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Reviews

The immediate and maintained effects of neurosteroids on GABA_A receptors

D. Belelli, J. A. Peters, G. D. Phillips and J. J. Lambert

Abstract

Allopregnanolone is an endogenous neurosteroid that acts in a rapid non-genomic manner to enhance the function of the GABA_A receptor (GABA_AR), the major inhibitory receptor in the mammalian central nervous system (CNS). Consequently, allopregnanolone elicits anxiolytic, antidepressant, anticonvulsant, analgesic and sedative effects. Endogenous allopregnanolone influences neural inhibition and behaviour, with perturbed neurosteroid levels implicated in depressive disorders. The approval of brexanolone (an allopregnanolone formulation) to treat postpartum depression (PPD) has encouraged optimism that they may provide a new approach to treat mood disorders. Elucidating how neurosteroids are distinguished from established GABA_AR-active drugs, e.g. benzodiazepines, may improve understanding of depressive disorders and aid drug development for psychiatric conditions. Here, we focus on research highlighting: (1) the acute interaction of neurosteroids with GABA_ARs incorporating the δ subunit (δ -GABA_AR) and (2) how neurosteroids may additionally act on a slower time scale to enhance expression of δ -GABA_ARs, thereby providing sustained therapeutic benefit.

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Introduction

In their seminal report of 1984, Harrison and Simmonds [1] revealed the potent positive allosteric modulatory (PAM) properties of alfaxalone, a synthetic steroidal

anaesthetic, upon the major inhibitory receptor in the CNS, the GABA_AR. Subsequent studies established that certain naturally occurring steroids, including allopregnanolone (a metabolite of progesterone) and 5 α -THDOC (a metabolite of deoxycorticosterone), in common with alfaxalone, acted to potently enhance GABA_AR function [2,3]. Soon it became evident that such endogenous steroids originated not only from endocrine glands but additionally were synthesised *de novo* in the CNS and peripheral nervous system, leading to their classification as ‘neurosteroids’ [4]. Administration of neurosteroids (e.g. allopregnanolone) to rodents produced anxiolytic, analgesic, anticonvulsant and sedative behaviours, with high doses inducing a state of general anaesthesia, a profile consistent with enhancement of GABA-ergic inhibition [2]. Collectively, these findings raised the intriguing prospect that neural inhibition may be ‘fine-tuned’ by such steroids acting in an endocrine, paracrine, or autocrine manner to consequently influence behaviour [5]. Importantly, the levels of these steroids are not static but may change dynamically in a variety of physiological and pathophysiological scenarios, including acute stress, the oestrous cycle, pregnancy, and certain depressive disorders [4,6*].

The GABA_AR is an established target for a variety of clinically important drugs that act to enhance GABA-ergic inhibition, e.g. benzodiazepines such as diazepam. However, given the extensive expression of this receptor throughout the CNS, it is unsurprising that the use of benzodiazepines, e.g. as anxiolytics, is associated with numerous side effects that limit their clinical utility [7,8]. The GABA_AR is a member of the Cys-loop transmitter-gated ion channel super-family and is composed of 5 transmembrane crossing subunits, drawn from an extensive repertoire of isoforms (α 1-6; β 1-3; γ 1-3; δ ; ϵ ; θ ; π ; ρ 1-3). This subunit diversity underpins the expression of 20–30 distinct receptor subtypes that differ in their physiological and pharmacological properties and crucially in their regional, neuronal, and subcellular location; for a detailed discussion, see Refs. [7–11]. These findings encouraged the quest for drugs that selectively influenced particular GABA_AR subtypes, in the expectation that they would deliver the desired behavioural effect, but with a reduced propensity for undesirable side effects. For example, in rodents, novel benzodiazepines that lack efficacy for the α 1 β γ 2 subtype but enhance the function of α 2 β γ 2

Abbreviations

BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CRF	Corticotrophin-releasing factor
GABA _A R	GABA _A receptor
δ-GABA _A R	δ-GABA _A receptor
GPCR	G-protein coupled receptor
(HPA) axis	Hypothalamic-pituitary-adrenal
MDD	Major depressive disorder
MSN	Medium spiny neuron
mPR	Membrane progesterone G-protein coupled receptor
NAM	Negative allosteric modulator
PV	Parvalbumin
PAM	Positive allosteric modulator
PPD	Postpartum depression
5α-THDOC	Tetrahydro-deoxycorticosterone
VTA	Ventral tegmental area

GABA_ARs, exhibit anxiolytic properties but are not sedative [7,8].

