Themed Section: Pharmacology of Cognition: a Panacea for Neuropsychiatric Disease?

REVIEW ARTICLE

Neurosteroid biosynthesis down-regulation and changes in GABA_A receptor subunit composition: a *biomarker axis* in stress-induced cognitive and emotional impairment

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By rapidly modulating neuronal excitability, neurosteroids regulate physiological processes, such as responses to stress and development. Excessive stress affects their biosynthesis and causes an imbalance in cognition and emotions. The progesterone derivative, allopregnanolone (Allo) enhances extrasynaptic and postsynaptic inhibition by directly binding at GABA_A receptors, and thus, positively and allosterically modulates the function of GABA. Allo levels are decreased in stress-induced psychiatric disorders, including depression and post-traumatic stress disorder (PTSD), and elevating Allo levels may be a valid therapeutic approach to counteract behavioural dysfunction. While benzodiazepines are inefficient, selective serotonin reuptake inhibitors (SSRIs) represent the first choice treatment for depression and PTSD. Their mechanisms to improve behaviour in preclinical studies include neurosteroidogenic effects at *low* non-serotonergic doses. Unfortunately, half of PTSD and depressed patients are resistant to current prescribed 'high' dosage of these drugs that engage serotonergic mechanisms. Unveiling novel biomarkers to develop more efficient treatment strategies is in high demand. Stress-induced down-regulation of neurosteroid biosynthesis and changes in GABA_A receptor subunit expression offer a putative *biomarker axis* to develop new PTSD treatments. The advantage of stimulating Allo biosynthesis relies on the variety of neurosteroidogenic receptors to be targeted, including TSPO and endocannabinoid receptors. Furthermore, stress favours a GABA_A receptor subunit composition with higher sensitivity for Allo. The use of synthetic analogues of Allo is a valuable alternative. Pregnenolone or drugs that stimulate its levels increase Allo but also sulphated steroids, including pregnanolone sulphate which, by inhibiting NMDA tonic neurotransmission, provides neuroprotection and cognitive benefits. In this review, we describe current knowledge on the effects of stress on neurosteroid biosynthesis and GABA_A receptor neurotransmission and summarize available pharmacological strategies that by enhancing neurosteroidogenesis are relevant for the treatment of SSRI-resistant patients.

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Abbreviations

3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; AEA, anandamide; Allo, allopregnanolone; AlloS, allopregnanolone sulphate; CB1, cannabinoid receptor type 1; dMPFC, dorsal medial prefrontal cortex; GABA_ARs, GABA_A receptors; ODNs, oligonucleotides; PAS, pregnanolone sulphate; PEA, palmitoylethanolamide; PES, pregnenolone sulphate; PMS, premenstrual syndrome; PTSD, post-traumatic stress disorder; PXR, pregnane xenobiotic receptor; SBSSs, selective brain steroidogenic stimulants; SPS, single prolonged stress; SSRIs, selective serotonin reuptake inhibitors; StAR, steroidogenic acute regulatory protein; THDOC, tetrahydrodeoxycorticosterone; THIP, 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol; TSPO, 18 kDa translocator protein

Introduction

Neurosteroids, including 5alpha-dihydroprogesterone (5alpha-DHP), **allopregnanolone** (Allo) and its stereoisomers, for example, pregnanolone (PA), are directly synthesized in the central nervous system by brain neurons (glutamatergic and GABAergic long-projecting neurons) (Baulieu and Robel, 1990; Agís-Balboa *et al.*, 2006, 2007) and act not only at classical **steroid hormone receptors** (that regulate gene expression and have long-lasting effects) but also rapidly modulate neuronal excitability by binding to membrane receptors and ion channels. For example, they are potent modulators of **GABA$_A$ receptors** (GABA$_A$Rs) (Belelli and Lambert, 2005). **Progesterone** or its neuroactive metabolite, Allo, when administered to rodents or humans, induces anxiolytic, sedative, anaesthetic, analgesic and anticonvulsant effects (Belelli *et al.,* 2009) by potently increasing Cl$^-$ ion flux induced by GABA binding at GABA$_A$Rs. Both neurosteroid levels and GABA$_A$R expression are subjected to physiological changes during pregnancy and the ovarian cycle (Concas *et al.,* 1998; Maguire *et al.,* 2005), or in pathological conditions caused by protracted or traumatic stress, such as anxiety, depression, and post-traumatic stress disorder (PTSD) (Rasmusson *et al.,* 2006; Romeo *et al.,* 1998; Uzunova *et al.,* 1998).

The upstream synthesis of Allo starts with the cholesterol's transport into the inner mitochondrial membrane of glial cells by the activity of the steroidogenic acute regulatory protein (StAR) and the 18 kDa translocator protein (TSPO) (Papadopoulos *et al.,* 2006), formerly called the peripheral mitochondrial benzodiazepine receptor (Costa and Guidotti, 1991). The enzyme cytochrome **p450scc** converts **cholesterol** into **pregnenolone**, the precursor for all neurosteroids. TSPO in glial cells is important in neurosteroidogenesis and is activated by selective ligands that initiate a cascade of neurosteroid biosynthetic events in several brain areas (Rupprecht *et al.,* 2009). Although this view has been suggested by an extensive number of studies, recent reports also suggest that TSPO is not essential for steroid hormone biosynthesis or viability (Selvaraj and Stocco, 2015; Selvaraj *et al.,* 2016). Pregnenolone can then be sulphated to **pregnenolone sulphate** (PES) and act not only at **NMDA and AMPA receptors** (reviewed in Smith *et al.,* 2014) but also at GABA$_A$Rs (Shen *et al.,* 1999) or can be taken up by neurons where it is further metabolized by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) into

progesterone. Then progesterone can be converted by 5α-reductase type I and 3α-hydroxysteroid dehydrogenase (3α-HSD) into Allo (Figure 1). Allo and its GABA$_A$R-active stereoisomer, PA can be further sulphated into Allo sulphate (AlloS) and PA sulphate (PAS) that act as inhibitors of NMDA receptors neurotransmission (Smith *et al.,* 2014).

The enzymes implicated in the biosynthesis of neurosteroids are not homogeneously expressed in the brain but are region- and neuron-specific (Agís-Balboa *et al.,* 2006). For example, 5α-reductase and 3α-HSD are highly expressed and co-localized in pyramidal neurons and granular cells in the cortex and hippocampus, and in pyramidal-like neurons in the basolateral amygdala (Agís-Balboa *et al.,* 2007). It has been suggested that Allo, synthesized in glutamatergic cortical or hippocampal pyramidal neurons or in granular cells of the dentate gyrus may be secreted by: (i) a paracrine mechanism, which would allow newly-synthesized Allo to exert an effect on GABA$_A$Rs located in the synaptic membranes of distal glutamatergic neurons, (ii) an autocrine mechanism, that allows Allo to bind to postsynaptic as well as to extrasynaptic GABA$_A$Rs located in the synaptic membranes of the same neurons in which it was produced, or (iii) Allo may diffuse into synaptic boutons of the cell bodies or dendrites of neurons in which it is synthesized, so that it reaches GABA$_A$Rs *via* intracellular pathways (Akk *et al.,* 2004; Agís-Balboa *et al.,* 2007). These mechanisms, by which neurosteroids are produced, secreted, and act at postsynaptic and extrasynaptic GABA$_A$Rs, have been supported by more recent studies in which the neural location of Allo has been explored using specific antibodies (Cook *et al.,* 2014).

