Neurosteroid interactions with synaptic and extrasynaptic GABA_A receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability

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Abstract

Rationale—Neurosteroids are steroids synthesized within the brain with rapid effects on neuronal excitability. Allopregnanolone, allotetrahydrodeoxycorticosterone, and androstanediol are three widely explored prototype endogenous neurosteroids. They have very different targets and functions compared to conventional steroid hormones. Neuronal GABA_A receptors are one of the prime molecular targets of neurosteroids.

Objective—This review provides a critical appraisal of recent advances in the pharmacology of endogenous neurosteroids that interact with GABA_A receptors in the brain. Neurosteroids possess distinct, characteristic effects on the membrane potential and current conductance of the neuron, mainly via potentiation of GABA_A receptors at low concentrations and direct activation of receptor chloride channel at higher concentrations. The GABA_A receptor mediates two types of inhibition, now characterized as synaptic (phasic) and extrasynaptic (tonic) inhibition. Synaptic release of GABA results in the activation of low-affinity $\gamma 2$-containing synaptic receptors, while high-affinity δ-containing extrasynaptic receptors are persistently activated by the ambient GABA present in the extracellular fluid. Neurosteroids are potent positive allosteric modulators of synaptic and extrasynaptic GABA_A receptors and therefore enhance both phasic and tonic inhibition. Tonic inhibition is specifically more sensitive to neurosteroids. The resulting tonic conductance generates a form of shunting inhibition that controls neuronal network excitability, seizure susceptibility, and behavior.

Conclusion—The growing understanding of the mechanisms of neurosteroid regulation of the structure and function of the synaptic and extrasynaptic GABA_A receptors provide many opportunities to create improved therapies for sleep, anxiety, stress, epilepsy, and other neuropsychiatric conditions.

Keywords

GABA; GABA_A receptor; neurosteroid; allopregnanolone; δ-subunit; tonic inhibition; phasic inhibition; extrasynaptic receptors

Introduction

Neurosteroids are steroids synthesized within the brain with rapid effects on neuronal excitability. The term *neurosteroid* was coined in 1981 by the French endocrinologist

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Étienne-Émile Baulieu to refer to steroids that are synthesized *de novo* in the nervous system from cholesterol independently of the peripheral steroidogenic endocrine glands (Baulieu, 1981). Subsequently, the term *neuroactive steroid* has been widely used to describe natural or synthetic steroids that rapidly alter the excitability of neurons by binding to membrane-bound receptors (Paul and Purdy, 1992; Kulkarni and Reddy, 1995; Reddy, 2003a). It has been known since the 1940s, from the pioneering work of Hans Selye, that naturally occurring steroids such as the ovarian steroid progesterone and the adrenal steroid deoxycorticosterone can exert anesthetic and anticonvulsant actions (Selye, 1941). Recognizing that some steroids could produce such acute central nervous system (CNS) effects, researchers at the pharmaceutical company Glaxo identified the synthetic steroid alphaxolone as having anesthetic properties. In the early 1970s, alphaxolone was marketed as a component of the intravenous anesthetic agent Althesin, which was withdrawn later due to solvent toxicity (Clarke et al., 1973). Several years later, the mechanism of action of alphaxolone was identified. Alphaxolone was found to enhance synaptic inhibition via an action on -aminobutyric acid (GABA) type A receptors in the brain (Scholfield, 1980; Harrison and Simmonds, 1984).

A major advance occurred when 5 -reduced metabolites of progesterone and deoxycorticosterone were found to enhance $GABA_A$ receptor function (Majewska et al., 1986; Kulkarni and Reddy, 1995). It was speculated that the anesthetic and anticonvulsant properties of progesterone and deoxycorticosterone, known since the time of Selye, were due to their conversion to allopregnanolone (3 -hydroxy-5 -pregnane-20-one) and allotetrahydrodeoxycorticosterone (3 ,21-dihydroxy-5 -pregnan-20-one; THDOC), respectively (Reddy, 2003b) (Fig.1). The androgenic neurosteroid androstanediol (5 -androstan-3 ,17 -diol) is synthesized from testosterone (Reddy, 2004ab; 2008; Reddy et al., 2005a). At the time, it was recognized that the enzymes required for the conversion of the steroid hormone precursors to their active 5 -reduced metabolites are present in the brain and therefore their synthesis occurs locally (Kulkarni and Reddy, 1995). There is now compelling evidence that all of the enzymes required for the biosynthesis of the neurosteroids from cholesterol are present in the brain (Do Rego et al., 2009). Therefore, allopregnanolone, THDOC, and androstanediol came to be referred to as *neurosteroids* (Reddy, 2011). Since neurosteroids are highly lipophilic and can readily cross the blood-brain barrier, neurosteroids synthesized in peripheral tissues accumulate in the brain. Pregnenolone, its sulfate ester, commonly referred as pregnenolone sulfate (PS) and dehydroepiandrosterone (DHEA) and its sulfate ester, DHEAS, are also present in the brain and could elicit rapid effects in enhancing neuronal excitability.

Increasing evidence points to the vast array of potent effects that endogenous neurosteroids have on $GABA_A$ receptors in modulating inhibition within the brain (Reddy, 2013a,b). Given that $GABA_A$ receptors are the main mediators of inhibition in the brain, properties that influence this inhibition exert significant regulatory control over neuronal excitability. $GABA_A$ receptors are membrane-bound, ligand-gated ion channels which, when activated by GABA, hyperpolarize neurons through influx of negatively charged chloride ions in the adult brain. At birth, the chloride driving force is positive such that GABA transmission causes depolarization. As the brain matures, a negative shift in the chloride ion reversal potential induces the electrochemical gradient to allow for inward current flow when the $GABA_A$ receptor channel is opened (Rivera et al., 1999). The hyperpolarizing current serves to reduce neuronal excitability and short-circuits action potential firing. Inhibitory $GABA_A$ receptors are heterogenic, possessing a high degree of variability in their structural features and subunit composition. In addition, there is a large catalog of agonists and antagonists for these receptors, and each ligand has distinct affinity and efficacy (Mitchell et al., 2008; Uusi-Oukari and Korpi, 2010). Inhibitory network control is essential to maintain proper

neuronal function for motor actions, cognition, autonomic activity, and nearly every other function of the brain.

This review aims to elucidate current perceptions on the interaction between neurosteroids and GABA$_A$ receptors. It aims to provide a critical appraisal of recent advances in the pharmacology of neurosteroids, in conjunction with emerging information on new mechanisms underlying the regulation of receptor subunit plasticity, tonic inhibition and their applications for improved understanding of normal and abnormal neuronal excitability conditions in the brain. For the purposes of this review, discussion will be centered on neurosteroid and GABA$_A$ receptor interaction as it pertains to limbic structures.

An overview of neurosteroid pharmacology

Emerging understanding of GABA$_A$ receptor interaction with neurosteroids has yielded many interesting developments in neuronal network physiology and clinical application. In the 1980s, alphaxolone and structurally similar steroids were found to enhance GABA agonist activity, and this action was unaffected by benzodiazepine antagonist activity (Harrison and Simmonds, 1984; Harrison et al., 1987). Since that time, both natural and synthetic neurosteroids have been identified which have more potent effects on GABA$_A$ receptors to enhance inhibitory activity within the brain. Consequently, it is clear that two distinct types of GABAergic inhibition –phasic or synaptic inhibition and tonic inhibition— control excitability in the brain (Fig.6). Phasic inhibition is attributed to the inhibitory postsynaptic current (IPSC) resultant from membrane receptor opening in response to rapid release of GABA across the synapse and binding GABA$_A$ receptors as a traditional neurotransmitter (Farrant and Nusser, 2005). Vesicular release of GABA into the synapse generates a local peak concentration of GABA that may reach millimolar levels. Tonic inhibition is persistent inhibitory current mediated by perisynaptic or extrasynaptic receptors in response to ambient or extracellular GABA (Glykys and Mody, 2007). Tonic current enables shunting inhibition to control gain of neuronal excitability (Mitchell and Silver, 2003). Both synaptic and extrasynaptic receptors possess sensitivity to neurosteroids and contribute to the overall significance of neurosteroid modulation of inhibition. Higher sensitivity to neurosteroid contributes to the extrasynaptic receptors' functional divergence from synaptic receptors in modulating inhibition (Bai et al., 2001; Belelli et al., 2009).

Neurosteroids play a significant role in the electrical and molecular control of neurons through GABA$_A$ receptor binding and interaction. Even in physiological concentrations, they are potent, allosteric modulators of GABA$_A$ receptors. Benzodiazepines, barbiturates, alcohols, and neurosteroids all interact with GABA$_A$ receptors to influence channel activity, but they have major differences in binding sites, potency and efficacy (Fig.2). Different types of positive allosteric modulators bind the GABA$_A$ receptor at unique sites. Each of the allosteric modulators can also modify the molecular components, location, and expression of receptors (Uusi-Oukari and Korpi, 2010). They are also involved in the plastic receptor expression changes that occur to adjust balance between inhibition and excitability. GABA$_A$ receptor assembly and turn-over occur through several regulatory mechanisms involving expressional and distributional changes to the synaptic and extrasynaptic membrane physiology (Luscher and Keller, 2004). Extrasynaptic receptors can be transported to synaptic sites in response to requirements for stability at the synapse (Bogdanov et al., 2006) and fine adjustment to recover inhibitory sensitivity through diffusional trafficking (Thomas et al., 2005).

Neurosteroid biosynthesis and regulation in the brain

Neurosteroid biosynthesis

Neurosteroids are synthesized *de novo* in the brain or derived in peripheral tissues from metabolism of classical steroids such as progesterone, deoxycorticosterone, and testosterone. Reduction reactions are catalyzed by 5 -reductase and subsequently 3 -hydroxysteroid dehydrogenase (or 3 -hydroxysteroid oxidoreductase, 3 -HSOR) (Fig.4). Allopregnanolone and THDOC are classified as pregnane steroids, and they are the most commonly studied endogenous neurosteroids (Reddy and Kulkarni, 2000; Belelli and Lambert, 2005). Allopregnanolone is synthesized from progesterone and THDOC is synthesized from deoxycorticosterone. Pregnenolone, progesterone, and deoxycorticosterone are pregnane steroid precursors and also exhibit neuroactive effects. The androstane class of neurosteroids, including androstanol and androstanediol, are derived from testosterone (Reddy, 2004b; Kaminski et al., 2006; Reddy, 2008). Both pregnane and androstane neurosteroids can positively potentiate $GABA_A$ receptor-mediated current and thereby enhance inhibitory function in neurons. Estradiol has neuroactive effects in modulating GABAergic substrates and regulation of neuronal plasticity to promote excitability, especially within the hippocampus (Wójtowicz et al., 2008; Herzog, 2009). Synthesis of estradiol can occur through the aromatase-driven modification of testosterone, which could serve as an endogenous regulator of neuronal excitability in men, as well as women (Herzog, 1999).

Neurosteroids reach neuronal $GABA_A$ receptors from the periphery or local brain tissue. Precursor steroids may enter the brain from the blood circulation and can be converted to neurosteroids (Agís-Balboa et al., 2006). In addition, neurosteroids are very lipophilic molecules and easily cross the blood brain barrier from peripheral sources. Subcutaneous injection of progesterone rapidly elevates plasma and brain levels of allopregnanolone in rodents, even in the case of animals in which the progesterone receptor gene is completely absent (Reddy and Mohan, 2011), indicating the efficiency of the neurosteroid biosynthetic system. The drug finasteride is used as a tool to inhibit endogenous neurosteroid biosynthesis, as it blocks the activity of 5 -reductase conversion of steroids into their 5 -dihydro-reduced form (Reddy, 2010; Reddy and Ramanathan, 2012).

In addition to peripheral production and humoral delivery, it is clear that neurosteroids can be formed locally in the brain from steroid hormone precursors. 5 -Reductase activity has been identified in both neurons and glia in rodent and sheep brain within regions such as the neocortex and hippocampus (Melcangi et al., 1998; Petratos et al., 2000). 3 -HSOR is also expressed widely in the brain (Khanna et al., 1995). In humans, both enzymes have been found in neocortex and hippocampus (Stoffel-Wagner et al., 2000; 2003; Stoffel-Wagner, 2001). Steroid precursors readily enter the brain, so pools of peripherally synthesized precursors are available for local neurosteroid biosynthesis. Taken together, all of the necessary enzymes required for steroid synthesis are region-specific, cell-specific, and available within the neural tissue, both in neurons and glia (Compagnone and Mellon, 2000). Therefore, *de novo* synthesis of neurosteroids may occur within the brain.

Regulation of neurosteroidogenesis and the role of TSPO in neurosteroid biosynthesis

The initial key step in steroidogenesis is the conversion of cholesterol to pregnenolone by the mitochondrial enzyme P450scc (cytochrome P450 cholesterol side-chain cleavage enzyme). Access of cholesterol to P450scc requires StAR (steroidogenic acute regulatory protein), which functions to transfer cholesterol from the outer mitochondrial membrane to the inner membrane where P450scc is located (Stoffel-Wagner et al., 2003). Translocator protein 18 kD (TSPO), formerly called peripheral or mitochondrial benzodiazepine receptor,

likely functions as a complex with StAR and VDAC (voltage-dependent anion channel) at the outer mitochondrial membrane, but this role is not completely clear (Korneyev et al., 1993; Jefcoate, 2002; Papadopoulos et al., 2006; Midzak et al., 2011b). The drug PK11195 is used to verify the TSPO mediated neurosteroid production. TSPO is highly expressed in tissue where steroidogenesis occurs including the brain (Papadopoulos et al., 2006). It transports cholesterol from the outer mitochondrial membrane to the inner membrane, and it promotes production of neurosteroid. Activation of TSPO by certain ligands facilitates increased neurosteroid production (Kita and Furukawa, 2008; Rupprecht et al., 2009). Other TSPO ligands may influence the synthesis of pregnenolone from cholesterol, and once synthesized, neurosteroids are likely released from microglial and glial cells in a paracrine fashion to act on GABA$_A$ receptor targets (Papadopoulos et al., 2006; Nothdurfter et al., 2012). While this assumption remains valid, reduced steroids are locally synthesized within neurons in a wide distribution throughout the brain (Saalmann et al., 2007). Additionally, recent findings suggest that neurosteroids or biochemically similar compounds may have a role in controlling their own biosynthesis through negative feedback loops to interact with TSPO and inhibit cholesterol utilization during steroidogenesis (Midzak et al., 2011a). Mechanisms pertaining to neurosteroid transport may be key in identifying how regional levels of neurosteroid are controlled within the brain to exert modulation over neural networks.