Given their physiological role as endogenous modulators of the GABA_AR, it is conceivable that neurosteroids, or closely related synthetic analogues, may offer advantages as drugs over other synthetic compounds, although attempts, over 30 years, to develop such steroids clinically, e.g. as anxiolytics, sedatives, general anesthetics, anti-convulsants, and analgesics has proven challenging [2–4]. However, the recent FDA approval of Zulresso (a formulation of allopregnanolone, also known as Brexanolone) to treat PPD has reinvigorated an enthusiasm to explore the therapeutic potential of neuroactive steroids [4,5,12–17]. Current clinical activity includes (1) investigating the use of synthetic neuroactive steroids as treatments for major depressive disorders (MDD) [12,14,15] and (2) the assessment of ganaxolone (a metabolically more stable analogue of allopregnanolone), which has recently achieved its primary endpoint in Phase 3 trial for treating CDKL5 Deficiency Disorder (CDD), a rare form of genetic epilepsy [18].

As highlighted above, a holy grail of GABA_AR neuropharmacology remains the clinical development of GABA_AR subtype-selective drugs. However, although neurosteroids such as allopregnanolone are highly selective for GABA_ARs, indeed enhancing their function at low nM concentrations, studies of recombinant GABA_AR subtypes expressed in host cells such as frog oocytes revealed limited selectivity across the various receptor isoforms [19]. Nevertheless, relatively minor populations of GABA_ARs, particularly those incorporating the δ-subunit, appear to play an important role in mediating the effects of neurosteroids upon neuronal

signalling and behaviour [19,20]. Furthermore, a recent study has revealed neurosteroids to discriminate across different subtypes of δ subunit-containing GABA_ARs (δ-GABA_ARs), dependent upon the isoforms of the α and β subunit - see below [21*]. Here we primarily focus on emerging literature exploring the role of δ-GABA_AR isoforms on neuronal signalling and the acute effects of neurosteroids on such dialogue. Additionally, we discuss how neurosteroids may act on a slower time scale to influence the expression of GABA_ARs, a form of neural plasticity, permitting the duration of their effects on neural signalling and behaviour to far exceed their lifetime as predicted by their pharmacokinetic profile. We will discuss recent studies that identify a critical role for phosphorylation of particular GABA_AR subunits in influencing both the acute and prolonged effects of neurosteroids on neural inhibition [21,22*].

Phasic and tonic inhibition mediated by synaptic and extrasynaptic GABA_ARs

As described above, in mammals, the diversity of GABA_AR subunit isoforms underpins the expression of 20–30 GABA_AR subtypes. Most native receptors are composed of two α, two β and one additional subunit, commonly a γ2 or a δ subunit [7–9]. The subunit composition not only influences the physiological and pharmacological properties of the receptor but additionally impacts upon their anatomical location in the CNS. Furthermore, within a neuron, subunit composition influences whether the receptors are clustered in synapses or expressed perisynaptically and extrasynaptically. Synaptic receptors usually contain a γ2 subunit and mediate fast, transient, phasic inhibition in response to the vesicular release of GABA, a form of inhibition ideally suited to modulate the temporal integration of excitatory and inhibitory signals and the gain of neuronal transmission [19,23,24]. Although the focus of this review is primarily on δ-GABA_ARs, neurosteroid modulation of phasic synaptic inhibition is also clearly important. A physiological example is provided by hypothalamic corticotrophin-releasing factor (CRF) neurons, where low nM concentrations of allopregnanolone enhance synaptic GABA_AR function, thereby reducing their firing rate [19,25]. In certain neurons, extrasynaptic or perisynaptic GABA_ARs mediate a more sustained tonic form of inhibition, better suited to regulate the offset of neuronal firing and excitability [7,8,26,27]. Tonic inhibition results from repetitive receptor activation by ambient concentrations of GABA or, caused by the spontaneous opening of the receptor [21*,26–28] – see below. Predominantly, these extrasynaptic receptors contain either the γ2 subunit, exemplified by the benzodiazepine-sensitive α5βγ2 receptor subtype [26], or benzodiazepine-insensitive receptors incorporating the δ subunit [28]. However,

studies of recombinant ($\alpha 1\beta 2\gamma 2$) GABA_ARs, representative of synaptic receptors, in the presence of low concentrations of GABA (0.5 μ M, designed to mimic putative ambient levels of this neurotransmitter) report a maintained, albeit small current, which is enhanced by neuroactive steroids [29,30]. If these features are replicated in neurons, then it is conceivable that such conductances may contribute to the effects of neurosteroids on neuronal excitability, particularly for those neurons that do not express δ -GABA_ARs. However, for neurons expressing δ -GABA_ARs, e.g. thalamic ventrobasal neurons that predominantly express synaptic $\alpha 1\beta 2\gamma 2$ GABA_ARs and extrasynaptic $\alpha 4\beta 2\delta$, a comparison of recordings from wild type and $\alpha 1^{-/-}$ mice revealed no significant contribution of $\alpha 1$ -GABA_ARs to tonic charge transfer [31].

