Title: Relating neurosteroid modulation of inhibitory neurotransmission to behaviour.

Delia Belelli¹, Grant D. Phillips¹, John R. Atack², Jeremy J. Lambert¹.

¹Neuroscience, Division of Systems Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY, UK.
²Medicines Discovery Institute, Cardiff University, Cardiff, CF10 3AT, UK.

Abstract.

Studies in the 1980s revealed endogenous metabolites of progesterone and deoxycorticosterone to be potent, efficacious, positive allosteric modulators (PAMs) of the GABA_A receptor (GABA_A R). The discovery that such steroids are locally synthesised in the central nervous system promoted the thesis that neural inhibition in the central nervous system (CNS) maybe “fine-tuned” by these neurosteroids to influence behaviour. In preclinical studies these neurosteroids exhibited anxiolytic, anticonvulsant, analgesic and sedative properties and at relatively high doses induce a state of general anaesthesia, a profile consistent with their interaction with GABA_A Rs. However, realising the therapeutic potential of either endogenous neurosteroids, or synthetic “neuroactive” steroids has proven challenging. The recent FDA approval of allopregnanolone (brexanolone) to treat postpartum depression has rekindled enthusiasm for exploring their potential as new medicines. Although neurosteroids are selective for GABA_A Rs, they exhibit little, or no selectivity across the many GABA_A R subtypes. Nevertheless, a relatively minor population of receptors incorporating the δ-subunit (δ-GABA_A Rs) appears to be an important contributor to their behavioural effects. Here, we will consider how neurosteroids acting upon GABA_A Rs influence neuronal signalling, how such effects may acutely and persistently influence behaviour and explore the case for developing selective PAMs of δ-GABA_A R subtypes for the treatment of psychiatric disorders.

Key Words

Neurosteroid; Allopregnanolone; GABA_A receptor. Postpartum depression; Major depressive disorder.
1) Introduction.

In 2019 the Food and Drug Administration (FDA) in the USA approved brexanolone (Zulresso) as the first specific treatment for the condition of post-partum depression (PPD). This exciting therapeutic breakthrough had its origins in seminal studies performed nearly 80 years earlier by Hans Selye, who demonstrated progesterone and related steroids caused sedation and at high doses induced a state of anaesthesia\(^1\). Brexanolone is a formulation of allopregnanolone, \((5\alpha\text{-pregnan-3\text{-\alpha}-ol-20\text{-one}})\), an endogenously occurring metabolite of progesterone\(^2\). Subsequent preclinical studies revealed the synthetic steroidal general anaesthetic alphaxalone (5\(\alpha\)\text{-pregnane-3\text{-\alpha}-ol-11,20\text{-dione}}) allopregnanolone and an endogenous metabolite of deoxycorticosterone (5\(\alpha\)\text{-pregnan-3\text{-\alpha,21\text{-dial-20\text{-one}}}, [5\(\alpha\)\text{-THDOC}]) to act as potent PAMs of the GABA\(_A\) receptor (GABA\(_A\)R)\(^3\text{-}11\). The GABA\(_A\)R is the major inhibitory receptor in the mammalian central nervous system (CNS) and is an established target for clinically important drugs of the benzodiazepine and barbiturate classes. Therefore, these observations provided a viable mechanism to explain the rapid acute effects of steroids such as allopregnanolone on brain activity. Indeed, in common with diazepam, upon administration these GABA\(_A\)R-active steroids produce anxiolytic, analgesic, anticonvulsant and sedative effects, but additionally at greater doses they induce a state of general anaesthesia\(^12,13\).

These observations raised the intriguing prospect that the activity of the major inhibitory receptor in the mammalian brain might be fine-tuned by endogenous levels of these pregnane steroids and consequently influence our mood and behaviour in both physiological and pathophysiological scenarios\(^{11}\). Initially, endogenous levels of GABA\(_A\)R-active steroids were considered to primarily originate from peripheral sources, such as the adrenal cortex and ovaries\(^{14}\). In this scenario it was proposed that these steroids would cross the blood brain barrier and act as endocrine messengers to influence neural inhibition. However, subsequently it was demonstrated that the enzymes required for synthesis of these pregnane steroids, derived either from cholesterol, or from peripheral precursors such as progesterone, are expressed within certain neurons and glial cells in the CNS\(^{15\text{-}18}\). Therefore, in addition to peripheral endocrine sources of the steroid, neurosteroids synthesised locally in the CNS could potentially act in a paracrine, or an autocrine manner, to influence neuronal signalling\(^{12,15}\). It soon became evident that the endogenous levels of these steroids are not static but, change dynamically in a variety of physiological conditions including during development, puberty, stress, during the ovarian cycle and in pregnancy\(^{14,19\text{-}24}\). Additionally, perturbation of the levels of neurosteroids are associated with various psychiatric conditions including schizophrenia, panic attacks, major
depressive disorders (MDD) and PPD\textsuperscript{23-28}. In this article neurosteroid refers to endogenous steroids synthesised either in the periphery for example by endocrine glands, or the CNS and the term neuroactive steroid refers to synthetic GABA\textsubscript{A}R-active steroids.

2) Neuroactive steroids and GABA\textsubscript{A}R subtypes.