Neurosteroidogenesis is altered in various neuropsychiatric disorders. An alteration in the concentrations of Allo, **tetrahydrodeoxycorticosterone** (THDOC), PA and progesterone has been identified in the serum, and CSF of patients affected by depression, PTSD, premenstrual syndrome (PMS), and addiction (Romeo *et al.,* 1998; Uzunova *et al.,* 1998; Pinna *et al.,* 2006a; Rasmusson *et al.,* 2006). Administration of selective serotonin reuptake inhibitors (SSRIs), such as the antidepressants **fluoxetine**, **fluvoxamine**, **paroxetine**, and **sertraline**, the serotonin-norepinephrine reuptake inhibitor, **venlafaxine**, or the norepinephrine reuptake inhibitor, **reboxetine**, and the atypical antidepressant, **mirtazapine** improve depressive symptoms by normalizing brain, plasma, and CSF levels of Allo in psychiatric disorders (Uzunov *et al.,* 1996; Romeo *et al.,* 1998; Uzunova *et al.,* 1998; Agís-Balboa
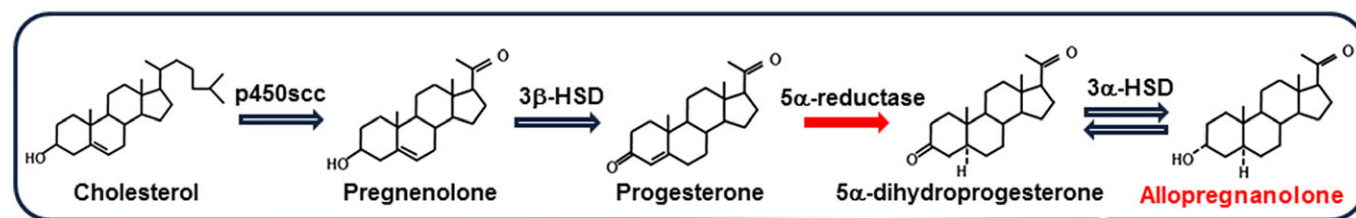
Figure 1

Biosynthesis of allopregnanolone. Neurons can synthesized allopregnanolone *ex novo* starting from pregnenolone, the precursor of all neurosteroids. Pregnenolone is metabolized into progesterone by 3β-HSD and progesterone can be further converted by the enzyme 5α-reductase into 5α-dihydroprogesterone (5α-pregnan-3,20-dione, 5α-DHP), and, finally, 5α-DHP is reduced into allopregnanolone (3α-hydroxy-5α-pregnan-20-one or 3α,5α-tetrahydroprogesterone) by the 3α-HSD enzyme.

et al., 2014; Schüle, 2014). Brain Allo levels are also decreased in mouse models of anxiety, depression and PTSD (Pinna *et al.,* 2004, 2006a; Uzunova *et al.,* 2006). In stressed rodents, we have found that 1/10 of the concentration of SSRIs that engage serotonin mechanisms, is needed to up-regulate Allo levels and improve behaviour (Pinna *et al.,* 2003, 2004). In patients, SSRIs are associated with high non-response rates (Block and Nemeroff, 2014; Otte *et al.,* 2016) when administered at doses that engage serotonergic mechanisms, while it remains unknown whether these 'high' doses still stimulate neurosteroid biosynthesis. Thus, one may wonder whether, when administering an SSRI, 'less is more' and a low dosage may offer a pharmacological advantage over a high dosage in the treatment of PTSD and depression. Hence, new clinical trials are required to test this hypothesis. Whereas treatment strategies specifically for the SSRI non-responder subpopulation have recently been suggested (Fava *et al.,* 2016), new therapies and novel biomarkers to guide selection of treatments for stress-induced psychiatric disorders are urgently required. Neurosteroid biosynthesis can be stimulated by several diverse receptors and neural systems, including the **pregnane xenobiotic receptor** (PXR) and the endocannabinoid system (Frye *et al.,* 2012; Vallée *et al.,* 2014; Vallée, 2016). The use of synthetic analogues of Allo also represents a strategy to reinstate functional GABAergic neurotransmission in PTSD or other neuropsychiatric disorders with altered neurosteroidogenesis.

This report reviews the neurophysiological role of neurosteroids acting as positive or negative modulators of GABA$_A$Rs, including when several GABA$_A$R subunit expression is changed due to stress. The down-regulation of neurosteroid levels found in PTSD patients can be modelled in rodent models of stress, including the socially isolated (SI) mouse and the single prolonged stress (SPS) mouse. This translational approach provides the ground for exploiting several neurosteroidogenic targets with drugs that improve emotional behaviour by enhancing Allo levels and thereby offer promising therapeutic tools for novel PTSD treatment. The emerging therapeutic role of sulphated pregnane steroids in neuropsychiatric disorders is also reviewed as a possible additional mechanism for neurosteroidogenic drugs to improve both emotional and cognitive behaviour. Taken together, deficits in Allo biosynthesis interacting with stress-induced changes in GABA$_A$R subtypes may represent a valuable *biomarker axis* for use in predicting, preventing, treating and monitoring symptoms of stress-induced disorders, including PTSD and depression.

Neurosteroid and stress modulation of GABA$_A$Rs

GABA$_A$Rs are probably the principal target of neurosteroids, including the endogenous neurosteroids, Allo and THDOC (Reddy, 2003; Belelli and Lambert, 2005; Belelli *et al.,* 2009). Allo plays a pivotal neurophysiological role by fine-tuning GABA$_A$Rs to agonists and allosteric modulators (Pinna *et al.,* 2000) by binding at two distinct residues on GABA$_A$Rs; a potentiation site is formed in a cavity within α-subunits and an activation site is located at the interface of α and β subunits

(Hosie *et al.,* 2006). By this mechanism, Allo may also play an important role in the regulation of emotional behaviour (Pinna *et al.,* 2009).

There are two types of GABA$_A$Rs that differ by subneuronal distribution, function, structure and pharmacology: synaptic and extrasynaptic receptors (Belelli and Lambert, 2005; Carver and Reddy, 2013). Synaptic GABA$_A$Rs contain mainly **α1**, **α2** and **α3** subunits, along with β subunit variants and the **γ2** subunit (Fritschy and Panzanelli, 2014), and are located at the synaptic membrane (Somogyi *et al.,* 1996; Essrich *et al.,* 1998), where they mediate a rapid and transitory inhibition (*phasic current*) following the intermittent release of high (millimolar) concentrations of GABA from the presynaptic terminals (Farrant and Nusser, 2005); importantly, these receptors are sensitive to benzodiazepines as well as Allo (Hájos *et al.,* 2000). GABA$_A$Rs that contain **α4** and **α6** subunits, often along with the **δ** subunit (instead of the γ2 subunit) are located at the peri- and extra-synaptic membranes (Nusser *et al.,* 1998; Wei *et al.,* 2003; Tretter and Moss, 2008; Fritschy and Panzanelli, 2014). Extrasynaptic GABA$_A$Rs containing α4/β/δ mediate a persistent inhibition (*tonic current*) under conditions of low (nanomolar) concentrations of ambient GABA (Mody, 2001; Semyanov *et al.,* 2004; Glykys and Mody, 2007). Deletion of the δ subunit (and the concomitant loss of α4 expression) significantly reduces tonic receptor activation in granule cells of the dentate gyrus (Peng *et al.,* 2002; Stell *et al.,* 2003). Reports also suggest that efficacy of δ-containing extrasynaptic receptors for GABA can be increased by neurosteroids (Stell *et al.,* 2003; Shu *et al.,* 2012). Remarkably, GABA$_A$Rs incorporating α4, α6 and δ subunits are highly sensitive to Allo (Farrant and Nusser, 2005; Belelli *et al.,* 2009). The modulation of GABA$_A$Rs subtypes by Allo is thus pharmacologically distinct from benzodiazepines, which fail to activate GABA$_A$Rs containing α4 and α6 subunits or extrasynaptic GABA$_A$Rs receptors containing δ subunits (Costa and Guidotti, 1996). Thus, these features make them an attractive pharmacological target after stimulation of neurosteroid biosynthesis, following administration of an Allo analogue, such as ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one), or the GABA$_A$R agonist, **4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol** (THIP), which shows selectivity for δ-containing receptors (Gulinello *et al.,* 2003; Belelli and Lambert, 2005; Herd *et al.,* 2013).