Known native δ -GABA_ARs isoforms include those incorporating the $\alpha 1$, the $\alpha 4$ and the $\alpha 6$ subunit, with the nature of the incorporated α and β subunit, also influencing the function of these receptors [21] - see below. It is now evident that δ -GABA_ARs play a more dynamic role in neuronal signalling than previously appreciated. For example, recent studies of dentate gyrus granule neurons demonstrate that activation by GABA of extrasynaptic or perisynaptic δ -GABA_ARs contributes to the duration of sIPSCs [32]. Furthermore, in the thalamus, high-frequency burst firing of presynaptic GABA-ergic nucleus reticularis neurons results in a spillover of GABA from the synapse to engage δ -GABA_ARs, thereby greatly prolonging phasic inhibition [19,33]. Extrasynaptic δ -GABA_ARs are not only expressed on principal neurons but are additionally located on particular GABA-ergic interneurons, where they can influence the release of GABA [19,28]. Examples include receptors composed of $\alpha 1$, β , and δ subunits expressed on hippocampal interneurons and δ -GABA_ARs expressed on interneurons and medium spiny neurons (MSNs) in the nucleus accumbens [19,34].

Many of the physiological properties of δ -GABA_ARs, differ from their synaptic counterparts, reflecting their distinctive roles in neural inhibition. Thus δ -GABA_ARs exhibit a high sensitivity and reduced desensitisation to GABA, which acts as a partial agonist at this receptor isoform. The expression of δ -GABA_ARs is highly plastic, being influenced by a variety of physiological stimuli and pathophysiological challenges [27] - see below. Pharmacologically, δ -GABA_ARs are insensitive to benzodiazepines, are selectively activated by low concentrations of THIP (gaboxadol) and GABA-evoked responses mediated by δ -GABA_ARs are enhanced by the selective PAM, DS2 [27,28,31,35]. Importantly, substantial evidence implicates δ -GABA_ARs in the behavioural actions of neurosteroids and synthetic neuroactive steroids (see below).

The interaction of neurosteroids with the δ -GABA_AR

As highlighted above, neurosteroid and neuroactive steroid (e.g. allopregnanolone and ganaxolone, respectively) modulation of GABA_ARs is mainly non-selective in functional studies of recombinant GABA_ARs, with only modest differences in potency and efficacy detected across a large spectrum of receptor subtypes [19,36]. A possible exception is provided by δ -GABA_ARs, where neurosteroids appear more effective. This apparent selectivity appears secondary to GABA acting as a partial agonist at this receptor subtype, thus affording a much greater degree of modulatory enhancement by the neurosteroid compared to other GABA_AR subtypes where GABA has greater efficacy [3,4].

In adding to the complexity, a recent study demonstrates that the subunit composition of the δ -GABA_AR influences spontaneous gating of the receptor channel complex, which, in turn, indirectly influences the functional effect of the neurosteroid [21*]. Traditionally, extrasynaptic GABA_ARs were primarily considered to be activated by ambient or spillover GABA from the synapse, but it is now evident that the channel pore of δ -GABA_AR may open in the absence of GABA [37], for example, in dentate gyrus granule cells of the hippocampus [38,39]. At the single receptor level, such openings are infrequent and of brief duration, although with the same single-channel conductance as GABA-evoked openings. Crucially, spontaneous openings can contribute to tonic currents, challenging the axiom that such currents are exclusively the provenance of spillover of GABA from the synapse or from glial or neuroglial cells [37]. Spontaneous activity of GABA_ARs is revealed pharmacologically by the use of a saturating concentration of the competitive antagonist gabazine (SR-95531) that selectively suppresses only GABA-evoked events and by picrotoxin that blocks all GABA_AR channel openings. Thus, the tonic current attributable to spontaneous openings corresponds to the change in holding current elicited by picrotoxin in the presence of gabazine [21,38–40]. Gabazine is preferable to bicuculline as a receptor antagonist since, in addition to competing competitively with GABA for binding to the orthosteric site of the receptor, bicuculline also acts as a negative allosteric modulator (NAM) capable of blocking both GABA-evoked and spontaneous channel activity [21,39,40]. Electrophysiological studies of rodent brain slices suggest the relative contribution of spontaneous versus GABA-gated activity to the tonic conductance is neuron dependent. In the absence of added GABA, the tonic current of both rat and mouse dentate gyrus granule cells appears to be primarily, if not solely, mediated by spontaneously active GABA_ARs, as defined by the lack of effect of gabazine [21,38]. However, in the presence of added GABA (5 μ M), gabazine does now

inhibit a component of the enhanced tonic current [38]. By contrast, the tonic current of thalamic relay neurons is gabazine sensitive, implying an ambient GABA-mediated component of the tonic current [21*].