The GABA\textsubscript{A}R is composed of five transmembrane crossing subunits, arranged in a pentameric structure around a central anion conducting pore region. Importantly, the GABA\textsubscript{A}R subunit family is diverse, grouped by sequence homology into the following categories: α1-6, β1-3, γ1-3, ρ1-3, δ, ε, θ, and π subunits. In the mammalian CNS this subunit repertoire underpins the expression of approximately 20 – 30 native GABA\textsubscript{A}R isoforms\textsuperscript{29-33}. The majority of GABA\textsubscript{A}Rs are composed of two α subunits, two β subunits and a γ subunit (predominantly, but not exclusively the γ2 subunit). For some receptors the δ subunit (δ-GABA\textsubscript{A}Rs) replaces the γ subunit.

The GABA\textsubscript{A}R subunit composition influences the functional properties of the receptor, including agonist affinity, deactivation, and desensitization. The various GABA\textsubscript{A}R isoforms exhibit a heterogeneous expression pattern in the CNS and indeed even their subcellular location within a neuron \textit{e.g.} synaptic \textit{vs} extra/peri-synaptic is influenced by subunit composition. Relatedly, the biochemical influence (\textit{e.g.} by kinases and phosphatases) upon the fundamental properties of the receptor including expression, trafficking and function is receptor isoform specific\textsuperscript{29,34-36}. Importantly, for developing new therapeutics particular pharmacological properties are receptor subtype specific\textsuperscript{29-33,37} (Section 4). Adding further complexity, GABA\textsubscript{A}R accessory proteins have been identified that influence not only receptor expression and location, but additionally their pharmacology\textsuperscript{38}.

Although the neurosteroids and neuroactive steroids (\textit{e.g.} alphaxalone) described above are highly selective PAMs of the GABA\textsubscript{A}R they do not discriminate across the numerous GABA\textsubscript{A}R isoforms\textsuperscript{12,37,39,40}. In this regard, their GABA\textsubscript{A}R profile resembles that of barbiturates, although interaction studies suggest the steroid effects to be mediated by a distinct site, or sites\textsuperscript{6-8}. Additionally, the GABA-ergic steroids do not act \textit{via} the benzodiazepine recognition site on the GABA\textsubscript{A}R\textsuperscript{6,7,11}. Distinct from benzodiazepines, neurosteroids enhance the function of GABA\textsubscript{A}Rs incorporating α4, or α6 subunits and are highly efficacious PAMs of GABA\textsubscript{A}Rs incorporating the δ subunit (δ-GABA\textsubscript{A}Rs), although this apparent functional selectivity appears to be a consequence of the limited efficacy of GABA acting at δ-
GABA_ARs^{40,41}. Note native δ-GABA_ARs may partner with either α4, α6, or α1 subunits and β subunits. The important issue of which GABA_AR isoforms mediate the various behaviours produced by neurosteroid administration is considered below (Sections 4,5).

3) GABA_ARs and neuronal signalling.

In the mammalian CNS the majority of neuronal GABA_ARs contain a γ2 subunit and are primarily expressed within the inhibitory synapse, where they mediate fast phasic inhibition in response to the vesicular release of GABA. Physiologically, this results in an inhibitory postsynaptic potential (IPSP), usually producing neuronal hyperpolarisation, or an inhibitory postsynaptic current (IPSC) when recorded under voltage-clamp conditions. To clarify the terminology, under voltage-clamp electrical stimulation of GABA-ergic interneurons will produce in the postsynaptic neuron a neurally evoked IPSC (eIPSC), IPSCs can occur spontaneously (sIPSC) i.e. without external stimulation and miniature IPSCs (mIPSCs) are recorded in the presence of tetrodotoxin (TTX) to block action potentials. Synaptic GABA_ARs are also expressed on certain GABA-ergic interneurons, where they can potentially influence GABA release on to principal neurons. For example, genetic deletion of the γ2 subunit of somatostatin interneurons, reduces the frequency of sIPSCs recorded from these hippocampal interneurons, but correspondently due to interneuron disinhibition increases the sIPSC frequency in CA1 principal neurons^{42}. Additionally, certain neurons express extrasynaptic γ2-GABA_ARs, that may be activated by ambient, or spillover concentrations of GABA, which under voltage-clamp conditions mediate a persistent tonic current. This is exemplified by the benzodiazepine-sensitive α5βγ2 isoform, which is densely expressed out with the synapse, particularly in certain neurons of the hippocampus^{43}. However, it is now evident that the neuronal location of this GABA_AR isoform is not static, being dynamically trafficked between synaptic and extrasynaptic locations^{44–46}.

By contrast to γ2-GABA_ARs, receptors incorporating the δ subunit, in place of the γ subunit, appear to be exclusively expressed out with the synapse in extra-synaptic, or peri-synaptic locations. Neurons expressing δ-GABA_ARs include cerebellar granule cells (α6βδ), thalamic relay neurons (α4βδ), hippocampal parvalbumin GABA-ergic interneurons (α1βδ), hippocampal dentate gyrus granule cells (α4βδ) and medium spiny neurons of the nucleus accumbens (α4βδ). In these neurons δ-GABA_ARs mediate an inhibitory chloride tonic current in response to ambient levels of GABA, or due to spontaneous opening of the δ-GABA_AR^{40,47–49}.
As described above, studies of recombinant GABA\textsubscript{ARs} reveal neurosteroids are not selective across GABA\textsubscript{AR} isoforms\textsuperscript{57}. In agreement, voltage-clamp recordings of neurons reveal neurosteroids to prolong the IPSC duration and enhance the tonic inhibition, irrespective of the synaptic and extrasynaptic GABA\textsubscript{AR} isoforms mediating phasic and tonic inhibition\textsuperscript{12,58}.

