally, PREGS enantiomers show no differences in effects on membrane capacitance, as expected for these lipophilic agents with the same physicochemical properties (Mennerick et al., 2008). In contrast, effects of DHEAS on $GABA_A$Rs surprisingly showed about 8-fold separation with higher potency of the natural enantiomer (Nilsson et al., 1998). The 5β-reduced sulfated steroid, PAS, is a negative modulator of $GABA_A$Rs and shows no enantioselectivity (Nilsson et al., 1998), although the unnatural enantiomer was about 2.6-fold less potent than PAS for inhibitory effects on NMDARs (Kapras et al., 2018). Effects of PREGS on activation of TRPM3 (Transient Receptor Potential Melastatin 3 channels) showed enantioselectivity favoring the natural enantiomer, while inhibition of PAORAC (proton-activated outwardly rectifying anion channels) showed no enantioselectivity, akin to GABA-gated chloride channels described above (Drews et al., 2014). Effects of sulfate enantiomers on other receptors and channels have not been reported to date.

PREGS enantiomers have been examined on synaptic transmission and in models of learning and memory and neurodegeneration. At hippocampal glutamatergic synapses, PREGS augments the frequency, but not the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in hippocampal cultures, and also decreases paired-pulse facilitation of evoked EPSCs, indicating a presynaptic increase in release of glutamate at these synapses. Enhancement of mEPSCs was mimicked by DHEAS but not by *ent*-PREGS, and was blocked by inhibitors of sigma 1 receptors (S1Rs), suggesting that PREGS acts on these receptors in an enantioselective fashion (Meyer et al., 2002).

Enhancing effects of PREGS on NMDARs and glutamate transmission are consistent with other studies that have examined effects of PREGS on memory, and the unnatural PREGS enantiomer has also been examined in several studies of memory and learning. Akwa et al. (2001) examined effects of the enantiomers in a spatial memory task involving two-trial recognition in a novel environment Y-maze test. In this study, both enantiomers enhanced memory performance following post-acquisition intracerebroventricular (icv) administration,

but surprisingly, *ent*-PREGS was about 10-fold more potent than PREGS. Furthermore, pro-memory effects of PREGS, but not *ent*-PREGS were blocked by the NMDAR antagonist, 2-amino-5-phosphonovalerate (APV), indicating that effects of the enantiomer were independent of NMDARs. Consistent with this, a subsequent study using the two-trial Y-maze discrimination task found that memory enhancement by PREGS, but again not *ent*-PREGS, was eliminated in mice with knockout of the obligatory GluN1 NMDAR subunit (Petit et al., 2011). These results indicate that there is reverse enantioselectivity for memory enhancement by PREGS in at least some tasks and that *ent*-PREGS can act by a mechanism independent of NMDARs.

The PREGS enantiomers have also been examined against amnesic effects of scopolamine (an anti-muscarinic drug) in a passive avoidance memory task. Here, both enantiomers blocked the effects of scopolamine, but PREGS was about 10-fold more potent than *ent*-PREGS following icv administration 30 min prior to training (Vallee et al., 2001). Mechanisms underlying the effects of the enantiomers in these scopolamine studies are unknown, but do not appear to involve well-known effects on either NMDA or GABA receptors. Also, in contrast to the studies outlined above, these results indicate clear enantioselectivity favoring the natural neurosteroid.

In a cell culture model of beta-amyloid (Aβ25–35) neurotoxicity in B104 cells, both enantiomers of PREGS and DHEAS diminished cell loss and stimulated neurite outgrowth (El Bitar et al., 2014). Natural PREGS was more potent than its enantiomer but DHEAS showed no enantioselectivity. The enantiomers were also studied in an in vivo model using icv administration of Aβ25–35 following pretreatment with the steroids. In this model, both *ent*-PREGS and *ent*-DHEAS, in contrast to the natural neurosteroids, effectively diminished Aβ25–35-induced memory impairment in spontaneous alternation and passive avoidance tasks when administered 6 h prior to Aβ25–35. The enantiomers also decreased lipid peroxidation, a marker of cell stress and injury (El Bitar et al., 2014). Mechanisms underlying these protective effects are unknown, but the results support the potential use of these enantiomeric steroids as neuroprotectants.