Neurosteroid synthesis in principal neurons

Emerging evidence suggests that production of neurosteroids in the brain occurs in principal neurons. In studies with rodent brain, in situ hybridization with mRNA probes to 5 -reductase and 3 -HSOR indicates that the two mRNAs colocalize to glutamatergic principal neurons and not GABAergic inhibitory neurons or glial cells within neocortex, hippocampus, amygdala, and other brain regions (Agis-Balboa et al., 2006). Immunohistochemistry with an antiserum against allopregnanolone, that also recognizes THDOC, confirms that the neurosteroids are concentrated in principal neurons, predominantly in cell bodies and thick dendrites (Saalman et al., 2007). The highly restricted distribution of neurosteroids to principal neurons suggests that they are mainly derived from local synthesis and not from the circulation, although it is clear that peripheral neurosteroids, as previously noted, readily cross the blood-brain barrier. It is remarkable that brain neurosteroids are localized to the neurons that contain their targets. This observation is consistent with the notion that neurosteroids function in an autocrine fashion in which they reach their targets by lateral membrane diffusion (Chisari et al., 2010). However, the rates of production and their specific control in different regions remain unclear.

GABA$_A$ receptor structure and subunit composition

The human brain contains about 100 billion neurons. An estimated 20–30% of the neurons in the CNS are GABAergic. Activation of neuronal GABA receptors typically results in hyperpolarization, and thus GABA is the major inhibitory neurotransmitter in the CNS. In the general scheme of feed-forward and feed-back interactions between principal and interneurons, inhibitory interneurons play critical role in regulating network excitability. By controlling spike timing and sculpting neuronal rhythms, inhibitory GABAergic interneurons play a key role in regulating neuronal circuits. Two pharmacologically distinct classes of GABA receptors have been identified: GABA$_A$ receptors (heteropentameric ligand-gated chloride channels) and GABA$_B$ receptors (heterodimeric G$_I$/G$_O$ protein-coupled receptors). There is little evidence that neurosteroids interact with GABA$_B$ receptors, which are not covered in this article.

GABA$_A$ receptors are pentameric in structure, and each of the five subunits has four transmembrane domains (M1-M4) (Fig.3). The pentamer arrangement of subunits forms a

selective channel for chloride ion passage when gated and opened by GABA. The second transmembrane domain lines the internal wall of the channel pore. There are 19 variants of $GABA_A$ receptor subunits ($_{1-6}$, $_{1-3}$, $_{1-3}$, , , , , $_{1-3}$), of which , , , and subtypes are most prominently studied (Sieghart and Sperk, 2002). The discussion is focused on receptors consisting of , , , and subunits as they pertain to neurosteroid interaction. $GABA_A$ receptors are typically composed of two subunits, two subunits, and the fifth subunit incorporated is most often , which is present in approximately 90% of $GABA_A$ receptors (Sieghart, 2006). The subunit can be assembled as the fifth subunit in the place of , and there is a high incidence of its occurrence within the cerebellum, thalamus, and hippocampus (Pirker et al., 2000). The functional stoichiometry forming the synaptic channel pore is 2 :2 :1 (Baumann et al., 2002; Ernst et al., 2003). Similarly, $_4$ $_2$ receptors only form functional channels with a stoichiometry of 2 :2 :1 (Shu et al., 2012). Due to the large degree of variety in receptor arrangements, it has been a large undertaking to further classify the physiology and pharmacology for different $GABA_A$ receptor pentamer isoforms.

The $GABA_A$ receptor subunit compositions and location are listed in Table 1. Approximately 60% of all $GABA_A$ receptors have the subunit combination $_1$ $_2$ $_2$, ~15–20% have the $_2$ $_3$ $_2$ combination, ~10–15% have the $_3$ $_x$ $_2$ combination, ~ 5% have the $_4$ $_x$ or $_4$ $_x$, combination, less than 5% have the $_5$ $_2$ $_2$ combination and, likewise, less than 5% have the $_6$ $_{2/3}$ $_2$ combination (Mohler et al., 2002; Rudolph and Knoflach, 2011).

Parsing isoform configurations at extrasynaptic/perisynaptic and synaptic sites is essential to understand GABAergic inhibition and its pharmacological modulation by various ligands. The distribution of $GABA_A$ receptors within the hippocampus is diverse, but there are expressional patterns that emerge from immunocytochemistry (Sperk et al., 1997). Synaptic $GABA_A$ receptors primarily consist of , , and $_2$ subunits. The -subunit expression within synaptic receptors varies, but they are typically benzodiazepine-sensitive (Hajos et al., 2000). Particular -subtypes contribute to synaptic or tonic inhibition differentially in hippocampal neurons (Prenosil et al., 2006). Extrasynaptic receptors are most often characterized by the inclusion of a subunit rather than , together with $_{2/3}$ and a particular isoform of the subunit, dependent on the tissue and region; $_4$-containing profiles are found in dentate gyrus granule cells (DGGC) and thalamic neurons, $_5$ is primarily located in CA1-3 neurons (although coupled with rather than), and $_6$ expression is pervasive within cerebellar granule cells (CGC) (Persohn et al., 1992; Benke et al., 1997; Pirker et al., 2000; Wei et al., 2003; Sun et al., 2004; Mangan et al., 2005; Zheleznova et al., 2009). These profiles mentioned are commonly found, but much cross-over exists in the variety of subunit assembly found within regions and cell-specificity of neurons.

In the hippocampus DGGCs, $_4$ receptors are expressed primarily extrasynaptically, while $_1$ $_2$ & $_2$ $_2$ receptors are located within the synaptic sites. However, $_1$, $_4$, $_2$, and are all present in the extrasynaptic or perisynaptic location in the hippocampus (Sun et al., 2004). According to immunostaining studies, a population of interneurons in the molecular layer of the dentate gyrus also express the -subunit, but the $_4$ subunit is not present (Sun et al., 2004). These receptors have been found to coexpress and colocalize $_1$ and subunits extrasynaptically with different kinetics and pharmacology than $_4$/$_6$ receptors (Glykys et al., 2007). Therefore, principal cells and interneurons in the hippocampus may have quite distinct extrasynaptic receptor composition. Further investigations into subunit-containing receptors have identified that $_2$ is a component of benzodiazepine-insensitive receptors contributing to extrasynaptic current in DGGCs, while $_3$-containing receptors are predominantly assembled in synaptic receptors associated with phasic current (Herd et al., 2008).

Pharmacological tools continue to develop in effort to explain the observed interactions between neurosteroids and receptors at the molecular level. It is important to supplement such evidence with principal brain network physiology in order to elucidate the properties of GABAergic inhibition. A recent study has rigorously investigated receptor isoforms that pertain to either synaptic or extrasynaptic current in order to identify properties of specific subunits that confer GABA potency (Mortensen et al., 2012; see Table 1).

Neurosteroid modulation of GABA$_A$ receptors

A variety of GABA$_A$ receptor modulatory neurosteroids are known to be synthesized endogenously (Fig.4). Neurosteroids are bimodal modulators of GABA$_A$ receptor function depending upon their structural features. The best recognized of these are the pregnane neurosteroids allopregnanolone, THDOC, and androstanediol. Generally, sulfated analogs, such as PS and DHEAS, as well as 3 -hydroxysteroids exhibit negative allosteric modulatory properties.

Positive modulation

Allopregnanolone, THDOC, and androstanediol are positive allosteric modulators of GABA$_A$ receptors, which modify the natural affinity and/or efficacy of GABA and thereby exert control over neuronal excitability. At concentrations in the range 10–500 nM, allopregnanolone and THDOC enhance the activation of GABA$_A$ receptors by GABA (Harrison et al., 1987; Kokate et al., 1994; Reddy and Rogawski, 2002). At higher concentrations, the steroids directly activate the receptor in the absence of GABA. Like other positive allosteric modulators of GABA$_A$ receptors, neurosteroids exert allosteric effects on these receptors such that there is enhancement of the binding of [^{3}H]flunitrazepam, a benzodiazepine receptor agonist, and [^{3}H]muscimol, a specific GABA-site agonist, as well as inhibition of the binding of [^{35}S]t-butylbicycloorthobenzoate (TBPS), a cage convulsant and noncompetitive GABA$_A$ receptor antagonist (Gee et al., 1988; Lan et a., 1991). Unlike benzodiazepines (which enhance the channel open frequency) or barbiturates (which increase the channel open duration), neurosteroid enhancement of GABA$_A$ receptors occurs in a hybrid fashion, increasing both the frequency and duration of the chloride channel opening (Twyman and Macdonald, 1992; Hosie et al., 2007; 2009; Lambert et al., 2009; Ramakrishnan and Hess, 2010). Thus, neurosteroids greatly enhance the probability of GABA$_A$ receptor chloride channel opening, thereby enhancing GABA$_A$ receptor-mediated inhibition.

The molecular nature of neurosteroid binding sites is under intense scrutiny. The effects of neurosteroids on GABA$_A$ receptors occur by binding to discrete sites on the receptor-channel complex that are located within the transmembrane domains of the   and   subunits (Hosie et al., 2007) (Fig.2). The binding sites for neurosteroids are distinct from the recognition sites for GABA, benzodiazepines, and barbiturates. Having the ability to potentiate GABAergic current at 10 – 500 nM concentrations and to autonomously, directly induce receptor channel opening at larger concentrations (> 500 nM) (Belelli and Lambert, 2005), neurosteroids appear to operate on a wider array of receptor isoforms and are thus less specific in binding as compared with benzodiazepines. In fact, the neurosteroid enhancement of binding is thought to be due to allosteric interaction with an altogether different site on the receptor. It has been proposed that neurosteroids may bind receptors from intracellular access or at a site within the neuronal plasma membrane (Akk et al., 2009). Nevertheless, the location of this site is uncertain and warrants further characterization. Neurosteroid binding interfaces are discussed in further detail throughout this review.

Consistent with potentiation effects at the cellular level, endogenous neurosteroids like allopregnanolone and THDOC are capable of sedative, anxiolytic, and anticonvulsant

behavioral effects on the CNS (Reddy and Kulkarni, 1997; Reddy, 2003a; Reddy and Zeng, 2007; Reddy, 2011). It is appropriate to consider the system-wide pharmacological effects of treatments that involve neuromodulatory agents. Because of the capacity of these steroids to affect the neuronal network in profound ways, behavioral measures are of importance to develop neurosteroids for therapeutic applications.

Structure-activity studies of neurosteroid potentiation of GABA-gated currents revealed unique structural features of neurosteroids (Harrison et al., 1987; Gee et al., 1988). A range of steroid structures have activity as positive modulators of GABA$_A$ receptors in line with the hydrophobic surface binding site model. Nevertheless, there are certain strict structural requirements for neurosteroid positive modulation. A hydrogen bond-donating 3 -hydroxy group on the steroid A-ring and a hydrogen bond-accepting group (typically a keto moiety) on the D-ring at either C20 of the pregnane steroid side chain or C17 of the androstane ring system are critical for positive modulatory activity at GABA$_A$ receptors (Purdy et al., 1990; Lambert et al., 2003). The orientation of the C5 hydrogen group only modestly influences potency (Kokate et al., 1994).

The structural configurations of   and   neurosteroid epimers have highly differential effects on GABA$_A$ receptor function, owing to the stereoselectivity of binding. The 5 -isomers of allopregnanolone and THDOC have GABA$_A$ receptor modulatory activity that is only modestly less potent than that of the corresponding 5 -epimers. Behavioral, electrophysiological, and pharmacological data substantiate that 5 -reduced steroids, but not 5 -reduced steroids possess a high level of enantiospecificity and selectivity in function of GABA$_A$ receptor modulation and anesthetic efficacy (Covey et al., 2000). 5 -Reduced neurosteroids have greater anticonvulsant activity in conjunction with inhibitory current potentiation compared to their 5 -epimers (Kokate et al., 1994).

The neurosteroids that have androstane skeleton and that lack the pregnane 17 -ethyl moiety, such as 5 -androstanediol, androsterone, and etiocholanolone, can be considered androstane neurosteroids. Androsterone and etiocholanolone also have GABA$_A$ receptor-positive modulatory activity and represent endogenous neurosteroids (Kaminski et al., 2005). Substantial amounts of androstenol, the 16-unsaturated form of 5 -androstanediol, are present in mammals, including humans. This compound is considered to be a pheromone that increases sexual receptivity in pigs and possibly other species. Androstenol also is a GABA$_A$ receptor-positive modulator that has similar efficacy but is modestly less potent than allopregnanolone (Kaminski et al., 2006).