Although fast phasic and tonic inhibition have generally been regarded as distinct forms of neuronal signalling, in dentate gyrus granule cells (DGGCs) extrasynaptic peri-synaptic δ-GABA\textsubscript{AR}S appear to contribute to the duration of IPSCs, thereby blurring that distinction\textsuperscript{38,58}. Studies of the thalamus, employing physiological rates of nucleus reticularis (nRT) stimulation reveal that synaptic and extrasynaptic GABA\textsubscript{AR}S can act in harmony to dynamically sculpt inhibition (Figure 1). Mouse thalamic ventrobasal (VB) neurons express both extrasynaptic α4β2δ receptors and synaptic α1β2γ2 GABA\textsubscript{AR}S, that mediate tonic and fast phasic inhibition respectively\textsuperscript{52,59}. The VB neurons are innervated by GABA-ergic nRT neurons, which can fire with high frequency bursts of short duration. Such intense nRT activity results in transiently high concentrations of GABA, that spillover from the nRT-VB synapse to activate not only postsynaptic α1β2γ2 receptors, but additionally extrasynaptic α4β2δ receptors, thereby greatly prolonging the neurally evoked IPSC (eIPSC)\textsuperscript{59} (Figure 1). The eIPSC duration is greatly prolonged by the δ-GABA\textsubscript{AR} selective PAM (DS2 – Section 7) and reduced in equivalent recording made in the α4\textsuperscript{−/-} mouse, confirming engagement of extrasynaptic α4βδ GABA\textsubscript{AR}S by synaptically released GABA\textsuperscript{59,60} (Figure 1).

The majority of in vitro electrophysiology studies have focussed on the role of δ-GABA\textsubscript{AR}S expressed on principal neurons, although in common with γ2-GABA\textsubscript{AR}S, they are additionally expressed on certain GABA-ergic neurons, where they may influence neurotransmitter release. For example, in the hippocampus receptors composed of α1, β and δ subunits are expressed on GABA-ergic interneurons and mediate a tonic current\textsuperscript{51,54,61} (Section 6). Additional pertinent examples occur in the ventral tegmental area (VTA) to nucleus accumbens, circuitry often referred to as a reward pathway. We reported that the medium spiny neurons (MSNs) of mouse nucleus accumbens, express extrasynaptic α4βδ GABA\textsubscript{AR}S that mediate a tonic conductance, that was greatly increased by gadoxadol (THIP), which at low concentrations acts as a selective δ-GABA\textsubscript{AR} agonist and by DS2, a selective δ-GABA\textsubscript{AR} PAM\textsuperscript{55,62-64} (see Section 7). However, in wild type mice, but not in δ\textsuperscript{−/-} or α4\textsuperscript{−/-} mice, these drugs additionally exerted an effect on the GABA-ergic input neuron to decrease the frequency of MSN sIPSCs\textsuperscript{55}. In wild type mice allopregnanolone (100 nM) similarly decreased vesicular GABA release onto MSNs\textsuperscript{65}. This disinhibition could be mediated by δ-GABA\textsubscript{AR}S located on other GABA-ergic MSNs, by
parvalbumin, choline acetyl transferase, or neuropeptide Y expressing GABA-ergic interneurons, all shown by immunohistochemistry to express the δ subunit. Neurosteroid-sensitive extrasynaptic δ-GABA<sub>A</sub>Rs are also expressed on GABA-ergic interneurons in the VTA and influence GABA release onto dopaminergic neurons (Section 5).

4) The behavioural effects of neurosteroids and GABA<sub>A</sub>Rs.

Neurosteroids are highly selective PAMs of GABA<sub>A</sub>Rs but, exhibit only limited specificity across the various receptor isoforms (Section 2). Given this promiscuous profile, which of the numerous GABA<sub>A</sub>R isoforms mediate the various behavioural effects of neurosteroids? Distinct from benzodiazepines, neurosteroids are efficacious enhancers of extrasynaptic δ-GABA<sub>A</sub>Rs (Sections 2, 3). Although caution is warranted in the interpretation of gene deletion studies e.g. 69, it is interesting that many of the behavioural effects of the neurosteroids are reduced in mice lacking the δ subunit, or the α4 subunit (a common partner of the δ subunit), for example the sedative and anxiolytic effects of neurosteroids 70,71.

For benzodiazepines, seminal studies of mice genetically engineered to express benzodiazepine-insensitive GABA<sub>A</sub>-R isoforms was instructive. This approach revealed the sedative effects of diazepam to be mediated by enhancing the function of GABA<sub>A</sub>Rs incorporating α1, β and γ2 subunits (Table 1) 72. Neurosteroids are also effective PAMs of this GABA<sub>A</sub>R isoform, but nevertheless the duration of sleep induced by allopregnanolone, or alphaxalone, is decreased in δ<sup>-/-</sup> subunit mice 70. Further implicating these extrasynaptic receptors, the sedative effect of the δ-GABA<sub>A</sub>R preferring agonist gaboxadol is impaired in δ<sup>-/-</sup> mice 49,73–75.