A recent study [21*] concluded that the presence of the $\beta 3$ subunit within heterooligomeric receptors is an important requirement for spontaneity. When $\alpha 4\beta 1\delta$, $\alpha 4\beta 2\delta$ and $\alpha 4\beta 3\delta$ were expressed in HEK293 cells, only the $\alpha 4\beta 3\delta$ construct exhibited spontaneous openings revealed by whole-cell voltage-clamp recordings. This β subunit isoform selectivity was traced to a 4 amino acid motif (GKER) located in the N-terminal extracellular domain [21]. Similarly, the $\beta 3$ subunit was a requirement for spontaneous activity of receptors incorporating the $\gamma 2L$ exchanged for the δ subunit. Notably, the spontaneous activity of constructs embodying the $\gamma 2L$ subunit was less than their δ subunit-containing counterparts, leading to the important suggestion that extrasynaptic δ -GABA_ARs, may have a much greater propensity for spontaneous gating than those within the synapse [21]. Moreover, the inclusion of the $\alpha 4$, or the $\alpha 6$ subunit, relative to receptors containing other α subunit isoforms such as $\alpha 1$, favoured spontaneous openings, a preference again governed by the N-terminal extracellular domain of the α subunit. Indeed, mutation of arginine 100 of the $\alpha 4$ subunit to its counterpart histidine 101 within the $\alpha 1$ subunit greatly reduced spontaneous gating of the $\alpha 4\beta 3\delta$ receptor [21*]. Intriguingly, the equivalent histidine residue in $\alpha 1$ -3 and $\alpha 5$ subunit-containing GABA_ARs is critical for high-affinity benzodiazepine binding, with benzodiazepine insensitive $\alpha 4$ and $\alpha 6$ subunits harbouring an arginine residue at this location [7,8].

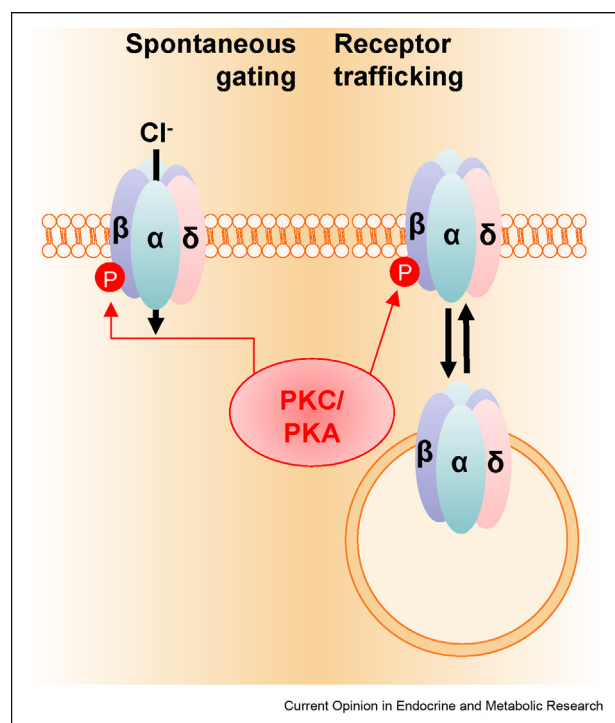
Although spontaneous in nature, the tonic current attributable to GABA-independent events is subject to significant modulation by phosphorylation of critical residues (S408 and S409), located on the large intracellular loop of the $\beta 3$ subunit [21*] – Figure 1. These residues are substrates for PKC, PKA and PKG [41], implying cellular signalling pathways engaged, for example, by G-protein coupled receptors (GPCRs) could influence spontaneous gating of these GABA_ARs (see below).