Taken together, these results indicate that *ent*-PREGS (and likely *ent*-DHEAS) have intriguing effects on brain function including memory enhancing actions and neuroprotection in several model systems. A summary of the effects of the sulfated neurosteroid enantiomers is shown in Fig. 4.

9. Summary & conclusions

While detailed studies of unnatural neurosteroid enantiomers are a work in progress, it is clear that these agents have potentially important effects on brain function that could be harnessed for treatment of a variety of neuropsychiatric illnesses. Several of these agents have potent neuroprotective actions that rival and, in some cases, exceed the effects of the natural neurosteroids. Some enantiomers also appear to have unique mechanisms compared to natural steroids (e.g. PREGS). As highlighted in this review, specific examples with neuroprotective properties include the enantiomers of progesterone, AlloP, 17β-estradiol and possibly PREGS. The enantiomers of the androgens, androsterone and etiocholanolone, are

potent positive allosteric modulators of $GABA_AR$s and could provide unique alternatives for treatment of disorders in which enhanced inhibition is desired, including epilepsy, sleep, anxiety and pain. Proof of concept studies in rodents support anticonvulsant properties of the androstane enantiomers (Zolkowski et al., 2014), but it is presently unknown whether they are neuroprotective.

Certain enantiomeric NAS have potentially important beneficial effects on learning and memory including prevention and/or reversal of memory defects in pathological conditions. Some of these effects may involve neuroprotective mechanisms and anti-cellular stress effects, including dampening of neuroinflammation and antioxidant properties. Given the importance of cognitive defects in driving disability and dysfunction in numerous neuropsychiatric illnesses, the enantiomeric steroids could play key roles in preventing or reversing these devastating outcomes.

Why enantiomers? Beyond potentially important effects on brain function outlined above, the enantiomers have certain properties that could enhance their attractiveness as therapeutics. These include a lack of agonist activity mediated by steroid nuclear hormone receptors as reported for the enantiomers of progesterone and 17β-estradiol. Additionally, the lack of effects of certain enantiomers such as *ent*-AlloP on GABA channels would be expected to avoid sedation and potentially adverse effects on cognition that can come with broadly enhanced GABAergic function. The enantiomers will also likely have major differences in their in vivo metabolism that could prolong half-lives as drugs and prolong key activities, as has been observed in studies of the anticonvulsant effects of certain *ent*-androgens (Zolkowski et al., 2014). Furthermore novel mechanisms of enantiomers may open new directions in therapeutics. This latter concept is not unprecedented and current efforts to understand enantiomeric metabolites of the rapid antidepressant, ketamine, already appear to offer potentially new approaches to treatment (Highland et al., 2021; Bonaventura et al., 2022).

Why not enantiomers? Beyond the fact that only limited information is presently available about the effects of unnatural neurosteroid enantiomers, there are several other considerations that could curtail their development as therapeutics. Enantiomeric steroids are generated by total synthesis and are not accessible at scale from naturally occurring steroids. Newer methods for their synthesis that have the potential to lower the cost for their commercial production have been recently reported (Kim et al., 2018). As noted above, *ent*-steroids are likely metabolized differently from natural steroids, but little is presently known about this important topic. Differences in the glucuronidation of 17β-estradiol, androsterone and etiocholanolone by human UDP-glucuronosyltransferases have been reported (Sneitz et al., 2011). Whether the enantiomers have additional off-target effects on metabolic or other endogenous pathways remains to be determined. The enantiomers will also have significant effects on cell membranes, but these effects are unlikely to differ from the natural steroids (Alakoskela et al., 2007; Mennerick et al., 2009), and studies to date indicate that enantiomers accumulate and dissipate similarly from cell membranes and intracellular compartments (Chisari et al., 2009, 2010; Jiang et al., 2016). To date there is no information about enantiomeric NAS in humans. Thus it is not clear what side effects will be encountered and how those side effects differ from natural neurosteroids. Future studies