Extrasynaptic receptors containing **α5** subunits are also involved in the modulation of tonic inhibition in the hippocampus, olfactory bulb and cerebral cortex (Caraiscos *et al.,* 2004; Glykys and Mody, 2006; Serwanski *et al.,* 2006; Bonin *et al.,* 2007; Glykys *et al.,* 2008; Fritschy and Panzanelli, 2014; Perez-Sanchez *et al.,* 2017). In fact, α5-containing extrasynaptic GABA$_A$Rs, similarly to δ-containing GABA$_A$Rs, are highly sensitive to low, persistent, ambient concentrations of GABA. Moreover, a study conducted on recombinant α5β2γ2 and α1β2γ2 GABA$_A$Rs in *Xenopus oocytes,* using a two electrodes voltage-clamp technique, showed a significantly lower level of desensitization in the α5-containing receptors. In addition, the potencies of Allo and THDOC to increase GABA response were significantly higher in the α5-containing receptors; however, their efficacies among the two receptors was not changed. In both α1- and α5-containing receptors, the efficacy of THDOC was higher

than that of Allo (Rahman *et al.,* 2006). Using patch clamp recording in the perirhinal cortex, Schwabe *et al.* (2005) showed that THDOC prolonged the time course and the amplitude of mono- and biphasic miniature inhibitory postsynaptic currents in rats that overexpress α2, α3 and α5 subunits. By comparison, THDOC was much more effective than Allo in inducing a tonic current in these rats.

The α5-containing GABA$_A$R subtypes in the hippocampus play an important role in cognition, learning and memory (Caraiscos *et al.,* 2004; Soh and Lynch, 2015). KO mice presenting a deletion of α5 subunit in the dentate gyrus are characterized by reduced tonic inhibition of granule cells without any change in fast phasic inhibition. As a result, α5-KO mice show impairments in cognitive tasks (Engin *et al.,* 2015). Recently, α5 positive selective allosteric modulators have been synthesized and they improved hippocampal-dependent memory in aged rats with identified cognitive impairment (Koh *et al.,* 2013).

Importantly, GABA$_A$Rs can also be negatively modulated by steroids that are sulphated at the C3 position, including PES and **dehydroepiandrosterone sulphate** (DHEAS). Although the precise site for sulphated steroids has not been established, it appears that PES acts at GABA$_A$Rs *via* a membrane sensitive site rather than a lock and key site positioned in the receptor complex (Shen *et al.,* 1999; Akk *et al.,* 2001). This view supports two distinct binding sites for neuroactive steroids that mediate positive (e.g. Allo, PA and THDOC) and negative (e.g. PES and DHEAS) GABA$_A$R modulation (Rahman *et al.,* 2006; Wang *et al.,* 2008). The inhibitory site for sulphated steroids is distinct from the site where **picrotoxin** acts and sulphated steroids seem to induce inhibition of GABA action by enhancing GABA$_A$R sensitization (Eisenman *et al.,* 2003).

Furthermore, the 3β-hydroxysteroids, including (3β,5α)-3-hydroxypregnan-20-one, (3β5β)-3-hydroxypregnan-20-one) and (3β5β)-THDOC can noncompetitively antagonize the potentiation on GABA$_A$Rs induced by their 3α-diastereomers, including Allo (3α,5α)-3-hydroxypregnan-20-one, PA (3α,5β)-3-hydroxypregnan-20-one, or 3α5α-THDOC (3α,5α)-3,21-dihydroxypregnan-20-one and 3α5β-THDOC (3α,5β)-3,21-dihydroxypregnan-20-one that positively modulate GABA$_A$Rs (Wang *et al.,* 2002). The 3β-hydroxysteroids are also able to significantly reduce the potentiation induced by high concentrations of barbiturates. The profile of block is similar to that exhibited by sulfated steroids that block GABA$_A$Rs (Wang *et al.,* 2002).

Collectively, these observations are highly significant in designing therapeutic strategies to counteract cognitive and emotional deficits resulting from a GABA signal transduction dysfunction observed after protracted stress, as well as behavioural impairment related to excessive GABA-mediated inhibition.

Depression and PTSD and other stress-related disorders are accompanied by changes in GABAergic neurotransmission. For example, patients with major depression show decreases in GABA levels (Luscher *et al.,* 2011). Postmortem studies show reduced number of GABAergic interneurons in the cortex and hippocampus of patients with mood disorders (Benes *et al.,* 2008). Up-regulation of genes governing expression of GABA$_A$R subunits, such as α5 and δ, was also shown in prefrontal
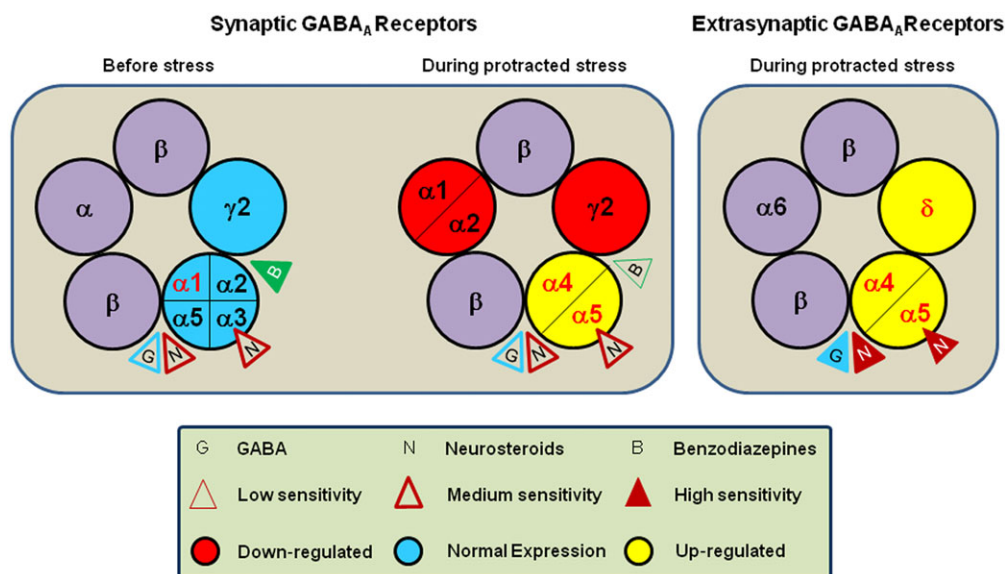
cortex subregions of depressed patients (Choudary *et al.,* 2005; Klempan *et al.,* 2009). A study of the brains of depressed suicide victims has demonstrated a marked up-regulation of *GABRA5* (α5) and *GABRG2* (γ2) genes in the prefrontal cortex and inferior temporal cortex while *GABRG1* (γ1) was downregulated in the prefrontal cortex (Sequeira *et al.,* 2009). Altered abundance of α1, α3, α4 and δ subunit mRNAs in the frontopolar cortex of suicide victims was also shown (Merali *et al.,* 2004).