The anxiolytic-like effects of benzodiazepines in mice are primarily mediated by α2βγ2 GABA<sub>A</sub>Rs 72. Neuroractive steroids are efficacious enhancers of this GABA<sub>A</sub>R subtype, and their anxiolytic effects are blunted in a mouse engineered to express a point mutation (α2<sup>Q241M</sup>), that imparts neurosteroid insensitivity to α2-GABA<sub>A</sub>Rs 76 (Table 1). Indeed, such mice exhibit an anxiogenic phenotype, suggesting that endogenous neurosteroid levels, acting via α2-GABA<sub>A</sub>Rs are sufficient to influence such behaviour 76. However, the anxiolytic-like effect of the synthetic steroid ganaxolone, as assessed in the elevated plus maze, is impaired in the δ<sup>-/-</sup> mouse 70. Voltage-clamp recordings of dentate granule cells obtained from the α2<sup>Q241M</sup> mouse demonstrated the effect of 5α-THDOC to prolong phasic inhibition was reduced, but intriguingly steroid enhancement of the resident tonic current was unexpectedly blunted,
suggesting the possible expression of a novel benzodiazepine-insensitive α2βδ, or α2β
GABA_R subtype.

Although there is some overlap of the behavioural effects of benzodiazepines and
neurosteroids, benzodiazepines are not considered clinically efficacious in the treatment of
depression\(^77\), perhaps reflecting their differential interaction with δ-GABA_Rs (Section 5.6).
A possible exception is the benzodiazepine sensitive α5-GABA_R\(^78\) (Section 6).

5) Neurosteroids and depressive disorders.

A variety of mood disorders are associated with, or accompanied by perturbed levels of the
GABA-ergic neurosteroids such as allopregnanolone\(^18\). Many animal models have focussed on
investigating the consequences of exposure to chronic stress as a vulnerability factor\(^79\).
Whereas acute stress in rodents induces an increase in the levels of GABA-ergic neurosteroids,
exposure to chronic stress produces a decrease\(^19,26,80,81\). Major depressive disorders (MDD) are
associated with low levels of allopregnanolone, that are increased by treatment with
antidepressants such as fluoxetine\(^82-85\). In animals, impairment of neurosteroid synthesis by
inhibition of the 5α-reductase enzyme with finasteride increases the incidence of depression-
like behaviours\(^81,86,87\). In clinical studies treatment of male pattern hair loss with finasteride is
associated with mood disorders in some men\(^80,88,89\).

As regards the GABA-ergic neurosteroids both pregnancy and postpartum are physiological
situations of particular interest as they are associated with considerable temporal changes in
neurosteroid levels. During pregnancy both progesterone and allopregnanolone levels increase
steadily, until approaching term, when they begin to decrease, with a dramatic decline evident
immediately following birth\(^80,90-93\). Studies of pregnant mice suggest elevated neurosteroid
levels are associated with decreased expression of GABA_Rs, an imbalance proposed to persist
abnormally postpartum in patients suffering from PPD\(^94-97\). Of the numerous GABA_R
subtypes there is a particular focus on δ-GABA_Rs. The expression of δ-GABA_Rs is highly
plastic, changing in a variety of scenarios including during the ovarian cycle, upon puberty, in
response to stress, in rodent models of anorexia nervosa and of fragile X syndrome\(^22,98-103\).

Rodent studies implicate δ-GABA_Rs to play an important role throughout pregnancy and
postpartum. During pregnancy in mice there is reduced staining for the δ subunit on
hippocampal principal neurons and on GABA-ergic interneurons, which reverts to pre-
pregnancy expression levels postpartum. The global deletion (δ−/−), or reduction (δ+/−) of extrasynaptic δ-GABA_ARs disrupts the temporal decrease and subsequent recovery of δ-GABA_A expression that occurs naturally during the latter stages of pregnancy and postpartum. Importantly, postpartum homozygous (δ−/−), or heterozygous (δ+/−) mice exhibit “depression-like” and abnormal maternal behaviours resulting in a reduced survival of their offspring. This phenotype is rectified in heterozygous δ+/− mice by administration of the δ-GABA_AR preferring agonist gaboxadol. Similarly, a synthetic neuroactive steroid (SGE-516), improved “depression-like” behaviours and maternal care in both heterozygous (δ+/−) and homozygous (δ−/−) mothers, whereas a benzodiazepine was inert in this respect. However, note the behavioural efficacy of SGE-516 evident in the δ−/− mice suggests involvement of an additional target/mecchanism to δ-GABA_ARs.

As described above δ-GABA_AR expression is not limited to the hippocampus. Investigating in rodents whether plasticity of δ-GABA_AR expression in response to stress, pregnancy and postpartum occurs elsewhere in the brain may improve understanding of the putative role of this GABA_AR subtype in these conditions. For example, extrasynaptic δ-GABA_ARs of VTA interneurons, mediate a neuroactive-steroid-sensitive tonic current. These receptors influence GABA release onto dopamine neurons and consequently dopamine release into the nucleus accumbens. Indeed, allopregnanolone influences dopamine release into the accumbens, an effect influenced by the oestrous cycle. Altered expression of these neurosteroid-sensitive δ-GABA_ARs in this “reward pathway” could conceivably contribute to aspects of the behaviours associated with depression e.g. anhedonia. In support, GABARD gene expression in the VTA was reduced in mouse models of physical and emotional chronic stress and changed during the oestrous cycle.