The effect of the neurosteroids allopregnanolone and tetrahydro-deoxycorticosterone (5 α -THDOC) is influenced by spontaneous gating of the GABA_AR. Greater concentrations of allopregnanolone than those required for enhancement of GABA-mediated responses are well known to directly activate the GABA_AR complex. Importantly, low concentrations (100 nM) of allopregnanolone, or 5 α -THDOC, in the absence of GABA, acted as apparent ‘allosteric agonists’ of spontaneously open $\alpha 4\beta 3\delta$ recombinant receptors, mechanistically by prolonging the duration of spontaneous opening events [21]. By contrast, at such low concentrations,

allopregnanolone had no such effect on equivalent $\alpha 4\beta 2\delta$ GABA_ARs. The ‘allosteric agonist’ effect of allopregnanolone is prevented by a point mutation of the $\alpha 4$ subunit, known to impair neurosteroid binding [21,42]. In common with spontaneous gating, the ‘allosteric agonist’ action of neurosteroids is dependent on phosphorylation of the $\beta 3$ subunit S408 and S409 residues [21] implying cellular signalling pathways engaged, for example, by GPCRs, could influence spontaneous gating of these GABA_ARs. In the context of this review, a particular germane example may be provided by membrane progesterone GPCRs (mPRs), which were recently shown to be activated by certain neurosteroids [22] (see below).

In a neuronal context, the $\beta 3$ subunit facilitated spontaneous openings of GABA_ARs in hippocampal neurons in culture and *via* a shunting inhibition (increased

Figure 1



In hippocampal dentate gyrus granule cells, phospho-regulation by PKC and PKA of residues S408/9 of the $\beta 3$ subunit is reported to enhance cell surface expression of extrasynaptic $\alpha 4\beta 3\delta$ GABA_ARs. Allopregnanolone activation of membrane progesterone G-protein coupled receptors (mPR) may engage this kinase activation pathway to enhance tonic inhibition, a slow form of inhibitory plasticity to complement the immediate positive allosteric effect of this neurosteroid to enhance the tonic conductance. The change in extrasynaptic receptor expression may contribute to the reported prolonged beneficial clinical effects of brexanolone (allopregnanolone) in treating PPD. Additionally, phosphorylation of the $\beta 3$ S408/409 residues promotes spontaneous gating of extrasynaptic $\alpha 4\beta 3\delta$ GABA_ARs. Neurosteroids such as allopregnanolone act as apparent ‘allosteric agonists’ of spontaneously open $\alpha 4\beta 3\delta$ receptors, mechanistically by prolonging the duration of these events.

membrane conductance) reduced their excitability, reflected in a decreased input–output relationship to a depolarising stimulus. Utilising rodent acute brain slices, the contribution of spontaneous openings to the tonic current recorded from dentate gyrus granule cells of the hippocampus was much greater than that of thalamic relay neurons known to express extrasynaptic $\alpha 4\beta 2\delta$ GABA_ARs [21,31]. Although note that studies of $\beta 2$ subunit gene ‘knock-out’ and ‘knock-in’ mice reveal the presence of a population of extrasynaptic $\alpha 4\beta 2\delta$ GABA_ARs in dentate gyrus granule cells of mice [43]. These findings suggest that the underlying mechanisms whereby the neurosteroid primarily enhances tonic inhibition (facilitation of GABA-gated or spontaneous channel openings) will be neuron dependent – see above. In this context, given the reduced spontaneous channel activity of the $\alpha 1\beta 3\delta$ *vs* the $\alpha 4\beta 3\delta$ construct, it would be of interest to explore the contribution of spontaneous and GABA-gated currents to the effect of neurosteroids on extrasynaptic GABA_ARs expressed in hippocampal interneurons, where the δ subunit is partnered with the $\alpha 1$ subunit [34] - see also [44] and below. In conclusion, collectively, these findings imply that in certain neurons, their tonic currents *in vivo* may be subject to facilitation by neurosteroids that is independent of ongoing GABAergic synaptic activity and transmitter spillover.

Neurosteroid activation of progesterone GPCRs and δ -GABA_AR expression

In addition to the classical nuclear receptor for progesterone, neurons within the CNS also express G protein-coupled, cell surface mPRs, belonging to the progestin and adipoQ (PAQR) receptor family [45]. All five isoforms of mPRs (α , β , γ , δ , ϵ) are expressed in the brain, with the δ followed by the β variants exhibiting the greatest levels of overall abundance [45]. The δ receptor and the ϵ variant couple preferentially to the stimulatory G protein G_s, whereas the β isoform (in common with the α and γ isoforms) interacts preferentially with the inhibitory G_i protein [45]. In addition to progesterone, the GABA_AR-active neurosteroids allopregnanolone and 5 α -THDOC, activate mPRs and *via* metabotropic actions in dentate gyrus granule cells cause a slowly developing, but persistent enhancement of neuronal tonic currents mediated by δ -GABA_ARs, thereby complementing their immediate allosteric effect to enhance synaptic and extrasynaptic GABA_AR function [22,46] – Figure 1. This metabotropic regulation manifests as an additional enhancement of tonic currents that importantly persists following presumptive removal of the GABA_AR-active steroids from the preparation (e.g. a hippocampal slice) by prolonged incubation with a steroid-free medium. Potentiation of tonic currents thus outlasting the period of application has been demonstrated for allopregnanolone, 5 α -THDOC, zuranolone (SAGE217) and SAGE-516, but