are required to determine the translational potential of these compounds for the treatment of disorders where NAS have been suggested to have benefit, such as anxiety, depression, epilepsy, and others (Belelli et al., 2020; Goodchild et al., 2020; Johansson et al., 2016; Reddy and Estes, 2016; Zorumski et al., 2019).

In summary, enantiomeric neuroactive steroids have proven to be useful scientific tools in a wide range of studies of neuronal function, and have stepped out of the shadows of their natural partners to demonstrate major positive effects on their own. These findings raise intriguing possibilities about how the positive effects of these agents may be harnessed for therapeutic uses in neuropsychiatry.

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Data Availability

No data was used for the research described in the article.

Nomenclature

17β-E2	17β-estradiol
17PA	3α,5α-17-phenylandrost-16-en-3-ol
Aβ25–35	beta-amyloid 25–35
AlloP	allopregnanolone
CNS	central nervous system
DHEAS	dehydroepiandrosterone sulfate
ent	enantiomeric configuration steroid
EPSC	excitatory postsynaptic current
ERα and ERβ	estrogen receptor α and β
GABA	gamma aminobutyric acid
$GABA_AR$	gamma aminobutyric acid type A receptor
GPCR	G-protein coupled receptor
icv	intracerebroventricular
IL1β	interleukin 1β
MDD	major depressive disorder

mEPSC	miniature excitatory postsynaptic current
mPR	membrane progesterone receptors
NAM	negative allosteric modulator
NAS	neuroactive steroids
na	natural configuration steroid
NBD	7-nitrobenz-2-oxa-1,3-diazole
NMDAR	N-methyl-D-aspartate receptor
NPC	Niemann-Pick C disease
PAM	positive allosteric modulator
PAORAC	proton-activated outwardly rectifying anion channels
PAS	pregnanolone sulfate
PGRMC1	PROG receptor membrane component 1
PND 7	postnatal day 7
PPD	postpartum depression
PREGS	pregnenolone sulfate
PROG	progesterone
PRs	progesterone receptors
RGC	retinal ganglion cells
ROS	reactive oxygen species
S1R	sigma 1 receptor
TBI	traumatic brain injury
TBPS	tert-butylbicyclophosphorothionate
TNFα	tumor necrosis factor-α
TRPM3	Transient Receptor Potential Melastatin 3 channels
VDAC	voltage dependent anion channel.