Deletion of the δ-subunit has a considerable, albeit indirect impact upon the corticotrophin-releasing factor (CRF) neurons of the paraventricular nucleus (PVN). These neurons govern the hypothalamus, pituitary, adrenal (HPA) axis, play a major role in the stress response and their dysregulation is implicated in depressive disorders. During pregnancy and postpartum a failure to blunt the activity of the HPA axis may be a contributing factor to developing PPD. In CRF expressing neurons low nM concentrations of allopregnanolone prolong phasic inhibition and suppress action potential discharge. Although the δ-subunit is not expressed in these neurons (although see) we found the global deletion of this subunit (δ−/− mice) to increase CRF immunostaining and impair the effect of allopregnanolone.
to suppress CRF neuronal firing. The loss of the δ subunit resulted in a greatly increased synaptic and extrasynaptic glutamatergic drive to these CRF releasing neurons, producing a change in the excitatory and inhibitory balance, sufficient to nullify the neurosteroid effect.

Adversity in childhood is now recognised as an important factor in the likelihood of subsequently developing psychiatric disorders. Furthermore, prior early-life trauma associates with a failure in adulthood to respond to antidepressants. Intriguingly, mice previously subjected as pups to a limited nesting model of early life adversity (ELA), in common with δ- mice, presented with an increase in CRF staining in the PVN, an increased glutamatergic drive and an impaired effect of allopregnanolone to suppress CRF neuronal firing δ- mouse.

These findings are consistent with the dysregulation of the stress response in the pathophysiology of mood disorders. Determining if a similar perturbed profile occurs in these CRF releasing neurons in response to reduced δ-GABA<sub>A</sub>R subunit expression during pregnancy and postpartum would be of interest, particularly as neurosteroids and dysfunction of the HPA axis have been implicated in PPD. The imposition of the ELA paradigm upon δ- mice magnified the ELA behavioural phenotype, suggesting the expression of the δ-subunit to be stress protective. Further implicating the activity of the HPA axis in PPD, using DREADD technology, the chemogenetic activation of CRF neurons produced “depression-like” behaviours and impaired maternal behaviour of postpartum mice.

Collectively, these preclinical studies provided encouragement to explore the clinical utility of GABA-ergic steroids in the treatment of MDD and PPD, resulting in the FDA approval of brexanolone for this condition and ongoing clinical trials of SAGE 217 (zuranolone), an orally available synthetic steroid. Indeed, SAGE 217 is reported to show efficacy in a Phase 2 study of MDD and encouraging results in a recent Phase 3 study.

6) **Neurosteroids as antidepressants: possible acute and sustained mechanisms of action.**

The acute antidepressant actions of neurosteroids such as allopregnanolone are widely attributed to facilitation of GABA<sub>A</sub>R inhibition, with δ-GABA<sub>A</sub>Rs having gained prominence as a behaviourally relevant target. Indeed, polymorphisms of the GABARD gene are associated with childhood onset mood disorders. Attempts to unravel the relevant actions of neurosteroids in treating mood disorders has relied primarily on animal studies. Simplistically, enhancement of GABA-ergic transmission appears consistent with the hypothesis of a GABA-
ergic deficit in the pathogenesis of depression\textsuperscript{79,128}. Acutely, allopregnanolone prolongs fast phasic inhibition mediated by synaptic GABA\textsubscript{A}Rs and enhances tonic inhibition in those neurons expressing either extrasynaptic α5-GABA\textsubscript{A}Rs, or δ-GABA\textsubscript{A}Rs and may potentially prolong a slow form of phasic inhibition produced by the synaptic spillover of GABA occurring at relatively high frequencies of firing of the GABA releasing neuron\textsuperscript{59}.

The emergence of rapidly acting antidepressants, such as low dose ketamine, has further encouraged studies focussed on improving our understanding of the neural mechanisms that underpin the effect of such compounds upon mood. Somewhat surprisingly, given the GABA-ergic deficit hypothesis, disinhibition, resulting from a decrease in GABA release, has emerged as a putative mechanism, that may contribute to the reported rapid antidepressant effects of drugs such as ketamine and scopolamine\textsuperscript{129}. In the hippocampus these effects are proposed to be mediated by inhibition of NMDARs (ketamine) and muscarinic receptors (scopolamine) expressed upon GABA-ergic interneurons\textsuperscript{129}. In common, by reducing GABA release these drugs acutely impact upon the excitatory/inhibitory balance, thereby potentially triggering changes to downstream signalling pathways that persist following drug elimination (see below).