interestingly not ganaxolone, despite all agents acting allosterically to acutely enhance both phasic and tonic currents upon application [46]. The persistent increase in tonic activity has been attributed to phosphorylation of the $\beta 3$ subunit residues S408 and S409, facilitating trafficking to the cell surface of $\beta 3$ subunit-containing receptors, e.g. $\alpha 4\beta 3\delta$ GABA_ARs [46] – Figure 1. Furthermore, this metabotropic effect of 5 α -THDOC and allopregnanolone is not evident in dentate gyrus granule cells obtained from mice homozygous for the S408A and S409A phospho-null mutations. In apparent contradiction to the results of electrophysiological studies, ganaxolone binds with high affinity to mPR δ receptors, as determined by an ability to compete with [³H]-progesterone binding [47]. However, competition with the latter was incomplete, as is also the case for allopregnanolone, with a significant proportion of [³H]-progesterone binding remaining, complicating the interpretation of the assay. Nonetheless, ganaxolone acting *via* the mPR δ receptor in transfected 231-mPR δ breast cancer cells exerted agonist activity, evidenced by enhancement of cAMP production and attenuation of serum starvation-induced cell death [47].

Metabotropic regulation is pharmacologically isolated by ORG OD 02-0 (10-ethenyl-19-norprogesterone), a selective agonist of mPRs. This steroid is neither an activator of nuclear progesterone receptors, nor a PAM of representative synaptic (e.g. $\alpha 1\beta 2\gamma 2$), or extrasynaptic (e.g. $\alpha 4\beta 3\delta$) GABA_ARs [22]. Activation of mPRs by sustained exposure to ORG OD 02-0 has no acute effect upon GABA-ergic phasic or tonic inhibition recorded from dentate gyrus granule cells within hippocampal slices but does cause a delayed and persistent enhancement of the tonic current, with no effect on phasic inhibition [22]. The effect on tonic inhibition is crucially dependent upon phosphorylation by PKA and PKC of S408 and S409 within the intracellular domain of the $\beta 3$ subunit, as evidenced by suppression of enhancement by selective kinase inhibitors and the complete abrogation of the effect of ORG OD 02-0 upon dentate gyrus granule cells obtained from slices prepared from the $\beta 3$ S408A and S409A mutant mouse [22]. Given the key role played by phosphorylation of the $\beta 3$ subunit S408/9 residues in both the acute enhancement of spontaneous gating by allopregnanolone and the slower metabotropic effects of this steroid upon cell surface δ -GABA_AR expression, it is intriguing that ORG OD 02-0 has no acute effect on such tonic currents, hinting at further signalling complexity (Figure 1). Nevertheless, clinically it is plausible to postulate that following infusion of brexanolone (allopregnanolone), there is an immediate beneficial allosteric effect, followed by this slower metabotropic action of the steroid, which might help address the proposed reduced expression of δ -GABA_ARs associated with PPD and may underpin the sustained clinical benefit of steroid treatment, still evident up to 27 days following cessation of

drug administration [17]. However, note other mechanisms may influence neurosteroid-induced receptor plasticity. For example, allopregnanolone increases the levels of brain-derived neurotrophic factor (BDNF), and this neurotrophin is reported to facilitate the cell surface expression of both the $\alpha 4$ and δ GABA_AR subunits in the hippocampus and to cause a transient increase in phosphorylation of the $\beta 3$ S408/9 residues [19,48].

A variety of GABA_AR subunits contain consensus sequences required for phosphorylation by kinases (including PKC and PKA), with such post-translational modification influencing diverse receptor properties, including trafficking, channel function, and their interaction with associated proteins [41,49]. The selective effect of the progesterone agonist ORG OD 02-0 on δ -GABA_AR cell surface expression, with no apparent impact upon synaptic GABA_AR properties [22] may conceivably result from a local apposition of the δ -GABA_AR and the mPR, although alternatively note in a neuronal environment the presence of a kinase consensus sequence *per se* does not necessarily guarantee that this portion of the protein provides a kinase substrate [22,41].