The androgenic steroid testosterone differs from progesterone by virtue of a 17-hydroxyl group that replaces the 17-acetyl in progesterone. Testosterone is a substrate for both 5 -reductase and 5 -reductase isoenzymes (Reddy, 2004ab; Reddy, 2008). The product of 5 -reduction of testosterone, 5 -dihydrotestosterone, is hormonally more active than testosterone itself. However, subsequent 3 -reduction leads to 5 -androstanediol (5 -androstane-3 ,17 -diol). 5 - and 5 -androstanediol are further metabolized by 17 -hydroxysteroid dehydrogenase to androsterone and etiocholanolone, respectively. The synthetic enantiomers of androsterone and etiocholanone have been found to have more efficacy than their naturally occurring counterparts (Katona et al., 2008). This is in contrast to ent-pregnane steroids, which are less potent than allopregnanolone or pregnanolone. *In vitro* electrophysiology on CA1 pyramidal neurons demonstrates divergent androstane 3-epimer activity on GABA receptors as well; co-application of 3 -androstanediol (reduced by 3 -hydroxy dehydrogenase) and GABA induce a greater hyperpolarizing current than that of GABA alone, while 3 -androstanediol (reduced by 3 -hydroxy dehydrogenase) co-applied with GABA shows no significant effect in enhancing the GABA response (Reddy and Jian, 2010). In animal studies, half-maximal values for seizure protection in mice by

androstanediol are within the concentration range of androstanediol-induced potentiation (Reddy and Jian, 2010). The enhanced potentiation by 3 -androstanediol, rather than the 3 epimer, exhibits protective effects against kindled seizure excitability, and this confirm that the neuroactive effect of the neurosteroid are attributed to the epimer structure, connecting *in vitro* and *in vivo* findings across several studies and methods.

Neurosteroids act as autocrine factors in principal neurons or interneurons. Although neurosteroids are viewed as high potency modulators of $GABA_A$ receptors since they are effective at concentrations in the nanomolar range in aqueous solution, recent studies indicate that neurosteroid binding to the $GABA_A$ receptor is actually of low affinity (true membrane EC_{50}, ~1 mM) (Chisari et al., 2010). The high effective potency of neurosteroids results from partitioning of the lipophilic steroids within the plasma membrane, such that the concentrations accessible to the receptor are orders of magnitude greater. Neurosteroids access the $GABA_A$ receptor from the lipophilic plasma membrane. The nonspecific accumulation and removal of the neurosteroids from the membrane are the major factors determining the rates of neurosteroid action when applied to cells via aqueous solution; rates of binding and unbinding to the receptor are only secondary factors (Chisari et al., 2009). It is noteworthy that intracellular delivery through the plasma membrane is compatible with the autocrine mechanism discussed above, in which the neurosteroids act on the $GABA_A$ receptors in the same neurons in which they are produced (Saalmann et al., 2007).

Endogenous neurosteroids often have low availability due to rapid inactivation or conversion into other steroidal products. To overcome these limitations, synthetic neurosteroids analogs have been developed for therapeutic use (Reddy and Kulkarni, 2000; Fig.5). Analogs also serve as pharmacological tools to better understand specific ligand binding characteristics of neurosteroids based on their conserved lipophilic characteristics. Ganaxolone (3 -hydroxy-3 -methyl-5 -pregnan-20-one) is an analog of allopregnanolone which is altered to include a -methyl group at the C3 position (Carter et al., 1997; Reddy and Woodward, 2004). It possesses sedative and anticonvulsant effects and positively modulates $GABA_A$ receptors allosterically, similarly to allopregnanolone (Reddy and Rogawski, 2010). It is the only neurosteroid analog thus far to be considered in human clinical trials for the treatment of epilepsy (Nohria et al., 2010). Alphaxolone is a general anesthetic that has been well classified in $GABA_A$ receptor binding characteristics. The synthetic derivative minaxolone has been shown to induce an extrasynaptic, GABAergic conductance, similar to allopregnanolone at 1 μM concentrations (Mitchell et al., 2007). These analogs have the ability to potentiate $GABA_A$ receptors and induce sedative, anticonvulsant, anesthetic, or anxiolytic actions, very similar to the endogenous neurosteroids. Their positive interaction with receptors verifies that neurosteroid structural selectivity is reliant on conserved sites to bind and enhance GABAergic function.

Negative modulation

Sulfated neurosteroids such as DHEAS and PS can inhibit GABAergic activity and may serve as proconvulsants (Baker et al., 2010; Gartside et al., 2010). As negatively-modulating steroids, they have been shown to antagonize $GABA_A$ receptors in a noncompetitive fashion. They inhibit channel activity in either a closed state prior to GABA agonist activation or in an open state, blocking activity in instances where GABA is bound (Akk et al., 2001). In addition to GABAergic antagonism, PS and DHEAS have been implicated in potentiating N-methyl-D-aspartate (NMDA) receptors to induce excitability (Friess et al., 2000; Horak et al., 2004), and PS modulation of NMDA is regulated by phosphorylation pathways (Petrovic et al., 2009). Interestingly, PS does not exhibit an effect on the rapid kinetics of channel opening, closing, or desensitization in the presence of high concentrations of GABA (1 mM), however the inhibition of receptors occurs as a slow, gradual block (Akk et al., 2001). The exact mechanism of antagonism is not well understood in the presence of low levels of

extracellular GABA responsible for physiological tone. A high concentration of GABA reflects synaptic neurotransmission of GABA release, but may not explain a negatively-modulating mechanism at extrasynaptic sites of lower GABA concentrations.

While studies have thus far revealed that the sulfated neurosteroid binding site and mechanism of action differs from that of positively potentiating neurosteroids, pharmacological study of sulfated neurosteroids has been limited to the context of synaptic receptor interaction. In pharmacological studies, PS and DHEAS promote learning and memory (Reddy and Kulkarni, 1998a). Due to their intrinsic ability to block GABAergic inhibition, there is a basis for this group of neurosteroids to serve as a homeostatic balance to the positively potentiating class of endogenous neurosteroids. Recently, sulfated steroid antagonism of GABA$_A$ receptors was found to be similar on recombinantly expressed -containing and -containing receptors (Wang et al., 2002; Shu et al., 2012). Pregnane sulfated steroids display diastereoselectivity in antagonism as well.

PS and DHEAS are proconvulsant steroids and can induce seizures when administered systemically or directly into the brain (Reddy and Kulkarni, 1998b; Kokate et al., 1999; Williamson et al., 2004). These pharmacological effects are consistent with their negative modulation effect on the GABA$_A$ receptor function (Wu et al., 1991; Majewska, 1992). The proconvulsant actions of PS are evident at concentrations which are 100 to 500-fold higher than its levels in the brain. Thus, it is highly unlikely that endogenous PS by itself can trigger seizures. However, PS can decrease GABAergic inhibitory transmission at physiological concentrations via a presynaptic action (Teschemacher et al., 1997; Mtchedlishvili and Kapur, 2003). Allopregnanolone blocks the seizure facilitating effects of PS and DHEAS, and consequently, these sulfated neurosteroids could contribute to seizure susceptibility when allopregnanolone and THDOC levels are low.

3 -Hydroxysteroids possess interesting properties to negatively modulate GABA$_A$ receptors. Previous reports suggested that the 3 -diastereomer of pregnanolone, epipregnanolone, was found to act as a competitive antagonist for the neurosteroid site and this inhibited its ability to potentiate the receptor but displayed no antagonism at the GABA site (Prince and Simmonds, 1992). More recent studies have revealed that the 3 -hydroxy pregnane steroids act noncompetitively with their 3 counterparts, and furthermore act as noncompetitive GABA$_A$ receptor antagonists and share similar properties with the sulfated neurosteroids in terms of negative modulation (Wang et al., 2002). Both classes of antagonists block GABA$_A$ receptor in conditions of channel opening. 3 -Hydroxysteroids however, inhibit GABA$_A$ receptor activity in a manner independent of desensitization kinetics while PS aids in slowing the desensitization of receptors. In this context, PS is a more potent modulator than the 3 -hydroxysteroids (Wang et al., 2007).

(3 ,5)-17-Phenylandrost-16-en-3-ol (17PA) has been classified as a neurosteroid antagonist, displaying selective, negative modulation of 5 -reduced steroids with limited effect on 5 -reduced steroid, and no effect on barbiturate, or benzodiazepine potentiation (Mennerick et al., 2004). The evidence suggests that this compound may competitively antagonize the neurosteroid allosteric sites, explaining specificity of its actions. This antagonism serves to reduce allosteric potentiation of the gated receptor and inhibits the anesthetic effects of neurosteroid on the brain. 17PA exhibits higher affinity for inhibiting allopregnanolone and THDOC over alphaxolone (Kelley et al., 2007). Its diverse modulatory characteristics may be attributed to the heterogeneity of GABA$_A$ receptors and subunit configurations that dictate binding affinities for both the positive and negative neurosteroid modulators.

Other steroid metabolites could negatively modulate GABA$_A$ receptors through antagonism in reducing channel opening probability. 11-Deoxycortisol induces neuronal excitability in promoting status epilepticus by accelerating the decay time and reducing the amplitude and frequency of IPSCs that are conducted via GABA$_A$ receptors (Kaminski et al., 2011). This finding suggests a potent activity of steroids and their precursors whereby 11-deoxycortisol contributes to the dampening of GABAergic activity and could have an underpinning role in epileptogenesis.

There are a wide array of non-steroid antagonists, inverse agonists, and convulsants that also negatively affect GABA$_A$ receptor inhibition (Ebert et al., 1997; Rupprecht, 2003). They are typically used pharmacologically to study and contrast the neurosteroid effects on GABA$_A$ receptors. Bicuculline and SR95531 (gabazine) are competitive agonists which act on the GABA ligand binding site to block channel activity. Neither bicuculline nor gabazine act as competitive inhibitors of currents that are engendered by allosteric drugs, like alphaxolone or pentobarbital. They cannot fully block current elicited by these types of agents, but they reduce the channel opening probability once alphaxolone or pentobarbital has bound and thus may be classified as inverse agonists to these types of currents (Ueno et al., 1997). The β-carboline class of organic amines also has negative modulatory effect on GABA$_A$ receptors due to inverse agonist function. Some agents are able to interfere with receptors based on selectivity to specific receptor subunits or channel location (Ing and Poulter, 2007). L-655,708 is a partial inverse agonist for the α_5 subunit with selectivity over a limited concentration range (Quirk et al., 1996). L-655,708 has an intrinsic efficacy for the α_5 subtype rather than other α-containing isoforms, enhances long-term potentiation, but it does not display proconvulsant activity at the range of dose selective for α_5 (Atack et al., 2006). Furosemide noncompetitively blocks α_4/α_6 mediated currents, regardless of β_2 or γ subunits (Korpi et al., 1995; Korpi and Luddens, 1997; Bosman et al., 2002). Picrotoxin and other compounds act as noncompetitive antagonists for GABA$_A$ receptors, blocking the channel and inducing convulsive effects. Along with the cage compound *tert*-butylbicyclophosphorothionate (TBPS), picrotoxin has been shown to act at the channel pore-forming M2 domain to block inward chloride ion flux due to specific residues in that region (Jursky et al., 2000; Buhr et al., 2001; Sedelnikova et al., 2006). Negatively functioning agents can be investigated in conjunction with positive potentiating neurosteroids to understand the mechanisms underlying GABA$_A$ receptor interaction.

Physiology of neurosteroid interactions with GABA$_A$ receptors

Physiological potentiation by neurosteroids

Low nanomolar concentrations of neurosteroid found in the plasma (Wang et al. 1996) and brain are able to substantially potentiate GABA$_A$ receptor inhibition in the presence of ambient GABA (Lerma et al., 1986; Lambert et al., 1995). Neurosteroids prolong the decay of IPSCs in CA1 pyramidal cells, dentate gyrus principal neurons, cerebellar granule cells, Purkinje neurons, hypothalamus, and cortex (Lambert et al., 2003). It is shown that 60 nM allopregnanolone is sufficient to induce a hyperpolarizing shift in GABAergic tonic current within mouse DGGCs (Rajasekaran et al., 2010). Patch clamp recordings of hippocampal neurons demonstrate a concentration-dependent enhancement of GABA-activated, hyperpolarizing potentiation by allopregnanolone (Fig.7). The endogenous levels of neurosteroid vary within circulation and the brain and depend on endocrine fluctuations due to physiological changes like development, the menstrual cycle, or stress. However, concentrations are normally greater in the CNS due to *de novo* synthesis (Reddy, 2003). Levels of neurosteroid in plasma and brain have been estimated to be between 10-300 nM, owing to a variety of physiological and pathophysiological conditions (Belelli et al., 2009). At pharmacological application of 10-1000 nM, neurosteroids allosterically enhance GABA$_A$ receptor function in the presence of GABA. However, at greater concentrations reaching 1

μM and above, neurosteroids are able to directly activate the gating of the chloride channel without GABA (Reddy and Rogawski, 2002; Lambert et al., 2009). It is also possible for sub-micromolar levels of neurosteroid to directly gate GABA$_A$ receptors at a slower, gradual rate due to accumulation within the plasma membrane (Shu et al., 2004; Akk et al., 2009). Direct activation has interesting implications for therapeutic application of neurosteroid when acute control of excitability is necessary (e.g. status epilepticus). Conditions in which GABA transport and uptake are augmented may also influence extrasynaptic receptor interaction (Madsen et al., 2011). The ability of neurosteroid drugs and similar positive allosteric modulators to affect tonic inhibition also depends on the concentration of extracellular GABA and GABA$_A$ receptor occupancy (Houston et al., 2012).

Recent evidence suggests that allopregnanolone may potentiate presynaptic GABA$_A$ receptors, which serves to increase the frequency of spontaneous glutamate release in the hilar neuronal region (Kim et al., 2011). Therefore, neurosteroids may be responsible for presynaptic feedback control on the level of excitability and possibly even modulate tonic current presynaptically as well. Presynaptic GABA$_A$ receptors are pharmacologically distinct from extrasynaptic receptors and appear to largely contain subunits due to high benzodiazepine sensitivity and low zinc sensitivity (Han et al., 2009).