Furthermore, a PET and a structural MRI study of male Dutch veterans showed a significant reduction of [^{13}C] flumazenil binding in the cerebral cortex, hippocampus, and hypothalamus of PTSD patients (Geuze *et al.,* 2008). This is in accord with a previous study showing reduced [^{123}I] iomazenil uptake in Brodman's Area 9 in male Viet Nam veterans with PTSD (Bremner *et al.,* 2000). This evidence points to possible stress-induced changes in the expression of GABA$_A$R subunits at which benzodiazepines bind and act. Hence, identification and further characterization of these deficits could be valuable in selecting treatments to improve symptoms of PTSD and depression.

In preclinical studies, chronic cold stress in male rats induced a reduction of GABA levels, GAD expression, and **[^{3}H]GABA** binding in the cerebral cortex, olfactory bulb, hypothalamus and striatum (Acosta *et al.,* 1993). Numerous studies report altered GABA$_A$R subunit expression following chronic stress. In male mice, repeated swim stress induces a decrease of hippocampal α1 subunit expression (Montpied *et al.,* 1993). Chronic unpredictable stress decreases hippocampal δ subunit and increases hippocampal α5 subunit expression in male rats (Verkuyl *et al.,* 2004). Protracted social isolation decreases α1, α2 and γ2, and increases α4 and α5 GABA$_A$ subunit expression in the hippocampus and frontal cortex (Pinna *et al.,* 2006b; Matsumoto *et al.,* 2007). These changes are associated with altered pharmacological response to GABA$_A$R agonists, such as resistance to the sedative and anxiolytic effects of **diazepam** and **zolpidem** (Pinna *et al.,* 2006b; Nin *et al.,* 2011a), which bind with high affinity to α1, α2, α3 and α5 (diazepam) and α1 (zopidem) subunits. The binding site of benzodiazepines is located at the interface of α and γ2 subunits (Figure 2), and GABA$_A$Rs devoid of γ2 subunits show lower sensitivity to the pharmacological action of these drugs (Rudolph *et al.,* 1999). Thus, resistance to the sedative and anxiolytic effects of diazepam and zolpidem in SI mice could relate to down-regulation of α1, α2 and γ2 subunits and to up-regulation of GABA$_A$R subtypes containing α4 and δ subunits, which are insensitive to benzodiazepines (Rudolph *et al.,* 1999; Serra *et al.,* 2006; Nin *et al.,* 2011a). This is further supported by the finding that social isolation increases the expression of specific GABA$_A$R subunits mainly expressed extrasynaptically, like α4 and α5 subunits (Belelli *et al.,* 2009; Fritschy and Panzanelli, 2014; Deprez *et al.,* 2016). A study conducted in SI rats also showed up-regulation of hippocampal α4 and δ subunits, which confers higher sensitivity to **alcohol** (Serra *et al.,* 2006). Whether stress directly causes alterations in the expression of GABA$_A$R subunits or whether these changes are the effect of altered neurosteroid biosynthesis remains to be elucidated.

Altered expression and function of GABA$_A$Rs were observed after ethanol exposure that changes Allo levels in

Synaptic GABA_A Receptors

Before stress | During protracted stress

Extrasynaptic GABA_A Receptors

During protracted stress

G	GABA	N Neurosteroids	B Benzodiazepines

Low sensitivity | Medium sensitivity | High sensitivity

Down-regulated | Normal Expression | Up-regulated

Figure 2

Schematic representation of the GABA_AR. Structurally, GABA_AR is a macromolecular complex composed by five subunits that delimit a central chloride-permeable ion channel. The most expressed subunit configuration in the brain under basal condition includes two α, two β and one γ subunit (*see figure on the left*). GABA, by acting at two binding sites located between α and β subunits (Smith and Olsen, 1995; Baumann *et al.,* 2002), induces the channel opening, allows the chloride ion influx, and, ultimately, permits the inhibitory action on cellular membranes. Moreover, the GABA_AR has two different residues important for neurosteroid (e.g. allopregnanolone) action; the first is located between α and β subunits, and the second is formed by a cavity exclusively on α subunits (Hosie *et al.,* 2006). The binding sites for benzodiazepine are located between α and γ subunits (Sigel, 2002). Importantly, the different pharmacological profiles of benzodiazepines depend on the specific α subunit included in the GABA_AR. For example, the α1 subunits mediate the sedative, amnesic and partially anticonvulsant effects inducted by benzodiazepines (McKernan *et al.,* 2000); the α2 subunits are responsible for the anxiolytic effects (Löw *et al.,* 2000), and α3 subunits induce muscle relaxant and, partially, anxiolytic effects (Whiting, 1999). The α5 subunits are involved in the amnesic effects and tolerance induced by benzodiazepines, while GABA_ARs including α4 and α6 subunits are benzodiazepine-insensitive (Wisden *et al.,* 1991). Thus, different receptor configurations are associated to different pharmacological and functional properties. In particular, α/β/γ GABA_AR subtype, the most prevalent configuration expressed in the synaptic membrane region (mediating inhibitory phasic currents), is highly sensitive to benzodiazepines, and, in parallel, shows lower sensitivity both to GABA and neurosteroids (Nusser and Mody, 2002). On the other hand, the α/β/δ GABA_AR subtype, located in the extrasynaptic region (mediating inhibitory tonic currents), is not sensitive to benzodiazepines, and show low efficacy for GABA, and neurosteroids increase its agonist efficacy (Stell *et al.,* 2003; Shu *et al.,* 2012). The efficacy of neurosteroids is greatly enhanced for this receptor combination (Brown *et al.,* 2002; Nusser and Mody, 2002; Wohlfarth *et al.,* 2002) (*see figure on the right*). The physiological and pharmacological relevance of neurosteroid action on the different GABA_AR subtypes has been studied in relation with stress and several neuropsychiatric disorders. Importantly, the expression of specific subunits of GABA_ARs changes after prolonged stress. For instance, the α4, α5 and δ subunit expression is markedly increased, and the α1, α2 and γ2 is significantly decreased, both in the frontal cortex and hippocampus of SI mice, a putative mouse model of PTSD (Pinna *et al.,* 2006b; Pibiri *et al.,* 2008). Moreover, these effects are strongly related to a decreased pharmacological response to benzodiazepines (Pinna *et al.,* 2006b; Nin *et al.,* 2011a). On the other hand, the altered GABA_AR subunit composition, which results from protracted stress, favours high sensitivity for neurosteroids, neurosteroid-like molecules (e.g. the synthetic allopregnanolone analogue, ganaxolone) or alcohol (Pinna *et al.,* 2006b; Serra *et al.,* 2006). Collectively, these findings suggest that treatments that stimulate allopregnanolone biosynthesis may offer a therapeutic advantage for stress-induced psychiatric disorders, in which benzodiazepines are ineffective or in patients who show a poor or no response to SSRI antidepressants.

different brain regions (Cagetti *et al.,* 2003; Suryanarayanan *et al.,* 2011; Lindemeyer *et al.,* 2014). The expression of specific subunits of the GABA_AR also changes following fluctuations in neurosteroid levels induced by oestrous cycle, pregnancy, after delivery or by treatment with synthetic steroids, including oral contraceptives and anabolic androgenic steroids (Concas *et al.,* 1998; Maguire *et al.,* 2005; Pibiri *et al.,* 2006; Porcu *et al.,* 2012; Modol *et al.,* 2014; Locci *et al.,* 2017). GABA_ARs containing α4 subunits are overexpressed during progesterone withdrawal (Smith *et al.,* 1998) and after inhibition of 5α-reductase, which decreases brain Allo levels. Also a switch in the expression of extrasynaptic δ subunits and synaptic γ2 GABA_A subunits is observed across the oestrous cycle (Maguire *et al.,* 2005)

and in pregnancy (Concas *et al.,* 1998; Sanna *et al.,* 2009). Other findings suggest rapid, continuous and dynamic changes in the localization and distribution of synaptic and extrasynaptic GABA_ARs in the hippocampus (Jacob *et al.,* 2008). Thus, in response to stress and hormonal state, dynamic changes in the expression of GABA_ARs and related alterations in GABA_AR function and pharmacology mediated by inhibitory phasic *versus* tonic GABA currents is conceivable. Administration of molecules with high sensitivity for extrasynaptic receptors, like Allo, its analogues, or THIP – rather than administration of benzodiazepines – may be indicated when such switches in brain GABA_AR subtypes occur. Such agents that target subunits mainly expressed in extrasynaptic GABA_ARs, in fact, show anxiolytic

effects in SI mice, whereas benzodiazepines are ineffective (Pinna *et al.,* 2006b; Nin *et al.,* 2011a).