δ -GABA_ARs and the behavioural effects of neurosteroids

In vivo studies of mice incorporating a partial or full deletion of the δ subunit suggest that extrasynaptic δ -GABA_ARs play a key role in mediating the behavioural effects of neurosteroids. For example, the global deletion of the δ subunit impairs both the sedative and anxiolytic properties of the neuroactive steroid ganaxolone [20]. These observations could be considered surprising given the wealth of evidence implicating the $\alpha 1\beta\gamma 2$ and $\alpha 2\beta\gamma 2$ GABA_AR subtypes in the sedative and anxiolytic effects respectively of benzodiazepines [7,8,50]; however, see Ref. [6] and below. Interpretation of gene deletion studies may be confounded by compensatory mechanism. An example is provided by the CRH-releasing parvocellular neurons of the hypothalamus. By enhancing fast, phasic, synaptic inhibition, neurosteroids such as allopregnanolone potently suppress the firing of these neurons [25]. Although these neurons do not express δ -GABA_ARs, this physiologically important effect of the neurosteroid upon the hypothalamic-pituitary-adrenal (HPA) axis is blunted by the global deletion of the δ subunit [25]. The nullifying of the neurosteroid effect on CRF neuron firing resulted not from a change in GABA-ergic transmission, but from a greatly increased glutamatergic excitatory tone, i.e. an indirect consequence of δ -gene deletion. Intriguingly, the same glutamatergic perturbation in these neurons was manifest in a mouse model of early life adversity [25]. Given the essential role of these neurons in both acute and chronic stress, this perturbation of the inhibitory-excitatory balance may be particularly

pertinent to the role of neurosteroids in psychiatric disturbances associated with HPA dysfunction, e.g. depression and anxiety.

Expressions of the δ subunit and the $\alpha 4$ subunit are sensitive to dynamic changes in regions of the brain known to regulate the HPA axis, e.g. the hippocampus (see below), a plasticity evoked in various physiological and pathological scenarios (e.g. in response to chronic stress, during pregnancy and post-partum [16,27,51] – see below). Of particular relevance are the actions of neurosteroids in the hippocampus, both from a cognitive and regulation of mood perspective [52]. In the hippocampus, δ -GABA_ARs are expressed in both specific principal neurons, i.e. dentate granule cells, where they can influence neuronal excitability, and in interneurons, e.g. parvalbumin (PV) GABAergic interneurons, where, upon activation, they reduce GABA release onto principal neurons [19,51]. Intriguingly, even partial deletion of the δ subunit from PV interneurons in $\delta^{+/-}$ mice is sufficient to reduce the peak frequency of a specific neuronal oscillation in the γ frequency band, an activity associated with learning and memory. Such a deficit can be rectified by allopregnanolone administration in heterozygous $\delta^{+/-}$ mice [53,54]. Moreover, a similar decrease in the peak γ frequency was observed in female mice during the late stages of pregnancy, where allopregnanolone administration again reversed the deficit [53]. Relevant to these findings, reduced expression of the δ subunit is associated with late stages of pregnancy in rodents, and a lack of rebound receptor expression post-partum is suggested to contribute to human PPD [17,27]. As neurosteroid levels in both animals and humans change during pregnancy and post-partum, this dynamic interplay of steroid levels and δ -GABA_AR expression may be pertinent to post-partum mood disorders [55]. Similar considerations may be relevant to other stress-related mood disorders that are associated with plastic changes to δ -GABA_AR expression and neurosteroid levels [51].

Although much of the research focus has been on the postsynaptic effects of neurosteroids, the findings discussed above suggest that presynaptically both δ -GABA_AR mediated suppression of GABA release from interneurons and facilitation of this effect by allopregnanolone may be relevant. Note a recent electrophysiological study of human cortical GABA-ergic interneurons, classified by their firing properties, revealed diversity in their tonic currents and their response to allopregnanolone and to DS2, the δ -GABA_AR selective PAM. These pharmacological effects were mirrored by the expression density of the δ -subunit [44].