Within minutes of getting into circulation following systemic administration, neurosteroids enter the brain and modulate neuronal function. The acute effects of neurosteroids involve modulation of GABAergic inhibition, while chronic effects may involve modulation of steroid receptor and related pathways. Neurosteroids rapidly bind and immediately potentiate inhibitory GABA$_A$ receptors upon reaching the target neurons. Dynamic changes to GABA$_A$ receptor subunit composition provide a separate mechanism and slower time course for adjusting inhibitory inputs over hours to days. This is discussed in greater detail later in the review.

Extrasynaptic GABA

Extracellular concentration of GABA has been predicted to be 0.4 μM at resting membrane potential (−80 mV) and 3.1 μM during depolarization (+20 mV) (Attwell et al., 1993). Extracellular GABA in the rat hippocampus has been measured to be between 0.8 – 2.9 μM using microdialysis (Lerma et al., 1986). GABA transporters likely have an important and dynamic role in controlling extracellular levels of GABA responsible for tonic inhibition (Richerson and Wu, 2003). The prevalent perception is that surplus GABA spillover from vesicular synaptic release contributes to tonic current in neurons that contain extrasynaptic, -containing receptors like CGCs and DGGCs (Hamann et al., 2002; Wei et al., 2003; Glykys and Mody, 2007). This is challenged by the recent finding that extrasynaptic populations of receptors in CGCs and thalamic neurons have been shown to be desensitized to synaptic, transient spillover of GABA (Bright et al., 2011). There may be a role in certain neuronal networks for desensitization to high concentrations of GABA to stabilize inhibition, but this desensitization to synaptic release has not yet been observed in other brain regions. While this recent finding interestingly contrasts previously held views, determination of the precise mechanism of GABA$_A$ receptor modulation in the presence of spillover GABA and the effect of distance from the synapse on tonic function remains to be explained. The desensitization phenomenon may not be present in all areas where region-dependent tonic inhibition occurs. GABA reuptake may not be as efficient at the perisynaptic and extrasynaptic membrane, and it is reasonable that some GABA escapes into the extracellular fluid. Tonic inhibition proceeds only in the presence of GABA activation, as physiological levels of neurosteroid have not been found to induce receptor channel opening on their own.

Extrasynaptic, α_4-containing receptors contain properties that limit the capability of response to prolonged GABA and/or recurring stimulations that result in higher concentrations of GABA (Lagrange et al., 2007). Houston and colleagues report decreases in tonic conductance upon manipulation by lowering the rate of vesicular GABA release (Houston et al., 2012). Sources of ambient GABA have also been proposed to be from interneurons or astrocytes within the cerebellum (Cavelier et al., 2005). This is an alternative view to synaptic spillover based on reports that blocking vesicular release of GABA had no effect on tonic inhibition, but this has not been confirmed in all brain regions with prominent tonic inhibition (Rossi et al., 2003). Reverse uptake could result from astrocytic depolarization by glutamate released from nearby neurons (Attwell et al., 1993). Recent evidence suggests that glial and astrocytic cells may modulate release of GABA and reuptake of extracellular GABA to influence the overall tonic current in CGCs via GABA-permeable channels belonging to a bestrophin family of anion channels residing in the glial membrane (Lee et al., 2010). This finding, however, has been challenged by contrary evidence concerning the ability of Bestrophin1 channels to contribute to GABAergic tonic currents (Diaz et al., 2012).

Subunit property differences

Studies of recombinant GABA$_A$ receptor isoforms indicate that neurosteroids act on most subunit configurations (Puia et al., 1990; Brown et al., 2002). This distinguishes neurosteroids from benzodiazepines, which only act on GABA$_A$ receptors that contain γ_2 subunits and do not contain α_4 or α_6 subunits. In general, the specific α subunit type may influence neurosteroid efficacy, whereas the β subunit type may affect both the efficacy and potency of neurosteroid modulation (Lambert et al., 2003). The α_1-containing receptors display a greater potentiating current response to allopregnanolone than α_2-containing receptors (Puia et al., 1993), however, α_1 has a distribution generally contained within the rodent basal and septal forebrain, amygdala, and basal ganglia, and is not found within the hippocampus (Pirker et al., 2000). GABA is a relatively low-efficacy agonist of GABA$_A$ receptors containing the δ subunit rather than the more common γ_2 subunit, even though it binds with high affinity to such δ-subunit containing receptors (Brown et al., 2002; Glykys and Mody, 2007). These δ-containing receptors possess a significantly higher affinity for GABA than other receptor subtypes, and they are not desensitized in the constant presence of agonist (Saxena and Macdonald, 1994; Mody, 2001). Neurosteroids therefore can markedly enhance the current generated by δ-subunit-containing GABA$_A$ receptors even in the presence of saturating GABA concentrations. A recent study using concatemeric receptors expressed in oocytes reported that allopregnanolone and THDOC modulate δ-containing receptors and synaptic-like receptors at similar threshold concentrations (Shu et al., 2012). The study confirms the low-efficacy properties that drive δ-specific current gating. However, individual isoform expression is not representative of the complex composition of receptors on native neurons and does not necessarily reflect regional δ at dendritic locations responding to low, exogenous GABA. Furthermore, extrasynaptic GABA$_A$ receptors that contain the δ subunit are highly sensitive to neurosteroid-induced potentiation of GABA responses (Belelli et al., 2002; Wohlfarth et al., 2002; Meera et al., 2009).

There are evident differences in GABA$_A$ receptor synaptic strength that are regulated by specific γ-subunit isoforms (Ortinski et al., 2004). In comparison with $\gamma_1\beta_2$ receptors, $\gamma_2\beta_2$ receptors possess faster activation kinetics, slower deactivation kinetics, and prolonged channel opening (Lavoie et al., 1997). A substantial change from γ_1- to γ_2-containing receptors within hypothalamus has been previously shown to be associated with slower GABAergic postsynaptic current decay (Brussaard et al., 1997; Brussaard and Herbison, 2000). An γ_2-dominant population of synaptic receptors could increase net

inhibitory synaptic current while exhibiting differential neurosteroid affinity (Maitra and Reynolds, 1999). Herbison and colleagues report changes in allopregnanolone sensitivity coupled with this -subunit plasticity in the regulation of oxytocin across pregnancy. Subunit plasticity could also have an effect on receptor desensitization in presence of neurosteroid (Smith and Gong, 2005). In study of recombinant receptors, the $_2$ $_3$ $_2$ isoform exhibits the lowest GABA$_A$ receptor agonist binding affinity but a moderate degree of potency when compared to the other -containing isoforms; $_2$ $_3$ $_2$ also displays the highest binding affinity for the GABA$_A$ receptor antagonists gabazine and bicuculline (Ebert et al., 1997). The -subunit has recently been described to have an important role in specific targeting of GABA$_A$ receptors to synaptic versus extrasynaptic sites (Wu et al., 2012). Endogenous neurosteroids may differentially bind and regulate $_1$- and $_2$-containing synaptic GABA$_A$ receptor function.

While neurosteroids are able to modulate both synaptic and extrasynaptic receptors (Fig.6), there are clear differences in efficacy between -containing and -containing receptors, (Pillai et al., 2004) as well as differences between the composition of subunits (Belelli et al., 2002; see Table 2). The -containing receptors produce a much higher maximal response to neurosteroids (Brown et al., 2002). The -subunit-containing receptors possess unique functional actions and are found at dendrites, where there are low levels of ambient GABA (Wei et al., 2003; Semyanov et al., 2004). Therefore, receptors that are attuned to extrasynaptic concentrations of GABA can be modulated by exogenous agents like neurosteroids that can finely adjust and potentiate the level of tonic inhibition (Stell et al., 2003; Farrant & Nusser, 2005). Knockout studies reveal that $_4$ and $_5$ subunit-containing receptors mediate the majority of tonic inhibition in DGGC and CA1 hippocampal neurons, respectively (Caraiscos et al., 2004; Glykys et al., 2008). Similarly, neurosteroids have high affinity for the $_6$ -containing GABA$_A$ receptors, which are responsible for the tonic inhibition within cerebellar granule cells (Hadley and Amin, 2007). Other subunit isoforms may be expressed extrasynaptically and contribute to tonic inhibition, but this would account for a relatively small fraction of total tonic current. Co-expression of different subtypes with $_1$ and $_2$ delivers relatively little influence over endogenous neurosteroid modulation of GABAergic current (Belelli et al., 2002). In contrast, combinations of different subtypes with $_3$ $_{2S}$ present different degrees of pregnanolone potentiation; $_5$ isoforms have the greatest potentiation and $_1$ isoforms have the lowest potentiation (Smith et al., 2001). Interactions of $_4$-containing receptors with neurosteroid largely depend on adjacent, co-assembled subunits, as $_4$ profiles are more sensitive to neurosteroids than $_4$ receptors (Brown et al., 2002).

At the DGGC synapse, $_1$ and $_2$ display similar distributions when observed with synaptic marker colocalization, however $_1$ and $_2$ are also expressed extrasynaptically in these neurons (Sun et al., 2004). Receptors with $_2$ subunit may have a contributing role in tonic inhibition as neurosteroids have a greater effect on $_1$ $_1$ $_2$ than $_1$ $_1$ receptors; moreover, the particular isoform of appears to have little or no effect on the degree of modulation (Belelli et al., 2002; Lambert et al., 2003). Consequently, -containing receptors participate in a distinctive role to control the excitability of the hippocampus and other neuronal tissues by setting a baseline level of inhibitory current. Evidence continues to emerge that this type of neuromodulation exhibits a great amount of plasticity in the ability of neurons to reconfigure receptor populations on the membrane. Further insight into the composition of GABA$_A$ receptor profiles, neurosteroid pharmacodynamic activity, and regional brain inhibition provide meaningful explanations of the underlying modulatory controls intrinsic to GABA$_A$ receptor and neurosteroid interaction. Overall, the robust effect of neurosteroids is likely to be due to their action on both synaptic and perisynaptic/extrasynaptic GABA$_A$ receptors.

Potency of neurosteroids in seizure models

Allopregnanolone-like neurosteroids are powerful anticonvulsants. Exogenously administered neurosteroids, like other agents that act as positive $GABA_A$ receptor modulators, exhibit broad-spectrum anticonvulsant effects in diverse rodent seizure models (Reddy, 2010). Neurosteroids display rapid anticonvulsant activity and provide protection within minutes. Neurosteroids protect against seizures induced by $GABA_A$ receptor antagonists, including pentylenetetrazol and bicuculline, and are effective against pilocarpine-induced limbic seizures and seizures in kindled animals (Kokate et al., 1994; Belelli et al., 1989; Frye, 1995; Wieland et al., 1995; Reddy et al., 2004; 2010; Reddy and Rogawski, 2010). Like other GABAergic agents, they may exacerbate generalized absence seizures (Snead, 1998; Citraro et al., 2006). As shown in Table 3, the potencies of neurosteroids in models where they confer seizure protection vary largely in accordance with their activities as positive allosteric modulators of $GABA_A$ receptors. Thus, allopregnanolone is roughly equally potent as THDOC, but androstanediol and androsterone are somewhat less potent (Reddy, 2004a; 2004b; Kaminiski et al., 2005).

Like other GABAergic agents, neurosteroids are inactive or only weakly active against seizures elicited by maximal electroshock. Neurosteroids are highly active in the 6-Hz model, a better paradigm in which limbic-like seizures are induced by electrical stimulation of lower frequency and longer duration than in the maximal electroshock test (Kaminiski et al., 2004). Androstanediol, but not its 3 -epimer, produced a dose-dependent suppression of behavioral and electrographic seizures in the mouse hippocampus kindling (Reddy and Jian, 2010). The estimated concentrations of androstanediol producing 50% seizure protection in the kindling model (~10 μM) are within the range of concentrations that potentiate $GABA_A$ receptor function in CA1 pyramidal neurons. Neurosteroids are also highly effective in suppressing seizures due to withdrawal of $GABA_A$ receptor modulator drugs including neurosteroids and benzodiazepines, and also due to other types of agents such as ethanol, which may act in part through $GABA_A$ receptors, and cocaine, which does not (Tsuda et al., 1997; Devaud et al., 1996; Reddy and Rogawski, 2000a; 2001; Reddy et al., 2012).

Lack of tolerance

Benzodiazepines exhibit pharmacodynamic tolerance during prolonged use. In contrast to benzodiazepines, where utility in the chronic treatment of epilepsy is limited by reductions in efficacy over time, anticonvulsant tolerance is not obtained with neurosteroids (Kokate et al., 1998; Reddy and Rogawski, 2000b). Thus, neurosteroids have the potential to be used in the chronic treatment of epilepsy, and this has also been evident in clinical trials (Nohria et al., 2010). The mechanisms responsible for tolerance to benzodiazepines are not known. However, factors such as uncoupling of the allosteric linkage between the GABA and benzodiazepine sites and changes in receptor subunit turnover with switching of subunits may be contributing mechanisms (Bateson, 2002). Neurosteroids do not act on the benzodiazepine site of $GABA_A$ receptors, and they are able to modulate all isoforms of $GABA_A$ receptors, even those that contain benzodiazepine-insensitive $_4$ and $_6$ subunits or do not include the obligatory $_2$ subunit required for benzodiazepine sensitivity. Surprisingly, chronic neurosteroid exposure does lead to cross tolerance to benzodiazepines (Reddy and Rogawki, 2000b). Thus, it appears that the same plastic changes that underlie benzodiazepine tolerance are brought into play by chronic neurosteroid exposure. However, neurosteroids act at distinct sites on $GABA_A$ receptors and exhibit effects on the full range of $GABA_A$ receptor isoforms. Therefore, the potentials side effects of neurosteroids are mostly related to their GABAergic effects (Reddy, 2013b).

GABA$_A$ receptor pharmacology and allosteric interactions

The positive or negative modification of GABA$_A$ receptors by allosteric modulators depends on their structural features. The affinity and efficacy define their overall profile. In study of the allosteric interaction of neurosteroids on receptors, an accurate understanding of the various pharmacopore interactions on isoforms of GABA$_A$ receptors is essential. Specific binding sites and overlap between pharmacological features are useful in examination of the neurosteroid pharmacopore, which is less clear than most of the other GABA$_A$ receptor modulators.