These converging findings suggest that mouse stress models with altered GABA$_A$R subunit composition can be used to study drugs that target extrasynaptic GABA$_A$Rs that have been up-regulated in response to stress. Drugs acting at such receptors would be expected to alter GABAergic tonic inhibition in a manner that improves cognitive capacities, which are critical to the reprocessing of traumatic events and recovery from PTSD and depression.

Neurosteroid down-regulation: From mouse models to psychiatric disorders

Deficits in neurosteroid levels are strongly associated with major depression and PTSD (for a review please see Zorumski *et al.,* 2013). Patients with depression show serum, plasma, CSF, and brain reductions of Allo levels and/or biosynthesis (Romeo *et al.,* 1998; Uzunova *et al.,* 1998; van Broekhoven and Verkes, 2003; Agís-Balboa *et al.,* 2014; Porcu *et al.,* 2016). Likewise, depression during pregnancy and postpartum is associated with altered Allo levels (Nemeroff, 2008). Progesterone levels are subjected to a rapid decline immediately after parturition; thus, progesterone (and Allo) 'withdrawal' may result in anxiety and depressive symptoms (Smith *et al.,* 2007). Also, patients who take drugs that inhibit endogenous Allo levels, for example, 5α-reductase inhibitor, **finasteride**, can exhibit cognitive dysfunction, depressive symptoms and suicidality (Altomare and Capella, 2002; Caruso *et al.,* 2015; Welk *et al.,* 2017). The concentration of Allo in the CSF is 40–60% lower in patients affected by unipolar major depression and premenopausal women with PTSD, respectively, with the lowest levels found in the PTSD patients with comorbid depression (Uzunova *et al.,* 1998; Rasmusson *et al.,* 2006). Chronic treatment with fluoxetine or fluvoxamine restored plasma and CSF Allo levels in association with improvements in depressive symptoms (Romeo *et al.,* 1998; Uzunova *et al.,* 1998). Thus, the normalization of Allo may mediate the anxiolytic and antidysphoric pharmacological effects of SSRIs. Patients with PMS show alterations in progesterone-derived neurosteroid plasma levels during the luteal phase (Rapkin *et al.,* 1997; Sundström *et al.,* 1997; Bicíková *et al.,* 1998; Girdler *et al.,* 2001), and like depression, anxiety and mood swings in PMS are improved by increasing Allo concentrations (Bäckström *et al.,* 2003). Smith *et al.* (1998) have suggested that PMS may be explained by progesterone withdrawal at the end of the luteal phase; however, symptoms are often present earlier in the luteal phase, suggesting that deficits in Allo synthesis, as observed among women with PTSD, may be responsible for such symptomatology.

Studies conducted on the oestrous cycle of female rats have demonstrated that the drastic decrease of progesterone concentrations during diestrous is associated with an overexpression of extrasynaptic α4β1δ GABA$_A$Rs in the periaqueductal grey matter, which mediates the anxiolytic and mood regulating effects of Allo in this oestrous phase (Lovick, 2006). Increased anxiety-like behaviour during the diestrous phase in rodents is prevented by short-term non-

serotonergic low dose treatment with fluoxetine, which may counteract the rapid physiological fall in Allo (Lovick, 2013).

Brain levels of Allo are reduced in male rodent models of stress-induced behavioural dysfunction, including the SI mouse (Pibiri *et al.,* 2008) and SPS (Zhang *et al.,* 2014), which are suitable preclinical translational approaches to model the behavioural alterations observed in patients affected by anxiety, depression and PTSD (Pinna, 2010; Nin *et al.,* 2011b). These models allow investigations into the molecular mechanisms of GABA$_A$R neurotransmission dysfunction that results from: (i) stress-induced changes in GABA$_A$Rs and (ii) the reduction of Allo brain concentrations (Pinna, 2010). In SI male mice, the reduction of Allo is a result of decreased expression of 5α-reductase type I in the pyramidal-like neurons of the basolateral amygdala, in the hippocampal CA3 pyramidal neurons and the glutamatergic granular cells of the dentate gyrus, as well as in cortical pyramidal neurons of layers V-VI (Agís-Balboa *et al.,* 2007). The expression of the enzyme 3α-HSD, as well as that of TSPO was not altered in SI mice (Dong *et al.,* 2001).

This evidence thus suggests that social isolation in male mice specifically alters the p450 enzyme pathway involved in Allo biosynthesis. Allo biosynthesis was not affected by social isolation alone in female mice, unless they were ovariectomized and replaced with **testosterone** (Pinna *et al.,* 2005). These findings are strikingly consistent with the sex specificity of the Allo synthetic pathway dysfunction in humans. In premenopausal women with PTSD, a synthesis blockade of Allo was found at 3α-HSD (Rasmusson *et al.,* 2006). New CSF data from trauma-exposed men with and without PTSD reveals a PTSD-related p450 enzyme blockade at 5α-reductase (Rasmusson *et al.,* 2016). Of note, Gillespie *et al.* (2013) reported an association between PTSD risk in males and a polymorphism in the **5α-reductase 2** gene. Although focus in the field of neuropsychiatry has been, for the most part, on brain 5α-reductase 1, 5α-reductase 2 is highly expressed peripherally and specifically in the zona glomerulosa of the adrenal cortex (Eicheler *et al.,* 1994), a possible significant source of another GABAergic neuroactive steroid that changes during stress and accesses the brain, THDOC (Purdy *et al.,* 1991). Thus, the extent of involvement and manner by which deficient expression of 5α-reductase 1 and maybe 2 contribute to neuropsychiatric disorders with a down-regulation of Allo and other neurosteroid (i.e. THDOC) levels (e.g. in PTSD) are yet to be clarified.

To elucidate the hypothesis that Allo represents a putative biomarker for stress-induced PTSD (Dong *et al.,* 2001; Pinna, 2010), we established in male mice the impact of social isolation on reducing brain levels of Allo and on several emotional behaviours (Pibiri *et al.,* 2008; Nelson and Pinna, 2011). These mice show an increase in aggressive behaviour, anxiety-like behaviour and exaggerated contextual fear responses compared to group-housed mice (Pibiri *et al.,* 2008; Nin *et al.,* 2011b). Interestingly, a down-regulation of corticolimbic Allo levels associated with behavioural deficits, such as enhanced anxiety-like behaviour and exaggerated contextual fear responses, were also observed in the SPS mouse model of PTSD. These PTSD-like behaviours were improved by normalizing cortical Allo levels after stimulation of TSPO (Zhang *et al.,* 2014, 2016).