Regarding mood disorders, the nucleus accumbens is another neuroanatomical relevant substrate with a prominent expression of the δ subunit. It is a crucial hub

of the reward circuit, which is implicated in the anhedonia associated with depression and other stress-related mood disturbances [56]. We previously reported the tonic current of MSNs, which is mediated by $\alpha 4\beta\delta$ GABA_ARs to be increased by activation of the dopamine (D1) GPCR [57]. Accumbal expression of extrasynaptic δ -GABA_ARs is again not limited to the principal cell, i.e. the GABAergic MSNs, but extends to a variety of interneurons modulating the activity of MSNs, including the PV, choline acetyltransferase and neuropeptide Y expressing GABAergic interneurons [57]. In common with the hippocampus, in the nucleus accumbens, allopregnanolone acts post-synaptically *via* the $\alpha 4\beta\delta$ GABA_AR subtype to enhance a tonic conductance and upon presynaptic δ -GABA_ARs, but in this case with the $\alpha 4\beta\delta$ subtype, to reduce the frequency of sIPSCs recorded from MSNs [57]. Within the reward circuit, an additional tonic δ -GABA_ARs mediated conductance, sensitive to neurosteroids, regulates GABA release from interneurons in the ventral tegmental area (VTA), thereby consequently influencing dopamine release in the nucleus accumbens [58]. How actions on δ -GABA_ARs in the nucleus accumbens and VTA may interact in physiological and pathophysiological scenarios is not known, but intriguingly, expression levels of the δ subunit in the VTA are downregulated in a rodent model of chronic emotional stress [59].

A current challenge concerns understanding the interplay of post-synaptic δ -GABA_ARs to produce tonic inhibition causing a neuronal offset in principal neurons and presynaptic δ -GABA_ARs acting to reduce GABA release from interneurons. Does the subunit composition of the presynaptic δ -GABA_AR influence the effect of the neurosteroid relative to its postsynaptic effect? For example, whereas in the accumbens, functional studies identify the $\alpha 4\beta\delta$ GABA_AR subtype to be expressed both pre-synaptically and post-synaptically, in the hippocampus, does the neurosteroid selectivity for $\alpha 4\beta 3\delta$ over $\alpha 1\beta 3\delta$ that is evident for recombinant GABA_ARs (see above), translate to a preferential interaction with native postsynaptic over presynaptic δ -GABA_ARs? Indeed, the effect of DS2 is limited for GABA_ARs composed of $\alpha 1$, β , and δ subunits in comparison to those incorporating $\alpha 4$, β and δ subunits agent [60]. Note adding further complexity the $\alpha 2$ subunit has recently been suggested to be a putative partner with the δ subunit in dentate gyrus granule cells [6]. Finally, the nature of the β subunit isoform expressed pre-synaptically may be important given the impact of the $\beta 3$ subunit on spontaneous gating and consequently the effect of neurosteroids [21].

From a therapeutic perspective, the presynaptic inhibitory actions of neurosteroids on GABA release observed both in the hippocampus and accumbens are broadly consistent with the disinhibition hypothesis of rapid-acting antidepressants such as ketamine [61]. However,

their enhancement of tonic inhibition on principal glutamatergic neurons, e.g. dentate gyrus granule cells, is more difficult to reconcile with the disinhibition hypothesis of antidepressant actions.

Conclusion

The use of intravenous brexanolone (allopregnanolone) to treat PPD should encourage efforts to further explore the therapeutic potential of naturally occurring neurosteroids. Although effective, brexanolone requires prolonged intravenous infusion in a hospital setting and is associated with undesirable side effects, including sedation. Encouragingly, synthetic neuroactive steroids that have improved pharmacokinetics and oral availability are in advanced stages of development for the treatment of PPD and for MDD [14,62]. For complementing these clinical developments, preclinical studies are required to elucidate what features distinguish the GABA-ergic neurosteroids from other GABA_AR PAMs, e.g. the benzodiazepines in the treatment of depressive disorders? For example, although neurosteroids do not discriminate across GABA_AR subtypes, is their interaction with benzodiazepine-insensitive δ -GABA_ARs important, and if so, would other compounds that share this profile, such as low dose etomidate be effective? Relatedly, would a brain penetrant selective δ -GABA_AR PAM be efficacious [60]? The clinical studies of PPD report improvement of mood by brexanolone long after cessation of treatment [17]. Clearly, clarifying how neurosteroids induce changes of neural plasticity that maintain their beneficial effect is important. Are these effects mediated by an interaction with other steroid targets, such as the metabotropic progesterone receptor, or *via* changes in BDNF, leading to changes in δ -GABA_AR expression as outlined above? In this regard, studies of the immediate and prolonged behavioural effects of neurosteroids in the GABA_AR $\beta 3$ 408/9A knock-in mouse may be informative, where both their immediate effect on spontaneous gating of δ -GABA_ARs and their slower metabotropic effect upon δ -GABA_AR expression will be compromised. Collectively such preclinical studies, coupled with continued drug development and further clinical studies, should enlighten our understanding of the role neurosteroids play in depressive disorders.

Conflict of interest statement

The authors declare no conflict of interest.

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