Facilitation of GABA-ergic transmission by neurosteroids at first glance would simplistically appear inconsistent with a disinhibition hypothesis. However, δ-GABA\textsubscript{A}Rs are additionally located on GABA-ergic interneurons where they may influence GABA release. As described (Section 3) activation of δ-GABA\textsubscript{A}Rs expressed on GABA-ergic neurons of either the VTA, or the nucleus accumbens, reduced the spontaneous release of GABA (disinhibition) onto dopamine neurons and onto MSNs respectively, this effect in the accumbens being mimicked by allopregnanolone\textsuperscript{55,66–68}. Pregnancy in mice is associated with reduced δ-GABA\textsubscript{A}R expression in both hippocampal principal neurons (α4βδ) and GABA-ergic interneurons (α1βδ)\textsuperscript{104}. Genetic manipulations to delete expression of δ-GABA\textsubscript{A}Rs in hippocampal GABA-ergic interneurons resulted in an increased frequency of sIPSCs in both CA1 and DGGCs and consequently decreased neuronal excitability\textsuperscript{54}. Presumably, enhancement of the δ-GABA\textsubscript{A}Rs of GABA-ergic by allopregnanolone would decrease quantal GABA release onto hippocampal principal neurons \textit{i.e.} disinhibition\textsuperscript{130}. In mouse hippocampal slice recordings the selective genetic reduction (δ\textsuperscript{+/−}), or deletion (δ\textsuperscript{−/−}), of the δ subunit from parvalbumin interneurons influences the peak frequency of γ-oscillations, a network activity associated with learning and memory\textsuperscript{61}. This perturbation was corrected in the heterozygous (δ\textsuperscript{+/−}) mouse by allopregnanolone\textsuperscript{61}. A similar change of γ-oscillations is reported for equivalent recordings
made from pregnant wild type mice, perhaps reflecting the changed interneuron expression of δ-GABAARs104. As discussed above (Section 3) disinhibition of somatostatin (SST) interneurons, by the genetic silencing of their γ2-GABAARs resulted in an increased release of GABA onto CA1 principal neurons. Adding complexity, intriguingly the behaviour of these mice mimicked that produced by antidepressant and anxiolytic drugs in tests of depression and anxiety42. In the hippocampus GABA released from SST + interneurons will activate α5-GABAARs. Given the decreased expression of SST associated with MDD there is interest in exploring the clinical utility of benzodiazepine PAMs, or negative allosteric modulators (NAMs) in the treatment of mood disorders and associated cognitive dysfunction78. Whether in this pathway neurosteroid facilitation of α5-GABAAR function, either pre- or post-synaptically contributes to the effects of allopregnanolone remains to be explored. Clearly, further investigation is required to better understand how influencing quantal GABA release and postsynaptic enhancement of GABAAR function by neurosteroids might integrate to impact upon neuronal signalling. Furthermore, it would be instructive to extend the investigation of the effects of neurosteroids on GABA release to additional neural circuits considered pertinent in mood disorders, such as those involved in the “reward pathway”. Although the immediate effects of neurosteroids on neural inhibition may be relevant to acutely influencing depressive behaviour, how they act to maintain improved mood following elimination of the drug from the body remains to be clarified. A variety of putative targets and mechanisms have been implicated to contribute41,131,132. Here we will focus on the neurotrophin BDNF and the putative involvement of a progesterone G-protein coupled receptor (p-GPCR). Allopregnanolone has been shown to increase BDNF levels133 (see below). This neurotrophin is implicated in essential functions within the CNS, including neuronal maturation, neurogenesis, synapse formation and synaptic plasticity134–136. Abnormal BDNF signalling is associated with a depressive phenotype in both animal models of mood disorders and in humans137–139. Furthermore, in rodent models a variety of treatments for depression, including electroconvulsive therapy, classical antidepressants such as fluoxetine and the rapidly acting antidepressant ketamine, restore BDNF signalling in anatomically relevant neuronal substrates of the limbic system, *e.g.* the hippocampus140. Providing further support, viral-mediated deletion of BDNF in the dentate, but not in the CA1 area of adult rodent hippocampus, appears crucial for the effects of classical antidepressants141.
The effect of drugs such as fluoxetine and ketamine upon BDNF was assumed to be a downstream effect resultant from actions on their upstream targets e.g. the serotonin transporter and the NMDA receptor respectively. However, both classical and rapidly acting antidepressants including fluoxetine, imipramine, ketamine, esketamine and the behaviourally active ketamine metabolite 2R,6R-HNK have recently been shown to bind directly to the TrKB receptor at clinically appropriate concentrations\textsuperscript{142}. Their binding promotes TrKB translocation and stabilisation to the cell surface, thereby increasing receptor availability for BDNF\textsuperscript{142}. Additionally, there is a separate cholesterol interaction domain located on the transmembrane region of the receptor and cholesterol can also facilitate cell surface expression of TrKB\textsuperscript{142}. Clearly it would be of interest to further explore the steroid structure activity relationship of this interaction and to determine whether neurosteroids such as allopregnanolone, or its’ precursors, can also interact with this site.

Associating GABA\textsubscript{ergic} neurosteroids and this neurotrophin, a single \textit{i.p.} administration of allopregnanolone to rodents increases BDNF levels in the hippocampus and amygdala\textsuperscript{133}. An additional potentially relevant anatomical substrate is the prefrontal cortex. Thus, in rodent prefrontal cortex a BDNF deficit induced by depressive-type manipulations\textsuperscript{143} is rectified by local infusion of allopregnanolone into this area\textsuperscript{144}. During pregnancy serum levels of BDNF decline, followed by a subsequent rebound postpartum, an effect probably related to a decrease in the levels of cortisol, a known negative modulator of BDNF\textsuperscript{145}. Implicating this neurotrophin in the development of PPD, in both the 3\textsuperscript{rd} trimester and in post-partum, serum BDNF levels and depressive symptoms are inversely related\textsuperscript{145,146}. Providing a tentative link to neurosteroids, levels of both allopregnanolone and BDNF are similarly dysregulated in rodent models of chronic stress and depressive disorders in humans\textsuperscript{119,147–152}. Similarly, in PPD the dysregulation of allopregnanolone levels appears temporally associated with the perturbed levels of BDNF described above\textsuperscript{145}. Furthermore, in common with neurosteroids\textsuperscript{153} dysregulation of BDNF signalling accompanies the exposure to early-life adversity, a recognised contributor to the likelihood of subsequently developing of mood disorders\textsuperscript{154–156}.