Using SI mice, we similarly observed that systemic administration of Allo or equimolar concentrations of its

analogue, ganaxolone reduces behavioural dysfunction related to corticolimbic Allo deficits (Pinna and Rasmusson, 2014). In addition, administration of SSRI antidepressants that at low doses, devoid of serotonergic mechanisms, act as *selective brain steroidogenic stimulants* (SBSSs) and up-regulate Allo levels, improve behaviour in Allo deficient rodents, whereas it shows no steroidogenic effects in group-housed mice (Pinna *et al.,* 2003, 2009). While it is not completely clear why SSRI treatment is associated with low pharmacological response rate at doses that one would expect to enhance Allo levels, some speculations can be drawn. First, SSRI treatment resistant depression may not be a result of a simple serotonin deficit but rather by an excess of midbrain peri-raphe serotonin and resulting deficits at the level of frontolimbic projection sites, which ultimately may compromise serotonin-mediated neuroplasticity (Coplan *et al.,* 2014). Second, it is also conceivable that too high doses of SSRIs may even mask beneficial drug effects that are often associated with severe side effects included in the spectrum of serotonin syndrome and that include agitation, mental confusion, increased reflexes, tremor, sweating and even seizures in the worst cases (Volpi-Abadie *et al.,* 2013; Coplan *et al.,* 2014; Ferri, 2016). Third, the neurosteroidogenic effect of these drugs at low doses that correlate with improved behaviour (Pinna *et al.,* 2003, 2004; Uzunova *et al.,* 2006; Lovick, 2013) makes one wonder whether 5-HT reuptake inhibition may even be necessary or sufficient to explain SSRIs pharmacological action to improve dysphoria, anxiety and other mood disorders in depressed patients (reviewed in Pinna *et al.,* 2006a, 2009). Finally, it is important to elucidate in clinical trials whether neurosteroidogenic low SSRIs doses offer a pharmacological advantage over large SSRIs doses in the treatment of PTSD and depression. Due to lack of controlled clinical trials to resolve this issue, these findings have inspired studies in which low, non-serotonergic but neurosteroidogenic doses of fluoxetine have been suggested for the treatment of PMS (Lovick, 2013). Moreover, administration of low fluoxetine doses may be valuable to treat depression in teens, as it may avoid the increased rate of suicide observed when depressed adolescents are administered higher serotonergic doses of SSRIs (Rahn *et al.,* 2015).

Resistance to currently prescribed antidepressant drugs

Mental and substance use disorders are major contributors to the global burden of disease (Whiteford *et al.,* 2013), and major depressive disorders represent a public problem affecting approximately 16% of adults in the United States. Several SSRIs and other antidepressants can counteract depressive symptoms. However, in spite of the rapid evolution of pharmacological therapies, epidemiological evidence demonstrates that only 50% of treated depressed patients respond positively to first-line therapy with antidepressants, while more than one third develop resistance to antidepressants (Kemp *et al.,* 2008). Moreover, half of patients treated with SSRI antidepressants fail to reach a full remission (Golden *et al.,* 2002; Rush *et al.,* 2006; Kemp *et al.,* 2008). The causes of resistance to antidepressants can be multiple and include pharmacokinetic factors or comorbidity with other mental disorders (El-Hage *et al.,* 2013;

Willner *et al.,* 2013). Another major negative aspect of these therapies is their delayed onset of therapeutic action (4–6 weeks) (Belzung, 2014).

PTSD is another prevalent disorder with no specific treatment. The efficacy of the SSRIs, sertraline and paroxetine, the only drugs currently approved by the FDA for the treatment of PTSD, rarely exceed 40–60%; further, only 20–30% of SSRI-treated PTSD patients do not relapse. Exposure to combat situations results in a particularly severe form of PTSD that tends to be chronic, disabling and heavily comorbid with depression and suicide (Prigerson *et al.,* 2001). Veterans affected by PTSD are generally resistant to SSRI therapy (van der Kolk *et al.,* 1994), leaving limited available options for their treatment. Studies suggest that certain types of trauma or traumas in a crucial period of an individual's life can predict which subpopulation of PTSD and/or depressed patients will respond successfully to treatment with SSRI antidepressants. Recent studies in the field have found, for instance, that a history of early-life trauma may predict a failure to respond to antidepressants during adulthood. Williams and collaborators, as part of the international Study to Predict Optimized Treatment for Depression, a randomized clinical trial with enrolment from December 2008 to January 2012 at eight academic and nine private clinical settings in five countries, have conducted a study to establish the role of early-life trauma as a predictor of acute response to antidepressants. They found that exposure to several types of traumatic events before the age of 18 (overall trauma 'load' and specific type of abuse) was significantly more likely to result in major depression. Abuse in general but – most notably – abuse occurring at 7 years of age or younger predicted a lower response to 8 weeks of SSRI antidepressants. Hence, specific types of early-life trauma and in particular physical, emotional and sexual abuse when they occurred at 7 years of age or before may dictate a patient's future response to antidepressant therapy (Williams *et al.,* 2016).

High resistance to SSRIs invites the development of new approaches for the treatment of non-responsive patients. In preclinical studies of stress-induced PTSD and depression, it would be important to determine variability among animals or groups that are more responsive from others that are less responsive to the beneficial effects of SSRIs. The non-responders or less responsive may offer an important subpopulation in which to explore new pharmacological agents that may improve behaviour acting at different neurosteroidogenic receptors (e.g. TSPO and **cannabinoid receptors**) than SSRIs'. These animals may also be studied using agents that may act directly and selectively at specific $GABA_AR$ subunits (e.g. selective agonists for α4 or α5), given that stress appears to up-regulate these subunits.

Neurosteroidogenesis: A putative target for development of new pharmacotherapies

Numerous preclinical studies have suggested a number of brain neurosteroidogenic targets for new anxiolytic and PTSD treatments (Rupprecht *et al.,* 2009; Pinna, 2014). One of the best characterized is probably the TSPO (Costa *et al.,* 1994;

Papadopoulos *et al.,* 2006), that, according to traditional views, serves as the upstream starting point for the neurosteroidogenic cascade. Transfer of cholesterol from the outer to the inner mitochondrial membrane occurs through the 'transduceosome' complex. This requires the cytosolic StAR to interact with the outer mitochondrial membrane proteins, TSPO, and the voltage-dependent anion channel (VDAC). Cholesterol transfer occurs at contact sites that are peculiar for bridging the membranes of VDAC and inner mitochondrial membrane adenine nucleotide translocase. Immunoblot and mass spectrometry analyses revealed that the 800-kDa complex is responsible for steroid production. The 800-kDa complex offers the micro-environment needed for cytochrome P450 enzyme CYP11A1 activity and for which the StAR mobilizes the cholesterol bound at the 800-kDa complex, which results in increased steroidogenesis. Thus, a multimeric protein complex, which extends from the outer to the inner mitochondrial membrane is implicated for the hormone-induced import, segregation, targeting, and, finally, metabolism of cholesterol (Rone *et al.,* 2012).