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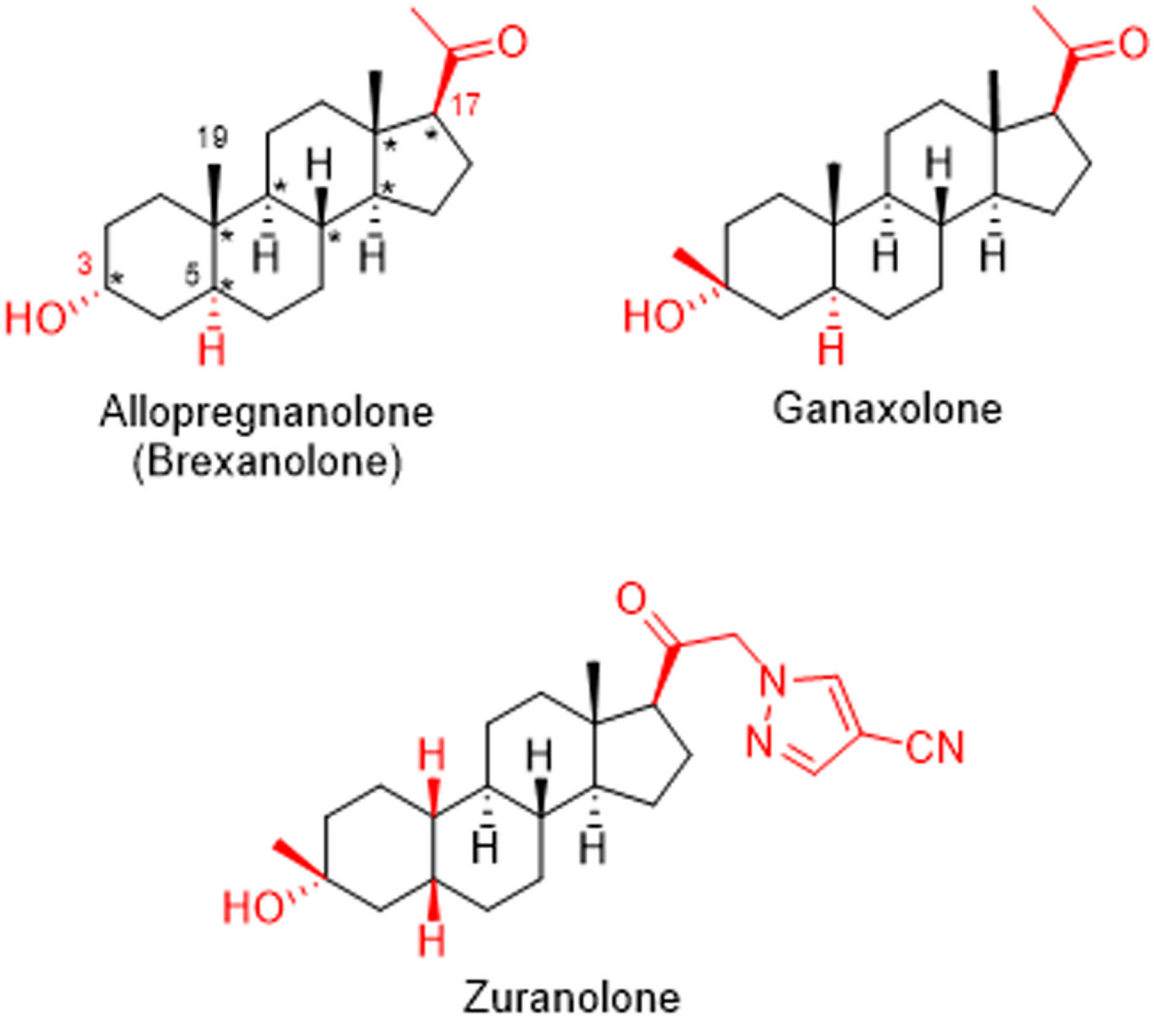

Fig.1.

Neuroactive steroids in clinical use or development. The eight chiral centers in allopregnanolone (brexanolone) are marked with asterisks. By convention, dashed and solid wedges designate the α and β configurations of substituents at chiral stereocenters. Important structural features described in the text are highlighted in red font.