Intriguingly, BDNF is reported to increase the cell surface expression of both α4 and δ GABA\textsubscript{A}R subunits in hippocampal neurons\textsuperscript{157,158}. This finding maybe highly pertinent given the hypothesis that the enhanced levels of allopregnanolone during pregnancy reduces the expression of GABA\textsubscript{A}Rs, including extrasynaptic δ-GABA\textsubscript{A}Rs, which may remain depressed in patients suffering from PPD\textsuperscript{93–97,131}. In a further potential association, antidepressants such
as fluoxetine are effective in both restoring BDNF levels and stimulating neurosteroidogenesis, the latter at doses lower than those required for blocking 5HT reuptake into neurons\textsuperscript{159–161}.

The putative interplay of neurosteroids and BDNF may extend to hippocampal neurogenesis\textsuperscript{162}. In various animal models of depression both the production and survival of new-born dentate gyrus granule neurons (DGGNs) is reduced, a deficit corrected by antidepressant treatment\textsuperscript{163}. As described above, BDNF plays an important role in neurogenesis. For example, ablation of the TrkB receptor, prevents both the effect of BDNF on neurogenesis and the behavioural effects of antidepressants in animal models\textsuperscript{162,164}. Allopregnanolone may also play a role in neurogenesis. For example, stimulation of neurosteroidogenesis in the dentate gyrus is directly associated with the proliferation of progenitor cells\textsuperscript{165}. Furthermore, in vivo administration of allopregnanolone rescues cell survival by restoring cell proliferation in the subventricular zone of the dentate gyrus in a rodent model of social isolation, an effect probably mediated by BDNF\textsuperscript{166}.

**Metabotropic progesterone receptors:**

Allopregnanolone not only binds to GABA\textsubscript{A}RS, but can additionally activate members of a family of progesterone G-protein coupled receptors (p-GPCRs) expressed on the plasma membrane\textsuperscript{167–169}. Steroid activation of these receptors promotes cell surface expression of extrasynaptic (α4βδ) GABA\textsubscript{A}Rs. In this regard the synthetic steroid ORG OD 02 is of interest as it selectively activates the p-GPCR, but distinct from allopregnanolone has no direct effect upon GABA\textsubscript{A}Rs\textsuperscript{169}. In contrast to allopregnanolone, in DGGCs this steroid had no immediate effect upon GABA-ergic inhibition, but in common with allopregnanolone produced a delayed and maintained increase of the tonic current resulting from increased cell surface expression of extrasynaptic (α4βδ) GABA\textsubscript{A}Rs. As highlighted above for BDNF, it is conceivable that following infusion of brexanolone (allopregnanolone) this metabotropic effect of the steroid may help to counteract the proposed reduced GABA\textsubscript{A}R expression associated with PPD. Note as exemplified by ganaxolone, not all neuroactive GABA-ergic steroids exhibit this metabotropic effect upon GABA\textsubscript{A}R trafficking\textsuperscript{170}. Given the similar acute effects of ganaxolone and allopregnanolone upon GABA-ergic inhibition a comparison of the behavioural effects of these two steroids may prove instructive in evaluating the importance of this metabotropic effect on GABA\textsubscript{A}R trafficking.

7) **Exploring therapeutic opportunities beyond neurosteroids.**
If δ-GABA_ARs are important in mediating the behavioural effects of neurosteroids would small molecule non-steroids, with a similar receptor profile, be viable in the treatment of mood disorders? Clinically approved drugs such as the general anaesthetic etomidate are relatively selective PAMs of GABA_ARs, including δ-GABA_ARs, but interact with many GABA_A isoforms^{37,49,59}. Clearly, in common with allopregnanolone, their use would be associated with side effects such as sedation. Importantly, would their acute effects on GABA-ergic transmission trigger the forms of neuronal plasticity implicated in maintaining the behavioural effects of allopregnanolone (Section 6)? For example, such compounds would seem unlikely to activate p-GPCRs to elicit sustained changes in GABA_AR expression^{169,171,172}.

Presuming the primacy of δ-GABA_ARs as the main neurosteroid target a logical approach would be to develop a δ-GABA_AR selective PAM, as exemplified by DS2^{59,63,64}. Although selective functionally for both recombinant and native δ-GABA_ARs, mutagenesis studies have highlighted key amino acid interaction sites with DS2 located not on the δ subunit per se, but residing in transmembrane pockets, formed between the α subunit (in this study α4) and the β subunit^{173}. Unfortunately, DS2 has limited brain penetration^{64} but ongoing analogue studies may resolve this current limitation^{174,175}. The development of brain penetrant δ-GABA_AR selective compounds would be invaluable in clarifying the role of these receptors in the behavioural effects of neurosteroids but may additionally pave the way for novel small molecule therapeutics with an improved side effect profile.