New molecules that bind and potently stimulate TSPO are able to induce a downstream increase in Allo concentrations in mouse hippocampus and cortex. TSPO activating drugs, such as AC-5216/XBD173 and **etifoxine** are able to exert significant anxiolytic effect in animal models of PTSD (Rupprecht *et al.,* 2010). Importantly, the behavioural effects induced by these drugs seem to be directly related to increases in brain Allo levels; indeed, pretreatment with finasteride drastically suppresses their anxiolytic effect (Schüle *et al.,* 2011). These findings have been demonstrated also in studies conducted with anxious patients (Rupprecht *et al.,* 2010; Schüle *et al.,* 2011). Chronic administration with YA-IPA08, a selective TSPO activator, induced a marked reduction of anxiety and contextual fear resulting from exposure to the SPS, an effect inhibited by pre-injection with the TSPO antagonist, **PK11195** (Zhang *et al.,* 2014). In addition, the administration of YA-IPA08 normalized Allo levels in the prefrontal cortex and serum of PTSD-mice in whom Allo levels were decreased by SPS exposure, an effect that was completely blocked by PK11195 (Zhang *et al.,* 2014).

The PXR represents another neurosteroidogenic target with potential to modulate emotional behaviour. PXR is a ubiquitous nuclear receptor that acts as a classical transcription factor that enhances the expression of several major families of genes such as the cytochrome p450 enzymes (Frye *et al.,* 2014a). PXR mediates the production or metabolism of various neurobiological factors (Kliewer *et al.,* 2002). The expression of PXR changes during the oestrous cycle in the adult female rat; indeed, the abundance of both PXR mRNA and protein is higher during proestrous than in diestrous 1 or in male rats (Frye *et al.,* 2012). A recent study has demonstrated that infusion of the PXR antisense oligonucleotides (ODNs) in the VTA, a brain area in which this receptor is highly expressed, induces a drastic reduction of time spent in the open arms of the elevated plus maze; of note, infusion of the antisense ODNs targeted against PXR outside the VTA does not produce the same effect. Thus, specific down-regulation of the activity of PXR in the VTA is associated with an increase in anxiety-like behaviour (Frye *et al.,* 2012). Interestingly, Allo binds directly at PXR. In addition, antagonizing TSPO with PK11195, which

significantly decreases Allo in the VTA, inhibits sexual behaviour, an effect reversed by Allo administration, but not by Allo co-administered with PXR antisense ODNs (Frye *et al.,* 2014b). This evidence suggests that PXR, acting upstream of TSPO, may modulate neurosteroid-mediated behavioural effects.

The endocannabinoid system is a newly discovered target for stimulation of neurosteroidogenesis. **Δ9-tetrahydrocannabinol**, the most important psychotropic compound found in *Cannabis sativa*, markedly increases the synthesis of pregnenolone by the direct activation of the **cannabinoid receptor type 1** (CB1) (Vallée *et al.,* 2014; Vallée, 2016). There are several similarities between the endocannabinoid and neurosteroid systems. Levels of both Allo and **anandamide** (AEA) are decreased in a variety of animal models of anxiety and depression and in patients with depression and PTSD (Romeo *et al.,* 1998; Uzunova *et al.,* 1998; van Broekhoven and Verkes, 2003; Rasmusson *et al.,* 2006; Hill *et al.,* 2008, 2013). Expression of CB1 receptors is high in targets for neurosteroidogenesis (e.g. pyramidal neurons) in brain areas involved in emotional behaviours such as hippocampus, frontal cortex and amygdala (Katona, 2009). The role of the endocannabinoid system in the modulation of emotional behaviours, and specifically in the attenuation of exaggerated fear responses, is underscored by the extensive literature addressing the therapeutic effects of AEA or other CB1 agonists (Lisboa *et al.,* 2010; Jenniches *et al.,* 2016). Moreover, recent studies have demonstrated that **cannabidiol**, another main active compound of cannabis, blocks reconsolidation of recent and older contextual fear memories by a CB1 receptor-mediated mechanism (Stern *et al.,* 2012). Similar effects were observed when reconsolidation of reactivated memories was blocked with midazolam, a benzodiazepine that, importantly, acts at TSPO to induce Allo biosynthesis (Stern *et al.,* 2012). The endocannabinoid, **N-palmitoylethanolamide** (PEA) also stimulates Allo biosynthesis by binding to intracellular **PPARα**, a class of nuclear hormone receptor involved in various cellular and molecular mechanisms (Sasso *et al.,* 2012). Administration of PEA induces antidepressant effects in a similar manner to the SSRI/SBSS, fluoxetine (Yu *et al.,* 2011). This evidence thus highlights the existence of a fine network of relationships between the neurosteroid and the endocannabinoid systems; and the development of cannabinoid-like molecules with neurosteroidogenic effects could lead to new depression, anxiety and PTSD therapeutics.

Finally, in patients for whom the administration of an SBSS (i.e. a molecule that induces steroidogenesis) is ineffective because neurosteroidogenesis is greatly impaired, an alternative therapeutic strategy may be the use of Allo 'substitutes' (Gulinello *et al.,* 2003), such as ganaxolone, or other molecules such as **topiramate** that directly activate extrasynaptic GABA$_A$Rs (Berlant, 2004). For example, treatment with pregnenolone appears to have the potential to improve emotional regulation by increasing Allo levels. Sripada *et al.* (2013) showed that intravenous administration of pregnenolone reduced activation of brain areas involved in negative emotions and increased activity in the dorsal medial prefrontal cortex (dMPFC). It also enhanced connectivity between the amygdala and dMPFC in association with reduced self-reported anxiety and improved performance on

an emotion regulation task. Remarkably, activities such as brief but vigorous acute exercise has been shown to raise Allo levels among responsive individuals (Scioli-Salter *et al.,* 2015) to levels shown by Sripada *et al.* (2013) to enhance amygdala-prefrontal cortical coupling in association with improved emotion regulation. After contextual fear conditioning in SI mice, a single dose of ganaxolone (an Allo analogue) administered after the first session of extinction training steeply and significantly reduced behavioural freezing on the following day. Freezing remained low in these mice over the remaining extinction training sessions and did not return *via* 'spontaneous recovery' after the passage of time, as it did in vehicle-treated rodents that extinguished slowly to the same low freezing endpoint (Pinna and Rasmusson, 2014). This study suggests that Allo administered to Allo-deficient mice prevents reconsolidation of fear and/or enhances extinction retention. Hence, the use of Allo or synthetic analogues of Allo, acting preferentially at extrasynaptic GABA$_A$Rs, may represent a valid means of augmenting exposure based cognitive treatments for PTSD. Remarkably, a rapid remission of post-partum depression achieved at 60 h and mainted at 30 day follow-up was recently induced in 70% of patients treated with a two-day course of intravenous Allo (SAGE-547) compared to 10% who received placebo (Herper, 2016; Kanes *et al.,* 2016; Meltzer-Brody 2016).

Together this evidence suggests that alterations in Allo biosynthesis and changes in GABA$_A$R subunit expression contribute to disorders of mood and cognition during stress and changes in reproductive state. Behaviour can be improved by administration of neurosteroids or drugs that may stimulate neurosteroidogenesis.