Neurosteroid interactions

While there have been early attempts to determine binding patterns for neurosteroids, the true ligand-binding properties for neurosteroid interaction remain unclear. Neurosteroids have a distinguishable binding sites from benzodiazepines, which act only on GABA$_A$ receptors containing γ_2 subunits and those that do not have α_4 or α_6 subunits assembled. Neurosteroids have been shown to possess a different binding site from barbiturates, though they share various similarities in receptor-mediated functions. The lipophilic properties of neurosteroids influence potency and affinity for GABA$_A$ receptors (Chisari et al., 2009). Molecular evidence shows that a C3 -hydroxyl group and a C20 position ketone group on pregnane neurosteroids or a C17 ketone on androstane neurosteroids are essential for binding affinity (Mitchell et al., 2008). This suggests specificity in a binding pocket and interaction with certain residues yet to be clarified. Although the exact location of neurosteroid binding has not been mapped, it has been proposed that there are two distinct sites for neurosteroids that act as positive modulators: one for allosteric enhancement of GABA and another for direct activation of the receptor, as well as an antagonistic transduction site for sulfated neurosteroids involving the M2 region of the subunit (Akk et al., 2001; Lambert et al., 2003; Hosie et al., 2006; 2007). Using site-directed mutagenesis, it has been shown that a highly conserved glutamine at position 241 in the M1 domain (toward the intracellular side) of the subunit plays a key role in neurosteroid modulation of GABA responses and is believed to contribute to the binding site for modulation (Hosie et al., 2009). Additional nearby residues in the M4 domain of the same subunit (tyrosine 410 and asparagine 407, toward the extracellular side) have also been proposed to contribute to the binding site.

Other investigators have found that mutations in serine 240 and tryptophan 245 of the subunit interfere with neurosteroid potentiation. Multiple studies with structurally diverse steroids have led to the conclusion that the steroid binding pocket on the subunit is more correctly viewed as a hydrophobic surface that can accommodate steroid molecules of different structures (Akk et al., 2009). Direct activation of the receptor, in contrast, has been proposed to be due to steroid binding at a site on the interface between and subunits formed by a threonine at position 236 in the subunit and a tyrosine at position 284 in the subunit (Hosie et al., 2007). However, more recent models of the GABA$_A$ receptor have questioned whether these residues reside at the – subunit interface. A photo-incorporable analog of the anesthetic etomidate appears to bind at the interface, but binding of this ligand is not competitively inhibited by neurosteroids (Li et al., 2009).

The subunit has been less prominently studied than subunit in regard to neurosteroid binding. Early reports suggested that the subunit does not appear to possess an influential role in neurosteroid interaction with GABA$_A$ receptors (Lambert et al., 2003). However, δ_2 subunits are preferentially assembled in extrasynaptic channels with high neurosteroid affinity and δ_2 deficiency reduces tonic inhibition, while γ_3 subunit-containing receptors are mostly synaptic, based on dentate gyrus investigation (Herd et al., 2008). Manipulation of the M1 domain of the δ_2 subunit has an effect on the spontaneous activity of extrasynaptic

receptors (Baker et al., 2010). The extracellular domain loop 9 of the $_2$ subunit has been shown to steady the closed-state gate of the $_1$ $_2$ $_{2S}$ synaptic GABA$_A$ receptors, and this has indirect consequences on receptor efficacy in GABA activation and the allosteric modulation by propofol and pregnanolone (Williams et al., 2010). A recent report identified a novel $_3$ subunit transmembrane domain involved in neurosteroid binding (Chen et al., 2012). While isoforms have been found to influence tonic current (Herd et al., 2008), it is still unclear if they significantly affect neurosteroid modulation.

The subunit is integral to the high sensitivity of neurosteroid-receptor interaction (Brown et al., 2002); it is not germane to the actual neurosteroid binding site for opening of the channel but rather is involved in the transduction of the gated response subsequent to initial binding (Hosie et al., 2009). Channels of $_4$ $_2$ do not differ in GABA potency from channel pentamers composed of $_4$ $_2$ without a third subunit type (Mortensen et al., 2012). Moreover, receptors modified to contain a single functional GABA agonist site and/or a single proposed allosteric site for steroidal binding are adequate in eliciting potentiation by allopregnanolone, yielding to the potent sensitivity of neurosteroid activity on receptors (Bracamontes et al., 2011). While this recombinant GABA$_A$ receptor study was conducted to disassociate the two known sites for neurosteroid binding, the physiologically existent receptors have much more diverse kinetics relative to direct and allosteric neurosteroid binding, and the details surrounding those binding targets remain unclear.

The -subunit does however, possess an intrinsic property within receptors to increase channel opening probability in potentiating current. Therefore, the - interface without may permit neurosteroid affinity, but the subunit incorporation provides a potentiating shift for longer channel opening and gating of current (Bianchi et al., 2003). Mutation in the M2 region of -containing receptors decreases the potency of THDOC and the positive modulator tracazolate, which typically potentiate GABA$_A$ receptors at low efficacy (Zheleznova et al., 2008). In addition, the S238-V264 domain of the subunit (including M1 and the intracellular loop connecting to M2) has been identified to influence the high level of agonist sensitivity to $_4$ $_3$ receptors apparent in both measures of efficacy and potency (You and Dunn, 2007). Binding of receptor does not likely occur at these subunit domains by either GABA or neurosteroid, but these domains nevertheless have profound transducing effects on channel gating.

Despite the fact that neurosteroid function diverges from the classical steroid role of diffusion across the membrane to act through nuclear receptors, the lipophilic structure of neurosteroids permits their movement in cells and the plasma membrane similarly to other steroids. Potentiation of GABA$_A$ receptor channels occurs upon application of allopregnanolone to the inner leaflet of transfected membrane in an inside-out patch configuration (Akk et al., 2005). In conjunction with other data showing the ability of neurosteroids to reach GABA$_A$ receptors through lateral diffusion or from intracellular paths, this substantiates that neurosteroids may be able to potentiate current through routes besides extracellular access which is representative of most ligand-gated ion channels (Akk et al., 2009). Access via a hydrophobic pocket of the receptor within the plasma membrane bilayer therefore remains a feasible biochemical explanation for neurosteroid binding.

Benzodiazepine interactions: Comparison with neurosteroids

Benzodiazepines non-selectively and allosterically bind to GABA$_A$ receptors containing the $_1$, $_2$, $_3$ or $_5$ subunits and thereby potentiate synaptic GABAergic inhibition. They have been in clinical use for decades and are still among the most widely prescribed drugs for the treatment of insomnia, anxiety, epilepsy, and status epilepticus. However, their use is limited by side effects such as sedation, cognitive impairments, tolerance and the risk of drug dependence. Due to their wide array of differential binding affinity, benzodiazepine-site

interactions with $GABA_A$ receptors are quite dynamic in comparison to neurosteroid allosteric interactions.

$GABA_A$ receptors have a separate allosteric binding site for the benzodiazepines located at the - interface, which is an altogether distinct site from the barbiturate binding interface as well (Sancar and Czajkowski, 2011). Thus, for each -containing receptor, there is one ligand binding site for benzodiazepine molecules. The pharmacology is mainly governed by and subunit isoforms at the binding pocket and has been well classified through classical 1,4-benzodiazepine study. Furthermore, subunit diversity has been implicated for the varying anticonvulsant, anxiolytic, or anesthetic activities by diazepam and similar benzodiazepines on $GABA_A$ receptors (Rudolph and Mohler, 2004; Olsen and Sieghart, 2009). Representative of subunit heterogeneity, zolpidem binds with high affinity for receptors with $_1$, partial agonism for $_2$ and $_3$, and low affinity for $_5$ (Smith et al., 2001). Although not a benzodiazepine itself, zolpidem is an imidazopyridine which binds to $GABA_A$ receptors in the same allosteric location as benzodiazepines and is functional only on receptors with $_2$ subunits (Wulff et al., 2007).

Ro 15-1788 (flumazenil, benzodiazepine antagonist), Ro16-6028 (bretazenil, partial agonist) and Ro15-4513 have been shown to have high affinity at $_4$ and $_6$ receptors that are otherwise insensitive to diazepam and imidazenil (Wisden et al., 1991; Knoflach et al., 1996; Wafford et al., 1996; Benke et al., 1997). In addition, flumazenil and Ro15-4513 modify the efficacy of $_4$ and $_6$ receptors to act as positive allosteric modulators. These compounds differ from 1,4-benzodiazepines, which only bind to $GABA_A$ receptors containing either $_1$, $_2$, $_3$, or $_5$ subunits. Ro15-4513 is a partial inverse agonist that binds to the - benzodiazepine site of $GABA_A$ receptors (Benson et al., 1998). It has high binding affinity and efficacy for modulating GABAergic current of $_4$ $_3$ $_2$ receptors, but does not directly modify or affect $_4$ $_3$ channel activity (Brown et al., 2002). A recent autoradiography study reported that $_{4/6}$ $_3$ receptors also have a high-affinity binding site for Ro15-4513 (Hancar et al., 2006). DGGC tonic current enhancement by Ro15-4513 is signified by decline of its potentiating effects in $_4$ knockout mice (Liang et al., 2008). These findings expand on the fact that -containing receptors are located extrasynaptically as well, albeit in lower quantities.

Receptors containing $_4$ or $_6$ subunits are insensitive to classical 1,4-benzodiazepines, and interestingly, receptors with these isoforms are most commonly found in extrasynaptic receptors. Chimeric $GABA_A$ receptor mutations have confirmed that specific residues in the -subunit impart altered binding affinity that affects benzodiazepine sensitivity (Derry et al., 2004). The receptor structure fits a motif which functionally divides receptors not only based on location relative to the synapse, but substrate-ligand interface affinity as well. Generally, receptors containing $_1$, $_2$, $_3$, or $_5$ subunits are diazepam-sensitive whereas receptors containing $_4$ or $_6$ subunits are diazepam-insensitive. This information suggests that receptor subtype-selective compounds could overcome the limitations of classical benzodiazepines such as sedation. Subtype-specific agents are undergoing clinical studies as non-sedative anxiolytics (Rudolf and Knoflach, 2011). In contrast to benzodiazepines, neurosteroids exhibit preferential modulation of extrasynaptic $_4/$ -containing receptors.

GABA site agonist interactions

It is generally accepted that GABA binds to the - interface to allow channel opening such that two GABA molecules may gate the receptor, given that each receptor has two - subunit interfaces. The two - interfaces differ in local environment based on the adjacent subunits (and or and) and therefore display some differences in agonist preferences (Baumann et al., 2003). Muscimol is a potent agonist for the GABA binding site, and is often used to quantify pharmacology regarding allosteric binding of $GABA_A$ receptors.

4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (gaboxadol; THIP) is a hypnotic agent and a partial agonist for GABA$_A$ receptors with a higher selectivity for -containing receptors (Ebert et al., 1997; Wafford and Ebert, 2006; Herd et al., 2008), but displays different degrees of agonist binding that is dependent on the   subunit isoform incorporated (Stórustovu and Ebert, 2003). The involvement of the -subunit in GABA$_A$ receptors confers a high gaboxadol potency (Meera et al., 2011). GABA and gaboxadol have higher potency on $_4$ $_3$ than $_4$ $_3$ $_{2L}$ recombinant receptors expressed in *Xenopus* oocytes (You and Dunn, 2007). Furthermore, gaboxadol has a higher efficacy than GABA for -containing receptors, but gaboxadol has lower efficacy than GABA for $_2$-containing receptors (Stórustovu and Ebert, 2006; Saarelainen et al., 2008). As potent receptor ligands, gaboxadol and muscimol exhibit different degrees of agonist response in interaction with extrasynaptic GABA$_A$ receptors. Gaboxadol is known to selectively enhance extrasynaptic function with high potency at $_4$ $_3$ and $_6$ $_3$, which display similar extrasynaptic functional properties (Brown et al., 2002); high concentrations of gaboxadol on $_4$ $_3$ receptors allow for longer and more frequent channel openings, while muscimol binding reduces the desensitization of the receptor (Mortensen et al., 2010). In comparison of $_4$ $_2$ and $_4$ $_3$ receptors, there does not seem to be a significant difference in the potency of GABA binding, signifying a less imperative role for   subunit variety to dictate agonist binding properties (Mortensen et al., 2012).

Other pharmacological ligands

The pervasive and abundant amino sulfonic acid, taurine, has been detailed as a partial agonist at GABA$_A$ receptors. Taurine also enhances GABAergic tonic current inhibition and displays pharmacological properties that are consistent with extrasynaptic, -containing receptors (Jia et al., 2008). The possibility of interplay between taurine and neurosteroids in the brain has implications in GABA$_A$ receptor plasticity and functional characteristics. Taurine may change   receptors to become low-efficacy, and in effect alter binding patterns to allow for neurosteroid to selectively bind and potentiate receptors that are primarily synaptic in location (Bianchi and Macdonald, 2003). In a similar manner, -alanine increases the efficacy of   receptors; altering concentrations of this amino acid can reduce neurosteroid selectivity for extrasynaptic receptors, having the greater effect of reduced baseline inhibition. Induction of seizure in rats with the convulsant pentylenetetrazol increases the levels of taurine in the hippocampus. In addition, these animals exhibit decreased extracellular levels of GABA, involving a shift in excitatory/inhibitory amino acid levels that could have profound effects on the overall neuronal circuitry and channel current (Szyndler et al., 2008).