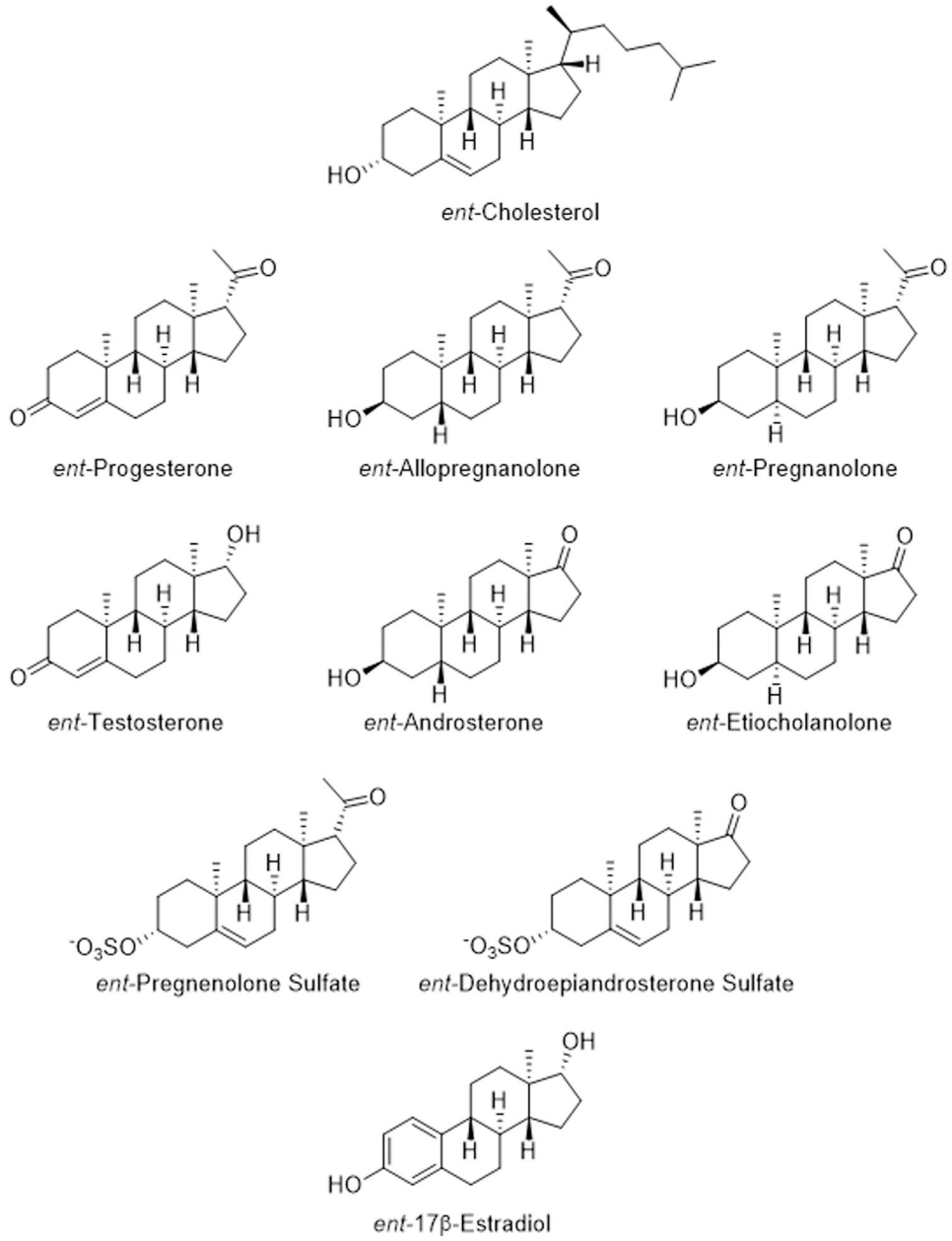

Fig. 2.

Structures of enantiomeric steroids. Stereocenters are denoted by solid (β configuration) or dashed (α configuration) wedges and are the opposite of the stereocenters in naturally occurring steroids. Comparison of the structures *ent*-allopregnanolone and naturally occurring allopregnanolone (Fig. 1) shows the opposite configuration of all eight chiral centers.