Emerging therapeutic role of sulphated pregnane steroids in neuropsychiatric disorders

Upon its production, following the translocation of cholesterol into the inner mitochondrial membrane, pregnenolone can be further converted into a number of pregnane steroids that ultimately produce Allo. It can be also converted into PES by sulphation of the C3 hydroxyl group by the enzymes sulphotransferase and sulphatase, which are expressed in various rodent and human brain structures. PES is expressed in the human and rodent brain and serum and its concentrations reach levels in the nanomolar range, (Reviewed by Gibbs *et al.,* 2006; Smith *et al.,* 2014). Whether these concentrations are consistent with a neurophysiological role for this neurosteroid as a putative endogenous neuromodulator remains a matter of debate and future studies are required to carefully examine this issue. Micromolar concentrations of PES negatively modulate GABA$_A$R, and based on receptor subunit composition, PES negatively or positively affects NMDA receptor-mediated neurotransmission (Majewska *et al.,* 1988; Jo *et al.,* 1989; Wu *et al.,* 1991; Malayev *et al.,* 2002; Smith *et al.,* 2014). PES preferentially potentiates NMDA receptors containing **NR2A** and **NR2B** subunits, whereas it elicits a negative modulation of **NR2C** and **NR2D**-containing receptors

(Malayev *et al.,* 2002). Importantly, PES shows affinity for an inhibitory site, which likely is also a binding site for other sulphated pregnane steroids (e.g. PAS) within the NMDA receptors (Horak *et al.,* 2006). Recent investigation on the role of PES at glutamate receptors has established its allosteric inhibitory modulation at AMPA receptors by binding both **GluA2** ATD and S1S2 domains (Cameron *et al.,* 2012). PES binding at the GluA2 subunit suggests a different binding domain from that of other sulphated neurosteroids. By such mechanisms, pharmacologically induced PES levels play an important neuromodulatory role at glutamate receptors in nanomolar to low micromolar concentrations. These are consistent with an agonist influence on LTP and memory. PES has reportedly showed neuroprotective effects on dentate gyrus granular cells (Yang *et al.,* 2012), enhances neurite outgrowth, reverses Aβ-induced toxicity, improves spatial memory (Xu *et al.,* 2012) and decreases immobility time in the tail suspension test (Dhir and Kulkarni, 2008). The effects of PES as a cognitive enhancer underline its therapeutic role in improving cognition deficits in schizophrenia and depression (Marx *et al.,* 2009).

Importantly, the inhibitory role at NMDA receptors has recently emerged for the endogenous neuroactive steroid, PAS. PAS has been shown to accumulate in plasma membranes, which provides the preferential route by which this neurosteroid accesses its binding sites located in NMDA receptors (Borovska *et al.,* 2012). PAS is particularly potent at inhibiting tonic rather than synaptically activated NMDA receptors. Synaptic activation of NMDA receptors is important for synaptic plasticity, learning and memory, and synaptogenesis, whereas tonic NMDA receptor activation is generally associated with excitotoxicity. Consistently, PAS's negative regulation of tonic NMDA neurotransmission has recently been reported to provide neuroprotection *in vivo* in a manner that is devoid of unwanted psychotomimetic effects (Vyklicky *et al.,* 2016). This feature is highly relevant for developing a novel class of steroid-based NMDA-inhibitor therapeutics, which lack the side effects that plague the use of classical NMDA receptor inhibitors, including ketamine and congeners. Based on this mechanism of action, a number of PAS analogues have been recently synthesized, including the amide-based analogues (Adla *et al.,* 2016) and pregnanolone hemipimelate that has no action at phasic and synaptic NMDA receptors but shows a strong selectivity (several folds higher than PAS) in inhibiting tonic-activated receptors (Vyklicky *et al.,* 2016).

The question of whether PES and even PAS or AlloS reach neurophysiologically significant brain levels has been matter of recent debate and Smith *et al.* (2014) examined this issue in terms of supporting a role for these sulphated steroids as endogenous neuromodulators. Precise quantification of PES has been difficult because of the methodology used in the past, which yielded a wide range of concentrations in the serum and brain. In the hippocampus, for example, in studies employing a direct detection by HPLC–MS/MS, nano-LC-ES or LC–MS, concentrations varied from under the threshold of detection and the low concentrations of ~50 pM to 26 000 pM (i.e. 10.25 ng·g^{-1}) (Liu *et al.,* 2003; Jäntti *et al.,* 2010; Rustichelli *et al.,* 2013; reviewed in Smith *et al.,* 2014). Other methods including enzyme-linked immunoassorbent assay, avoiding solvolysis, found brain concentrations less

than 0.15 ng·g^{-1} (Higashi *et al.*, 2003). In our laboratory we have established methods for the serum, plasma, saliva and brain quantification of neuroactive steroids (Uzunov *et al.*, 1996; Pinna *et al.*, 2000). Determination of sulphated steroids is performed indirectly after solvolysis, HPLC purification and separation of the steroids, which is followed by HFBA derivatization and gas-chromatography MS (GC–MS) quantification after adding deuterated internal standard for the steroids examined. We found that the mouse frontal cortex concentrations of PES, DHEAS, PAS, and AlloS were in the low ng/g range (2–20 ng·g^{-1}). Whereas PES levels were about 25% higher than pregnenolone levels in the frontal cortex, DHEAS, PAS and AlloS concentrations appeared to be 4, 2.5 and 1.5 times higher than those of the non-sulphated parental neuroactive steroids, respectively. Hence, endogenous PAS, DHEAS, PES and AlloS are highly measurable in the mouse frontal cortex. However, it remains to be clarified whether this expression level reaches neurophysiologically relevant concentrations to modulate NMDA receptor neurotransmission and by doing so, whether these sulphated neuroactive steroids are capable of improving cognitive functions in mouse models of neuropsychopathologies. Given that pregnenolone is preferentially metabolized into Allo and its parental neurosteroids or is converted into a sulphated class of steroids, the upstream activation of neurosteroidogenesis by targeting PXR, PPARα and CB1 receptors by increasing pregnenolone levels may also give rise to increased levels of PES, PAS and AlloS. This mechanism of pregnenolone's metabolism may have an impact on NMDA receptor neurotransmission. Altogether, we can draw a picture by which Allo and pregnanolone on the one hand and their sulphated congeners on the other may work in concert, by enhancing tonic inhibition with activation of extrasynaptic GABA$_A$R (Allo and PA) or by inhibiting tonic NMDA receptor neurotransmission (sulphated congeners), which may have an important role in improving cognitive and emotional deficits in various neuropsychiatric disorders. Research on the levels and biosynthetic regulation of PES, AlloS, and PAS during stress-induced neuropathologies and in rodent stress models may be helpful to understand new neuronal targets by which these neuroactive steroids can be elevated to improve symptoms.

Conclusions

Stress-induced down-regulation of Allo biosynthesis and changes in GABA$_A$R subtypes that create a receptor conformation with higher sensitivity to Allo invite the consideration of low Allo levels or enzymatic blocks in the Allo synthetic pathway as putative biomarkers for PTSD. The advantage of Allo biosynthesis as a biomarker for stress-induced disorders is that it can be strategically targeted by addressing several neurosteroidogenic mechanisms, thus limiting the odds of an unsuccessful treatment. It is plausible that neuroactive steroids themselves or drugs that stimulate neurosteroid biosynthesis upstream offer a valid therapeutic alternative for counteracting or even correcting biochemical alterations related to PTSD and depressive symptoms. This therapeutic approach may be a useful option for patients

who show: (i) compromised Allo biosynthesis, (ii) altered GABAergic neurotransmission, and (iii) relative resistance and/or the inability to respond to SSRI treatment. Perhaps then, diagnosis and therapeutic approaches could be guided by pretreatment verification of Allo levels, or identification of Allo biosynthesis blocks and changes in GABA$_A$R subunit expression as biomarkers of GABAergic function. Recognizing such dysfunction may not only increase treatment efficacy, but also prevent relapse – or even potentially prevent development of these stress-related disorders in the first place.

The use of pregnenolone or drugs that stimulate its levels is emerging as a key therapeutic approach that may originate both a downstream increase of PA and Allo, which by stimulation of GABA$_A$R may improve emotional behaviour, and at the same time may increase the sulphated forms of these steroids, including PAS and AlloS, that by inhibiting NMDA receptor tonic neurotransmission may provide neuroprotection and cognitive benefits in neuropsychiatric disorders.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c,d,e).

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

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