Transition metal zinc cations (Zn^{2+}) have a potent ability to inhibit GABA$_A$ receptor function. GABA$_A$ receptors exhibit differing sensitivities to Zn^{2+}, which can negatively modulate the receptors through noncompetitive antagonism. There are three sites that coordinate Zn^{2+} interaction including one within the ion channel, and two sites on amino-terminal residues on the   interface; for this reason, zinc potently inhibits subunits of   and   composition with high sensitivity while   isoforms have a lesser amount of interaction and sensitivity (Nagaya and Macdonald, 2001; Hosie et al., 2003). GABA$_A$ receptors responsible for tonic inhibition are highly sensitive to blockade by Zn^{2+} (Mortensen and Smart, 2010). Based on Timm's staining for heavy metals, zinc is pervasive throughout hippocampal mossy fibers which project from DGGCs to the hilus and CA3 regions. Endogenous Zn^{2+} is able to mediate GABAergic inhibition in mossy fiber regions (Ruiz et al., 2004). This leads to a rational assertion that Zn^{2+} may be homeostatically controlled in the hippocampus where neurosteroid levels are tightly regulated as well. Negative modulation by endogenous Zn^{2+} can differentially effect GABAergic inhibition and can be used to further understand neurosteroid modulation.

Electrophysiological basis of GABAergic tonic inhibition

GABAergic channel kinetics

The pharmacological modulation of $GABA_A$ receptors is measured by inhibitory current and channel kinetics, which provide important details to understand the differences between phasic and tonic receptors. Neurons containing highly expressed levels of receptors involved in tonic current have distinct, unique properties from phasic receptors (Stell and Mody, 2002). Single channel patches of neuronal membrane receptors possess channel gating properties ascribed to sensitivity and comparatively fast recovery of inward current. Synaptic inhibition is characterized by biphasic (fast followed by slow) desensitization and delayed deactivation of the channel's inward current in response to GABA, and this is typified by the population of receptors which are prominent at the postsynaptic membrane. In contrast, extrasynaptic receptors are exemplified by a single phase of decay, more rapid deactivation, and a much lesser degree of desensitization to GABA-induced current (Bianchi et al., 2001; Brown et al., 2002). Specific residues of the subunit M1 domain and N-terminus are necessary for the observed extrasynaptic desensitization kinetics, and mutations in the same region of $_{2L}$ result in increased fast deactivation (Bianchi et al., 2001). Endogenous neurosteroids modify neuronal $GABA_A$ current by increasing the channel opening duration, prolonging slow deactivation and delayed recovery from desensitization (Zhu et al., 1997; Wohlfarth et al., 2002).

GABA transport and reuptake

Since extracellular GABA is responsible for setting the inhibitory tone, GABA transport and its maintenance within brain has large bearing on the control of tonic current. There are four GABA transporters, GAT-1, GAT-2, GAT-3, and BGT-1. GAT-1 is the most well studied in conjunction with rodent models. Knockout of GAT-1 in mouse cerebellum produces adverse motor disability and tremors as well as increased tonic conductance in cerebellar neurons (Chiu et al., 2005). Depletion or inhibition of GAT-1 raises levels of GABA, and this has been explored as a therapeutic possibility for anxiolytic, depressive, and epileptic disorders (Kristensen et al., 2011). Tiagabine is a GAT-1 selective inhibitor which does not alter the anticonvulsant or behavioral effects of gaboxadol, but EF1502, which has broadened selectivity for inhibiting GAT-1 and BGT-1, results in a decreased anticonvulsant effect in mice (Madsen et al., 2011). The GABA transporters may serve roles beyond recycling GABA into the axon terminal for vesicular release. Transporters also display a significant role in modulating the level of baseline tonic current in the dendritic regions of the neuron. Furthermore, neurosteroids and allosteric modulators of tonic current undergo regulation of receptor binding sensitivity due to GABA transporters (Fleming et al., 2010). Altered sodium electrochemical gradients may contribute to ambient extracellular GABA by reversing the direction of GAT-1 transport (Attwell et al., 1993; Wu et al., 2006, 2007). In addition, GABA synthesis itself is significant in extrasynaptic function, as lack of GAD65 reduces tonic inhibition (Walls et al., 2010).

Neurosteroid affinity/efficacy for extrasynaptic receptors

$GABA_A$ receptor-mediated tonic currents rely on a mechanism of control derived from channel properties and other exogenous stimuli (McCartney et al., 2007; Ransom et al., 2010). Certain neuronal regions exhibit high degrees of tonic inhibition determined by channel opening kinetics, even though tonic inhibition is observed throughout the brain. Neurosteroids have been shown to selectively potentiate $GABA_A$ receptor inhibition in a fashion independent to subunit composition, as revealed by switching the polarity of efficacy in and populations (Bianchi and Macdonald, 2003). The intrinsic low-efficacy activity of physiological -containing receptors is exemplified by brief channel openings and low levels of desensitization. The low-efficacy characteristics enable

neurosteroids to increase the mean channel open time in their potent modulation of the receptor.

Neurosteroids acting on $_4$ $_3$ receptors displays a greater efficacy than on $_4$ $_3$ $_2$ receptors (Brown et al., 2002). Mutation of residue L286S of the subunit modifies GABA$_A$ receptors to higher efficacy and channel characteristics similar to that of -containing, synaptic receptors (Zheleznova et al., 2008). Furthermore, differences in gating are expressed through altering subunit isoforms. With a saturating concentration of GABA (1mM), $_1$ $_3$ channels desensitize slowly, deactivate rapidly, and exhibit peak-amplitude voltage-dependence. In contrast, $_6$ $_3$ channels have voltage-dependent desensitization and slower deactivation but do not show rectification (Bianchi et al., 2002). Therefore, it is not necessarily the subunit composition per se that confers the neurosteroid ability to modulate tonic current, but it is the channel efficacy properties of subunit pharmacore that confers preferential targeting by endogenous neurosteroids to potentiate GABA inhibitory function. As neurosteroids interact with extrasynaptic GABA$_A$ receptors, the high-affinity binding increases gating efficacy and is manifested as potent inhibitory function.

Tonic current inhibition in the brain

Tonic current is typically quantified through whole-cell, voltage-clamp recording of a brain slice preparation. Inhibitory activity is represented in terms of conductance which is normalized by cell membrane capacitance (pS/pF). This allows for control of variability based on the membrane surface area contributing to overall capacitance. Baseline currents are initially measured and averaged from epoch increments to signify GABAergic mediated current. Saturating concentrations of GABA$_A$ receptor antagonist bicuculline or gabazine (SR-95531) are then applied to the recording chamber to identify the loss of current due to blocking of the ion channels. Extrasynaptic channels have been previously classified as relatively insensitive or resistant to gabazine due to inability to inhibit GABA$_A$ receptors that are active in the absence of GABA (Bai et al., 2001; Yeung et al., 2003; McCartney et al., 2007). However, this data was based on pyramidal cell tonic currents, which display lower levels of chloride conductance than -containing DGGCs or CGCs, and gabazine antagonism is largely concentration-dependent (Stell and Mody, 2002; Stell et al., 2003; Houston et al., 2012). Upon pharmacological antagonism, the change in current from active to blocked condition is quantified to calculate the tonic conductance for the cell. The mean tonic inhibition detected is typically several-fold greater than the mean spontaneous IPSC mediated current (Farrant and Nusser, 2005). GABA transporter and uptake blockers, such as the GAT-1 inhibitor NO-711 may maintain or increase exogenous levels of GABA and may even enhance tonic inhibition without altering phasic inhibition (Nusser and Mody, 2002). Ionotropic glutamate channel antagonists are necessary in the external perfusion solution to block glutamateric receptor currents from obfuscating the chloride current recordings.

Extracellular concentrations of extrasynaptic GABA have been estimated to be approximately 160 nM in slice preparations (Santhakumar et al., 2006), which is significantly lower than *in vivo* determinations (Lerma et al., 1986). Most slice recording preparations incorporate a low effective concentration of GABA (1-3 μM) into the perfusion solution to establish GABAergic holding current for the cell, reflecting a physiological level of ambient GABA around the neuron but not so great a concentration that would elicit receptor desensitization. However, establishing a baseline current before applying GABA and subsequent antagonist introduces a more eloquent method for observing a shift in the holding current for deciphering tonic inhibitory activity. Sanna and colleagues demonstrate this method by establishing a baseline for 5 minutes, applying GABA to induce a shift in current, and thereafter the GABAergic current is blocked by adding bicuculline (Sanna et al., 2009). Therefore, in comparing ambient GABA-activated current and neurosteroid

potentiation of the receptors, the foundational baseline current may be ascertained before the inclusion of modulatory agents (Fig.6). In effect, this allows for channel conductance and kinetics to be compared across experimental conditions before GABA or allosteric binding and before channel receptor antagonism.

There are many variables to consider during the process of slice electrophysiology that may have influential effects on the recorded neurons. Specific GABA concentrations employed, temperature, age of the neurons, chloride gradients, and oxygenation conditions of the slice preparation can all have substantial effects on recordings (Glykys and Mody, 2007; Succol et al., 2012). Hypoxia can significantly affect the health of the neuron being patched. In hypoxic conditions within the tissue, the extracellular volume is reduced, and this increases concentrations of neuroactive chemicals within the fluid, altering functionality of receptors and regulatory molecules alike. GABA transporter and reuptake molecules also contribute to fluctuations within the slice, as their activity or attenuation can significantly change the inhibitory faculty of GABAergic neurons (Chiu et al., 2005). The range of physiologically observed tonic inhibition is highly sensitive as well. The degree of $GABA_A$ receptor tonic modulation is centrally affected by the concentration of neuroactive agent applied and the $GABA_A$ receptor profile from the neuron being recorded. Membrane depolarization has recently been shown to influence tonic current $GABA_A$ receptor potentiation. Rapid increase in tonic conductance was observed following depolarization of cultured, hippocampal neurons, exhibiting voltage-dependent properties of $GABA_A$ receptors and current independent of ambient GABA or inward anion accumulation (Ransom et al., 2010). Postsynaptic, metabotropic $GABA_B$ receptors enhance extrasynaptic currents of -containing $GABA_A$ receptors in DGGCs (Tao et al., 2013).

While the pharmacological properties governing tonic and phasic current are distinct, the precise attributes that confer their divergence are not well understood. The pharmacological tools that are able to selectively modulate $GABA_A$ receptors are limited by their degree of sensitivity to the receptor subunit binding. Receptor conformation may be assessed based on molecular assays and known agonist pharmacology for a receptor profile, but as of yet there is no devised way to isolate extrasynaptic receptors (primarily of the subunit) by pharmacology alone. AA29504 is a novel positive modulator with strong subunit specificity that may possess some utility in further understanding modulation of synaptic versus extrasynaptic currents (Vardya et al., 2012). The heterogeneity of $GABA_A$ receptors remains an obstacle to accurate delineation of receptor physiology *in vivo*.

Functional characteristics of δ-containing receptors

δ Knockout physiology

The subunit knockout mouse model allows for the disambiguation of properties influencing GABAergic tonic current. Not only do -containing $GABA_A$ receptors possess distinctive pharmacology, they possess unique current-gating properties that play a role in controlling neuronal excitability through transduction of the inhibitory chloride channels. The global knockout mouse was first created and described by Mihalek and colleagues in 1999, but at that time little was known about the extent of neurosteroid interaction with -containing $GABA_A$ receptor isoforms. The construction and classification of the knockout was seminal in discerning the true functionality of neurosteroids at $GABA_A$ receptors. The initial knockout demonstrated that removal of the subunit from receptors substantially diminishes neuronal sensitivity to neurosteroids (Mihalek et al., 1999). Alphaxolone potentiation is lessened in knockouts, accompanying reduction in hippocampal levels of the $_4$ subunit (Spigelman et al., 2003). Allopregnanolone exhibits diminished dose-dependent modulation of $GABA_A$ receptor currents in DGGCs of knockout mice (Fig.7). The hypnotic activity of gaboxadol is reduced when administered to knockout mice as

quantified by behavioral tests, signifying high affinity for gaboxadol at -containing receptors (Boehm et al., 2006; Meera et al., 2011). The   knockout mice display lower behavioral impairment and reduced binding sensitivity to muscimol, the high-affinity agonist for $GABA_A$ receptors (Chandra et al., 2010). These findings provide pharmacological and behavioral evidence of $GABA_A$ receptor sensitivity based on discrete subunit composition profiles. The   specific profiles confirm shifts in channel gating due to endogenous neurosteroid modulation (Wohlfarth et al., 2002), which is attenuated in the -deficient model (Stell et al., 2003).

Knockout constructs and electrophysiology have revealed that DGGCs and CGCs have high levels of $GABA_A$ receptors incorporating   subunit into configuration (Pirker et al., 2000; Peng et al., 2002). The combination of knockout studies, electrophysiological parsing of current properties, and immunochemistry specific to DGGCs has shown that $_4$ extrasynaptic receptors comprise a majority of the tonic current observed in these cells (Sun et al., 2004; Chandra et al., 2006; Herd et al., 2008). While the $_4$ receptor composition accounts for a large portion of the observed tonic current, other compositions including $_5$ $_2$ or $_6$ combinations in CA1 or CGCs, respectively, are located extrasynaptically as well and likely contribute to tonic current (Semyanov et al., 2004; Glykys and Mody, 2007). The $_5$-containing receptors are responsible for a minor residual tonic current in DGGCs of knockout animals (Glykys et al., 2008). In addition, dentate gyrus molecular layer interneurons possess $_1$ receptors (Glykys et al., 2007). Concomitant with   knockout characteristics, $_4$ knockout mice display decreased GABAergic potentiation by alphaxolone in DGGCs (Liang et al., 2008). This mirrors physiological deficits of neurosteroid potentiation in mice receptors lacking   subunits and provides evidence for neurosteroid selectivity for certain subunit compositions to modulate the receptor function.