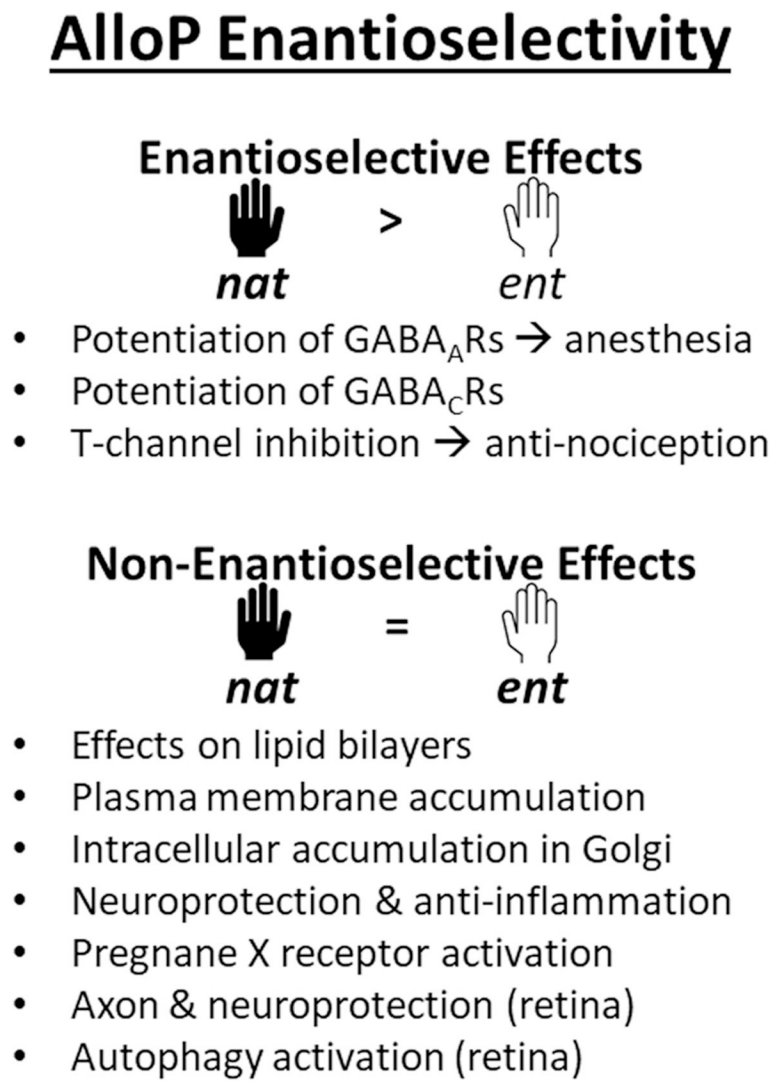

Fig. 3.

The figure summarizes various effects where the enantiomers of AlloP have been compared, including effects with enantioselectivity (*nat* > *ent*), and effects where the enantiomers are equivalent (nat = ent). See text for details and references.