8. Conclusion:

The approval of intravenous brexanolone for PPD has increased interest in exploring further the therapeutic potential of GABA-ergic neurosteroids in a variety of psychiatric and neurological disorders. Indeed, synthetic neuroactive steroids that unlike allopregnanolone are orally available with improved pharmacokinetics are currently in development, not only for treating PPD, but also for MDD^{125,176}. These exciting clinical developments should encourage further preclinical studies to better understand why these neuroactive steroids are efficacious. Questions that remain include: 1) What is the role of GABA_AR subtypes in mediating the acute behavioural effects of allopregnanolone? 2) What forms of neural plasticity underpin their prolonged effects upon mood and are they triggered downstream from GABA_ARs, or do they result from an action of the steroid on other targets such as the metabotropic progesterone.
receptor, or by producing changes in BDNF? Relatedly, given preclinical studies implicating benzodiazepine-insensitive δ-GABA_{A}Rs, would a selective PAM of this family of extrasynaptic receptors be efficacious? A recent mouse EEG study reported injection of SAGE-217 (Zuranolone) to increase power in the β frequency range\textsuperscript{172}. Complementary human and rodent EEG studies should be instructive. In particular, are the reported prolonged behavioural effects of brexanolone associated with a persistent characteristic EEG biomarker: do δ-GABA_{A}R selective PAMs produce a common or distinct EEG signature see \textsuperscript{61}.

These advances come at an opportune time with the development of new “rapid acting antidepressants” such as S-ketamine that recently received FDA approval for treating MDD and preclinical studies reporting efficacy of α_{5}-GABA_{A}R selective allosteric modulators\textsuperscript{78,177}. A comparison of how these drugs, which act via disparate molecular targets, share a common action upon mood should hasten a new dawn in our understanding and treatment of depression.
<table>
<thead>
<tr>
<th>Drug</th>
<th>GABA&lt;sub&gt;A&lt;/sub&gt; receptor subtype</th>
<th>Behaviour influenced</th>
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<tbody>
<tr>
<td>Benzodiazepine (diazepam)</td>
<td>α1βγ2</td>
<td>Sedation</td>
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<td>Anterograde amnesia</td>
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<td></td>
<td>Anticonvulsant</td>
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<tr>
<td>Benzodiazepine (diazepam)</td>
<td>α2βγ2</td>
<td>Anxiolysis</td>
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<td>Myorelaxation</td>
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<tr>
<td>Benzodiazepine (diazepam)</td>
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<td>Myorelaxation</td>
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<tr>
<td>Benzodiazepine (diazepam)</td>
<td>α5βγ2</td>
<td>Cognitive impairment</td>
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<tr>
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<td>Myorelaxation</td>
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<tr>
<td>Etomidate</td>
<td>β2-GABA&lt;sub&gt;A&lt;/sub&gt;Rs</td>
<td>Sedation</td>
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<td>Hypnosis</td>
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<td>Etomidate</td>
<td>β3-GABA&lt;sub&gt;A&lt;/sub&gt;Rs</td>
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<td></td>
<td>Immobility</td>
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<tr>
<td>5α-THDOC</td>
<td>α2-GABA&lt;sub&gt;A&lt;/sub&gt;Rs</td>
<td>Anxiolysis</td>
</tr>
</tbody>
</table>

Table 1 – The behavioural repertoire of benzodiazepines such as diazepam, the intravenous general anaesthetic etomidate and the neurosteroid 5α-THDOC, are mediated by different GABA<sub>A</sub> receptor subtypes (Modified from Rudolph and Knoflach, 2011<sup>72</sup>). See reference<sup>76</sup> for further information on 5α-THDOC and α2-GABA<sub>A</sub>Rs.
Figure 1. A) A diagrammatic representation of a synapse between a nucleus reticularis (nRT) GABA-ergic neuron and a ventrobasal (VB) thalamic relay neuron. Under conditions of brief high frequency burst firing of the nRT neuron GABA (red dots) levels are sufficient to not only activate synaptic GABA$_A$Rs ($\alpha_{12\gamma2}$) to cause fast phasic inhibition, but to activate GABA$_A$Rs ($\alpha_{4\beta\delta}$) located extrasynaptically. B) The representative traces are from recordings made from synaptically coupled nRT neuron in current-clamp (grey trace) and a voltage-clamped VB neuron of a wild type mouse (black) and superimposed an equivalent recording from and $\alpha_{4^-}$ mouse (blue). For each of the six action potentials recorded in the nRT neuron in this example, there is a corresponding fast, phasic eIPSC recorded in the VB neuron. However, upon cessation of nRT firing for VB neurons of WT, but not for the $\alpha_{4^-}$ VB neurons, there is an additional slowly decaying phasic current. In WT, but not $\alpha_{4^-}$ VB neurons this slow phasic current is prolonged by DS2, a selective $\delta$-GABA$_A$R PAM (not shown). Collectively, these observations suggest this slow response to be mediated by the spillover GABA from the synapse sufficient to activate extrasynaptic $\alpha_{4\beta2\delta}$ GABA$_A$Rs. See $^59$ for further details.
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