Mice deficient in   subunit also exhibit pathologically relevant changes in conjunction with reduced neurosteroid sensitivity. The GABA antagonist pentylenetetrazol administered to mice elicited a significantly greater number of clonic seizures in knockouts compared to control animals (Mihalek et al., 1999; Spigelman et al., 2002). This indicates a higher degree of acute seizure susceptibility marked by loss of -subunit-mediated tonic inhibition. Hippocampus kindling has not yet been implemented with the   knockout model to further typify brain excitation due to limbic epileptiform activity. Furthermore, DGGCs from knockouts demonstrate faster current decay rates of mIPSCs and evoked IPSPs, but there are no observed changes in frequency, rise time, or amplitude (Spigelman et al., 2002). DGGCs from   knockout mice have highly attenuated tonic current (Fig.8), and $_5$/   double knockout mice effectively lack any tonic inhibitory current within hippocampal neurons (Stell et al., 2003; Glykys et al., 2008, Fig.9). CA1 Pyramidal cells possess $GABA_A$ receptors with a lower amount of   subunit compared to DGGCs, (Peng et al., 2004) and the relatively low tonic conductances are not changed between wild-type and   knockout mice (Stell et al. 2003; Maguire et al., 2005). The -containing $GABA_A$ receptors have high sensitivity to Zn^{2+}, and wild-type mice DGGCs experience reduced spontaneous IPSC decay time in response to $ZnCl_2$ application. In contrast, spontaneous IPSC decay time is not affected by 10 μM $ZnCl_2$ in   knockout neurons (Saxena and Macdonald, 1996; Wei et al., 2003). Knockout mice have been reported to develop generalized, spontaneous seizures (Olsen et al., 1997). These findings hint at a reduced ability of the available $GABA_A$ receptors in the brain to control excitability, owing to an important niche role for -containing receptors.

δ Subunit expression in the hippocampus and modulation by neurosteroids

The expressional differences of   subunit between DGGCs and CA1 pyramidal cells may reveal an important key to understanding extrasynaptic receptor activity and function. The estrous cycle serves as a useful model because tonic inhibition changes are dependent on

cell-type and receptor-type specificity. Due to changes in steroid hormone levels during the estrous cycle, allopregnanolone levels and their interaction with $GABA_A$ receptors undergo plasticity, albeit differently in DGGC and CA1. This modulation is exhibited by alterations in neuron electrophysiology between estrus and diestrus. DGGC tonic conductance is increased in diestrous phase, but no cycle-dependent changes in conductance are observed in CA1 pyramidal cells (Maguire et al., 2005, Wu et al., 2013). Whole-cell, GABAergic currents from native, adult rat DGGCs are highly sensitive to positive modulation by allopregnanolone and negative modulation by PS (Mtchedlishvili et al., 2003). Tonic conductance in DGGCs is mediated by -containing $GABA_A$ receptors and is sensitive to neurosteroid, however tonic conductance in CA1 pyramidal cells is not mediated by -containing $GABA_A$ receptors and is not sensitive to neurosteroid (Stell et al., 2003). While pathophysiology dependent on -subunit receptors has yet to be confirmed, there is compelling evidence that heightened excitability and increased seizure susceptibility are prominent in conditions where -containing receptors are not present or down-regulated (Mihalek et al., 1999; Spigelman et al., 2002; Stell et al., 2003). Mutations to the   subunit structure found within human cases of epilepsy recapitulate functional changes that may increase excitability (Dibbens et al., 2004; Feng et al., 2006).There is ongoing work to understand how differences within -subunit expression modify neuroactive effects of neurosteroids.

The   knockout model remains a useful tool to develop current knowledge of how $GABA_A$ receptors interact with neurosteroids to modulate brain network functions. Moreover, neurosteroid effects on the brain may be tightly controlled through temporal mechanisms of development in addition to discrete neuronal cell types. Within the dentate gyrus of adult mice, $GABA_A$ receptors with $_4$ but not   subunits play a role in neurogenesis and early axonal/dendrite growth and migration, while $_2$ mediates late maturation of dendrites and pruning (Duveau et al., 2011). The tone of GABAergic current could therefore rely on spatiotemporal control of receptors since different isoforms influentially control neuronal maturation. A conditional knockout has yet to be devised for the   subunit and would aid in elucidating spatial and temporal receptor changes to regulate inhibitory functions.

Regulation and plasticity of subunit expression and function

Subunit expression differences within $GABA_A$ receptors are reflected by conditional changes such as development, stress, pregnancy, and ovarian/hormonal cycling and may be contextually relevant to further elucidating the function of neurosteroids on GABAergic neurons (Smith et al., 2007; Belelli et al., 2009). Studies in pathophysiology corroborate evidence that irregularly functioning states of neural networks like epilepsy involve changes in subunit expression. Many studies report compensational changes of subunit expression due to genetic, functional, or pharmacological changes to the control of GABAergic inhibition (Peng et al., 2002; Liang et al., 2008; Gangisetty and Reddy, 2010; Uusi-Oukari and Korpi, 2010; Suryanarayanan, et al., 2011; Kuver et al., 2012). Evidence for neurosteroid-dependent modulation of receptor plasticity is beginning to emerge. Therefore, in conditions where there is a substantial change in endogenous neurosteroid levels, receptor subunit reconfigurations could occur.

Initial electrophysiology studies on DGGCs in   knockout mice reported that there was no overarching change in synaptic inhibition, but an observed faster rate of decay for mIPSCs, suggesting that structural receptor modifications might occur (Mihalek et al., 1999). Molecular analysis of mRNA from   knockouts reveals a localized shift in which expression of $_2$ subunit increases and $_4$ subunit expression levels decrease in the forebrain, implicating functional compensation in response to the loss of   (Korpi et al., 2002; Peng et al., 2002). Hippocampal peptide levels of   knockouts also demonstrate that with a global

loss of   subunit, $_4$ levels are diminished, coupled to observed decreases in inhibition (Spigelman et al., 2003). In $_4$ knockout mice, $_2$ subunit levels increase within the hippocampus, further signifying a modulatory mechanism for balancing inhibition within this brain region (Liang et al, 2008).

While receptor expression data from knockout mice show signs of compensatory adaptation, the regulatory processes involved in subunit assembly and incorporation of receptors into the membrane remain to be discovered. It is possible that neurons with high extrasynaptic function depend on endogenous neurosteroids to adjust baseline inhibition. These neurons cannot rely on synaptic GABAergic transmission for inhibitory set point control due to the transient availability of synaptic GABA and the considerable distance from the synapse. However, extrasynaptic receptors are fine-tuned to allow modulation by secondary ligands that adjust channel gating. It appears that certain subunit isoforms are preferentially assembled together, and their location on neuronal membrane reflects the regulatory needs of the neuron (Bogdanov et al., 2006). Conditional changes that augment expressional regulation provide a larger understanding of functional maintenance of GABA$_A$ receptors and how neurosteroids influence this plasticity.

Stress

Stress has profound effects on the neuroendocrine release of steroids into the blood; elevations of corticosteroid promote increase in plasma and brain levels of THDOC and allopregnanolone (Purdy et al., 1991; Reddy and Kulkarni, 1996; Reddy and Rogawski, 2002; Reddy, 2006). Glucocorticoid metabolites can enhance allopregnanolone potentiation of GABA$_A$ receptors (Strömberg et al., 2005). Acute stress in animals has been shown to increase   subunit expression in conjunction with a rise in baseline levels of tonic inhibition (Maguire and Mody, 2007). This aspect of   plasticity is compounded by the fact that treatment of THDOC, released in conditions of high stress, can also increase   expression in a rapid manner (Smith et al., 2007). Following stress, THDOC induces a depolarizing shift in the GABA equilibrium potential which causes excitatory GABAergic transmission in corticotropin-releasing hormone neurons; acting on   -containing receptors, THDOC provides positive feedback for the hypothalamic-pituitary-adrenal axis response to stress (Sarkar et al., 2011). Acute swim stress in rats significantly increases plasma THDOC levels as well as the threshold of pentylenetetrazol to induce seizures (Reddy and Rogawski, 2002). Compensational adjustments have been proposed to serve as a protective mechanism for neurons to subsequent stress insults for brief, confined periods of time. In contrast, chronic stress has the effect of decreasing overall GABA receptor function which could be related to significant changes in the receptor expression profile (Serra et al., 2000). The introduction of chronic stress results in decreased global levels of neurosteroid in the system. Desensitization of neurons to neurosteroid over prolonged or constant releases of stress-activated biochemical may also possibly be reflected by GABA$_A$ receptor plasticity. Interestingly, social isolation-induced stress in rats has been found to increase extrasynaptic $_4$,   -containing receptors in the hippocampus (Serra et al., 2006). Isolation stress altered allopregnanlone sensitivity to GABA$_A$ receptor function and increased tonic inhibitory current the dentate gyrus.

Development

Formation of neural networks in the hippocampus has provided insight into receptor plasticity in the developing brain. In CA1 pyramidal neurons of embryonic and postnatal rats, a form of tonic inhibition is observed even before synapses are formed (Demarque et al., 2002). Interestingly, this mechanism of inhibition precludes the requirement of transient, synaptic GABA release to be governed by Ca^{2+} influx and GABA reuptake controlled by GAT-1. Tonic inhibition is also active at early developmental stages of the dentate gyrus and

is important in regulating the excitation/inhibition balance in the maturing hippocampus (Holter et al., 2010). Within rat hippocampus, $_4$ and subunit proliferation of expression occurs late in postnatal development and into adulthood (Laurie et al., 1992). By the time adult age is reached, synaptic and $_2$ containing receptors are already highly expressed and established within the brain. Both $_2$ and $_5$ subunits increase in the mouse hippocampus in the initial postnatal months of brain development but steady out during adulthood, whereas $_2$-subunit expression declines continuously in the adult and aging brain (Yu et al., 2006). This study implies that gene expression of $_2$ and $_5$ are induced and gene expression is down regulated due to the large changes across neural development in multiple brain regions. In DGGCs, the GABA$_A$ receptor-mediated currents have been found to increase in sensitivity to allopregnanolone as rats mature, wherein $_1$ expression also increases from birth to adulthood (Brooks-Kayal et al., 2001; Mtchedlishvili et al., 2003). There are also developmental changes from $_5$ subunits to $_1$, $_2$, and $_2$ in DGGC, linked to high zinc sensitivity of receptors, which subsequently declines and becomes channel-blocking as neurons mature (Galanopoulou, 2008b). As the brain establishes the structural and chemical foundations critical for neuronal activities, neurosteroid synthesis and modulation likely play a role in shaping function.

Traumatic brain injury

Post-traumatic epileptogenesis results from ischemic injury or mechanical forces that lead to the development of seizures. There are direct and indirect cellular pathways that are involved in controlling tissue damage triggered by traumatic brain injury (TBI), and the hippocampus is vulnerable to its adverse effects. Excitotoxicity is a well-studied change resultant from TBI, yet there are modulatory changes to brain inhibition as well. Consequent of traumatic insult, there are declining numbers of GABAergic interneurons and decreases in GABA$_A$ receptor-mediated phasic IPSC frequency, but preservation of tonic function (Pavlov et al., 2011). Within a rat model of TBI, GABA$_A$ subunits $_1$, $_3$, $_3$, and $_2$ undergo time-dependent expressional changes (Gibson et al., 2010). Another study reported a decrease in $_2$ expression to TBI, but examined a strong increase in subunit expression (Kharlamov et al., 2011). Perforant pathway lesion studies divulge a short-term decrease in -containing GABA$_A$ receptors followed by an increase in subunit and glutamate receptors 30 days post-lesion (Iwakiri et al., 2006). Functional studies reveal that TBI induces greater potentiation of DGGC tonic current through -specific application of gabaxodol, as well as decrease in mIPSC frequency (Mtchedlishvili et al., 2010). These studies suggest plasticity within hippocampal tissue for the maintenance of excitatory/inhibitory homeostatic balance.

Given that TBI results in excitotoxicity, cell death, and cognitive deficits within the hippocampus, neurosteroid modulation of GABA transmission may provide an important role in regulating the effects of trauma. Preclinical and clinical studies suggest that in the acute stage of injury, progesterone administration may limit or reduce tissue damage and can be neuroprotective of brain function in neuronal injury, in addition to reducing excitotoxicity and seizures (Sayeed and Stein, 2009). Within the dentate gyrus, treatment with progesterone after TBI reduces the increase of cell death due to injury to stabilize levels to that of control animals (Barha et al., 2011). Progesterone may influence neuronal injury directly or indirectly through metabolites (Reddy and Ramanathan, 2012). Allopregnanolone treatment has also been shown to aid in improving cognitive abilities and preventing neuronal loss post-trauma, suggesting a role for GABAergic transmission in neuroprotection (He et al., 2004). The progesterone's ability to modify injury and epileptogenesis are attributed partly to neurosteroids (Reddy et al., 2010; Reddy and Ramanathan, 2012).

Estrous cycle

Exploration into the fluctuations in steroid synthesis and circulation during the ovarian cycle has yielded critical information as to the function of neurosteroids on GABA$_A$ receptors. The ovarian cycle progresses with consequent endocrine changes in estradiol and progesterone levels. In the rodent estrous phase, progesterone levels are typically low and estrogen levels are high. During the diestrous phase, heightened levels of progesterone induce its metabolite, allopregnanolone, to increase during luteal surges within the ovary and plasma (Reddy and Kulkarni, 1999; Reddy, 2009a). GABA$_A$ receptor subunit gene expression is affected by oscillations in the ovarian cycle due to this rise in neurosteroid concentration not only in peripheral tissue, but also in the brain. This expressional response promotes gene activation and results in a change in amount of specific receptors that are functional on the neuronal membrane. During the diestrous phase, receptor protein levels of   subunit increase and $_2$ subunit decrease in the hippocampus compared to the estrous phase, and this is coupled to significant increases in GABAergic conductance in DGGCs (Maguire et al., 2005; Wu et al., 2013). Levels of $_4$ do not change significantly between these two phases of the cycle. The mirrored compensation of specific subunit isoform up-regulation in response to neurosteroid deficit in the CNS implicates a substantial system of neuromodulatory control of GABAergic inhibition. Over the course of the cycle, neither progesterone receptor antagonist RU846 nor estrogen receptor antagonist tamoxifen affect regulation of GABA$_A$ receptors in the brain, suggesting neurosteroid-dependent plasticity (Maguire and Mody, 2007). Control of GABA$_A$ receptor populations in puberty and into adulthood may be maintained by an overarching feedback mechanism involving the neuroendocrine axis balance of hormones. Therefore, developmental defects or pathophysiological onset of disorder within this system could greatly alter the excitability of the brain, such as in catamenial epilepsy (Reddy, 2013a).