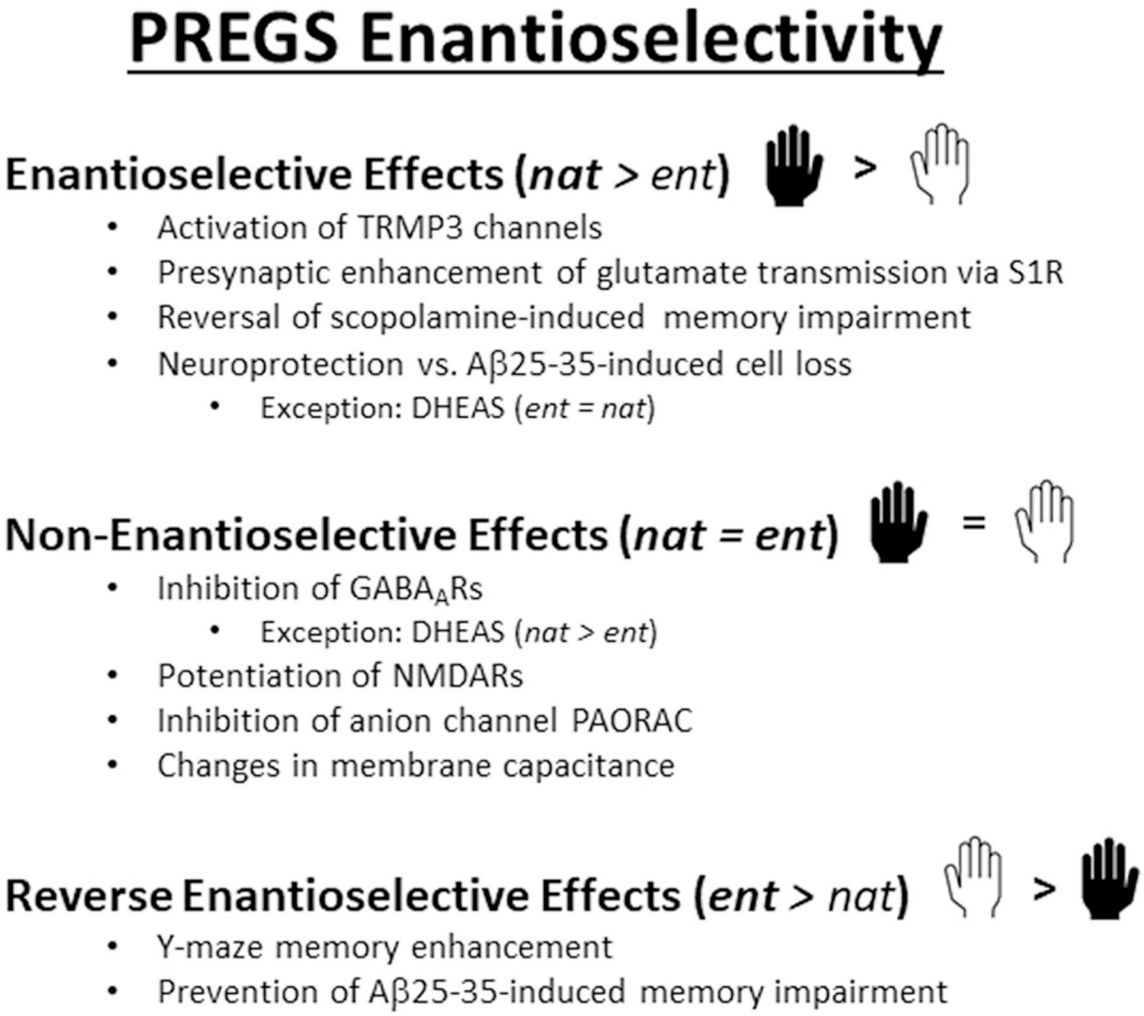

Fig. 4.

Various effects of PREGS and its enantioselectivity are depicted and highlight effects with enantioselectivity (*nat* > *ent*), no enantioselectivity (*nat* = *ent*) and reverse enantioselectivity (*ent* > *nat*). The figure also shows complex effects of the DHEAS enantiomers. See text for details and references.

Table 1

NAS Enantiomers & TBPS Binding.

Neurosteroid	Natural Neurosteroid TBPS IC_{50} (μM)	Enantiomeric Neurosteroid TBPS IC_{50} (μM)	Reference
Allopregnanolone	0.074 ± 0.007	1.91 ± 0.27	Katona et al. 2008
Alfaxalone	0.23 ± 0.02	5.15 ± 0.60	Unpublished
THDOC	0.039 ± 0.003	> 10	Unpublished
Pregnanolone	0.071 ± 0.018	0.28 ± 0.03	Katona et al. 2008
Androsterone	0.41 ± 0.13	0.31 ± 0.04	Katona et al. 2008
Etiocholanolone	4.15 ± 1.00	0.38 ± 0.06	Katona et al. 2008

The table displays IC50 values for the ability of NAS enantiomers to displace TBPS binding from rat brain membranes. Abbreviations: Alfaxalone (3α-Hy-droxy-5α-pregnane-11,20-dione); THDOC (3α,21-dihydroxy-5α-pregnan-20-one).