Pregnancy and parturition

The subunit compositions of GABA$_A$ receptors undergo drastic changes during pregnancy. Pregnancy-related fluctuations in neurosteroids in the plasma and the brain vary with respect to subunit expression and GABAergic function. Both progesterone and deoxycorticosterone brain and plasma levels are elevated during pregnancy, which in turn increase allopregnanolone, THDOC, DHEAS, and cortisol synthesis in circulation (Biggio et al., 2001; Paoletti et al., 2006).

Expression of $_2$ subunit mRNA is decreased within the hippocampus during rat pregnancy; finasteride treatment of pregnant rats prevents a significant reduction of $_2$ expression (Concas et al., 1998). Pregnancy in rats drastically increases the DGGC and CA1 expression of   subunits, coupled with the decrease in expression of $_2$, but $_4$ expression remains static during pregnancy (Sanna et al., 2009). A postpartum expressional upregulation of $_4$ and $_2$, but decrease in   is observed in the hippocampus. Subunit $_4$ increases only following delivery in parturition; this precise adjustment to subunit composition is corroborated by a notably intensified DGGC tonic inhibition in pregnancy which is subsequently reduced after delivery. The control animals used in these experiments by Sanna and colleagues were female rats in estrus. Furthermore, the allopregnanolone-induced changes to tonic current of DGGCs in pregnancy reflect the increase in   subunit-containing receptors. A large downward shift in the holding current occurs with an application of 1 μM allopregnanolone, which is well above the physiological levels found within the brain, but it is reasonable that hormonal levels during pregnancy may drive an increase in neurosteroid concentration above normal levels. These functional and structural observations on GABA$_A$ receptors during pregnancy coincide with neurosteroid mediation of the receptors, as treatment with finasteride ablates the aforementioned effects.

In contrast, a separate study involving pregnant mice observed decreases to both and $_2$ expression and decreases in tonic and phasic inhibition as a result of pregnancy, however this study used females in diestrus as controls (Maguire and Mody, 2008). The two studies employ as their controls differing estrous cycle conditions, which, given the differing expressional levels of expression, offer varying reference points for plasticity changes that occur to GABA$_A$ receptor subunits during pregnancy. Receptor plasticity remains a complex issue in identification of changes that influence excitability. Slice neurons examined from pregnant mice experience region-specific decreases in subunit expression owing to a compensational shift of the brain to control neuronal excitability, but knockout, pregnant mice do not present any changes to network excitability (Maguire et al., 2009). In the presence of allopregnanolone, both pregnant and nonovulatory, virgin wildtype mice show reductions in network excitability in dentate gyrus neurons. The dysregulation of -containing receptor function within the hippocampus can therefore be selectively altered by the levels of available neurosteroid (Belelli et al., 2009). These studies confirm that neurosteroids have an active role in modulating tonic current inhibition, preferential to -containing, extrasynaptic receptors.

Neurosteroid treatment

Treatment of animals with steroid exemplifies subunit expression compensation and plasticity. Administration of progesterone or pregnenolone selectively and substantially increases 3 ,5 -reduced neurosteroid levels in both rodents and humans and can be measured in blood serum by high throughput methods (Porcu et al., 2011). Gonadotropin treatment increases allopregnanolone levels (Reddy et al., 2012). Progesterone and allopregnanolone treatment can up-regulate $_4$ and in the hippocampus, documented in both *in vitro* and *in vivo* functional studies (Biggio et al., 2006; Uusi-Oukari and Korpi, 2010). Up-regulation of subunit in hippocampus after allopregnanolone treatment is significant for channel kinetics regarding potentiation; both efficacy and potency to gaboxadol increase following neurosteroid administration. This direct alteration of GABA$_A$ receptor plasticity reflects subunit targeting specificity in which is involved, but it also modifies the functionality of receptors where $_4$ and $_2$ are coexpressed.

In response to neurosteroid treatment, CA1 synaptic current decay time is reduced (Hsu et al., 2003). A brief 48-hour treatment with allopregnanolone highly increases the expression profiles of the $_4$ and composition and decreases expression of the typically synaptic $_1$ and $_2$ subunits within rat CA1 (Shen et al., 2005). However, -containing receptors are not as functionally significant at CA1 as compared to the dentate gyrus subfield, where expressional and functional changes to GABA$_A$ receptors have a higher impact on network excitability. Neonatal exposure to estradiol in female rats decreases brain levels of progesterone and allopregnanolone in addition to increasing the quantity of synaptic $_1$, $_2$, and $_2$ subunits with no change to $_3$, $_4$, $_5$, or subunits (Calza et al., 2010). An increase in anxiolytic behavior results when diazepam is applied to estradiol-treated animals, but there is little change by allopregnanolone. This estradiol-modulated, increased sensitivity exhibits subunit plasticity-directed specificity to allosteric ligands concerning broader GABA$_A$ receptor gating.

Neurosteroid withdrawal

Withdrawal from neurosteroid in the body and brain has striking impact on subunit plasticity within the hippocampus. Neurosteroid withdrawal, which occurs during the perimenstrual period, is linked to catamenial epilepsy and seizure susceptibility in women (Reddy et al., 2001; Reddy, 2013). In rat hippocampal cultures, withdrawal following chronic exposure to either progesterone or allopregnanolone causes a transient increase in subunit expression (Mostallino et al., 2006). Induced neurosteroid withdrawal following chronic doses of

progesterone or allopregnanolone in rodents has shown to increase the expression of α_4 subunit approximately 3-fold (Smith et al., 1998; Gangisetty and Reddy, 2009a; 2010). Progesterone and neurosteroid withdrawal models exhibit an up-regulation in α_4 and δ subunits with effects on current, pharmacology, and seizure susceptibility (Sundstrom-Poromaa et al., 2002; Reddy et al., 2012). Due to neurosteroid withdrawal-dependent increases of α_4 and δ subunits, fully kindled, epileptogenic mice experience enhanced sensitivity to the antiseizure effects of allopregnanolone (Reddy et al., 2012). There is no observed compensational shift in α_1, α_2, β_2, or γ_2 subunits in response to withdrawal. This may represent a divergent mechanism of expression for synaptic and extrasynaptic receptors.

Increase in seizure activity and excitation of the brain has differential effects on GABA$_A$ receptor physiology of CA1 and DGGCs (Gibbs et al., 1997). Progesterone withdrawal has been linked to α_4-dependent increase in CA1 pyramidal neuron excitability (Hsu and Smith, 2003). In CA1 subfield, GABAergic inhibition is primarily mediated by other subunits (Caraiscos et al., 2004; Prenosil et al., 2006). The α_4 subunit is more prevalent in dentate gyrus than in the CA1 pyramidal region (Pirker et al., 2000) and serves as a more crucial pharmacological target for tonic current modulation in the dentate gyrus. Alterations to receptor function within the dentate gyrus have severe effects on the control of epileptiform spread of hyperactivity (Coulter and Carlson, 2007). Extrasynaptic profiles of δ-containing receptors reflect considerable plasticity to neurosteroid withdrawal that influences their channel opening probability (Bianchi and Macdonald, 2003). Phasic and tonic GABA$_A$ receptors may rely on disparate mechanisms that control expression based on neuronal requirements to maintain network inhibition. Due to the high neurosteroid sensitivity to GABA$_A$ receptors mediating tonic current within the dentate gyrus, it is probable that neurosteroids drive expressional changes to regulate baseline inhibition.

Epilepsy

Epilepsy is a disease characterized by recurrent seizures resultant from aberrant hyperexcitability within the brain. While there are many types of epileptic seizures, certain types may be studied to elucidate the nature of the disease and formulate appropriate treatments in control of seizures. Receptor plasticity has been reported in several models of epilepsy, implicated to play a role in the molecular changes that promote seizure susceptibility. Alterations to the function of GABA$_A$ receptors through a pilocarpine-induced model of temporal lobe epilepsy (TLE) reveal that CA1 neurons from epileptic animals undergo a reduced maximal efficacy (decrease of 45%) to GABA with an enhanced potency, but DGGC neurons experience an increased efficacy (increase of 89%) to GABA without a change in potency, and an increased sensitivity to zinc blockade of current (Gibbs et al., 1997). Epileptiform bursting activity promotes cellular internalization of GABA$_A$ receptors and has consequent effects on synaptic GABA transmission in reducing inhibition (Goodkin et al., 2005). Both mature and newborn dentate cells undergo reductions in spine density and number in response to induced status epilepticus (Santos et al., 2011). Hippocampal neurons possess varying GABA$_A$ receptor compositional changes in response to hyperexcitability of the brain. The foundation for greater seizure susceptibility is maintained by the markedly large decrease in available neurosteroid in the brain, altered receptor expression levels on the neuronal surface, and reduced overall GABAergic inhibition.

Catamenial epilepsy is characterized by increased seizure frequency due to menstrual cycle-related hormonal fluctuations (Reddy, 2007; 2009a; 2012). Deficits in cyclical fluctuations in steroid hormones and receptor plasticity play a central role this condition. The seizures produced by the disease are believed to result from cyclic, temporal withdrawal during a deficit of circulating neurosteroid, which in turn increases seizure susceptibility in conjunction with a dearth of GABAergic inhibitory control over excitability (Reddy, 2004,

2009a; 2013a,b). The -containing receptors are crucial mediators of tonic inhibition and limbic epileptogenesis and are regulated within the estrous cycle (Reddy et al., 2012; Wu et al., 2013). In women with perimenstrual catamenial epilepsy, overall THDOC levels and DHEAS/cortisol ratio are reduced, but the pregnane steroids progesterone, pregnenolone, and allopregnanolone are not different compared to control groups (Tuveri et al., 2008). However, in the perimenstrual part of the cycle, the luteinizing surge produces a rapid flux in progesterone and its metabolites that afterward sharply decrease (Reddy, 2009a). Parturition is also associated with a substantial decrease in progesterone and allopregnanolone levels, and there is a postpartum increase in $_4$ and $_2$ subunit expression, but decrease in within the hippocampus (Sanna et al., 2009). Thus, epilepsy may cause a different compensational mechanism of $GABA_A$ receptor plasticity within the brain compared to the physiological condition of progesterone withdrawal in order to allow for parturition to proceed.

Treatment with neurosteroids has shown to reduce seizure activity in several epilepsy models (Lawrence et al., 2010; Reddy and Jian, 2010; Reddy et al., 2010). Using a rat model for TLE, Kapur and colleagues reported that allopregnanolone sensitivity was reduced, subunit was down-regulated in expression, but tonic inhibition remained intact in tissue isolated from epileptic rats (Sun et al., 2007; Rajasekaran et al., 2010). This is an interesting result and clinically relevant, but these findings have yet to be confirmed by an independent group. Noda model epileptic rats have decreased subunit expression in DGGCs along with decreased tonic current, smaller tonic response to allopregnanolone (Pandit et al., 2013). Furthermore, in Noda rat slices, allopregnanolone prolonged synaptic receptor decay in DGGC similarly to the normal Wistar strain counterparts. Pilot studies from our lab show that allopregnanolone retains its antiepileptic efficacy in this animal model of TLE and is successful in suppressing the occurrence of spontaneous seizures in chronically epileptic rats (Reddy et al., 2005c). Better discernment of the role of neurosteroid-receptor interactions in the context of epilepsy necessitate that *in vitro* studies are corroborated with *in vivo* studies that typify the whole network response to $GABA_A$ modulation.

The physiological, intrinsic low excitability of the dentate gyrus has an important role in the filtering of excitatory activity from the entorrhinal cortex and regulating propagation of current that passes through to other hippocampal neuronal tissue (Heinemann et al., 1992, Lothman et al., 1992, Coulter and Carlson, 2007). DGGCs from TLE rats have increased synaptic efficacy, exemplified by increase in number of postsynaptic channels, but not channel conductance (Otis et al., 1994). However, these changes to miniature IPSC amplitude do not take into account possible alterations in extrasynaptic $GABA_A$ receptors. Kainate treatment to induce seizures decreases frequency of miniature IPSCs, but does not change the frequency of action potential-dependent, spontaneous IPSCs (Shao and Dudek, 2005). While IPSC recordings do not provide a comprehensive view of how epilepsy modifies GABAergic inhibition, these findings denote compensatory effects within the hippocampus to maintain inhibitory current. Observed reductions in phasic inhibition in epileptic animals agree with evidence that $_2$-containing receptors may increasingly shift to extrasynaptic sites and assemble with $_4$ subunits. DGGC receptor plasticity in epileptic mice is reported to have static $_4$ expression, down-regulation, and $_2$ increase in dendritic areas of neurons (Zhang et al, 2007).

In contrast, epileptiform DGGCs have been found to display increased inhibition of mIPSCs and altered $GABA_A$ receptor sensitivity to zinc and benzodiazepine pharmacology, also associated with increased membrane receptors (Cohen et al., 2003). There are various contrasting findings regarding changes in dentate gyrus inhibition that could be interpreted by the location of the synaptic inhibition recording (Zhang et al., 2007). Pharmacological and electrophysiological evidence of pilocarpine-induced status epilepticus also suggests

enhanced tonic, GABAergic inhibition, largely modulated by L-655,708-sensitive α_5-containing receptors in lieu of -containing receptors (Zhan and Nadler, 2009). There is still considerable uncertainty regarding how receptor physiology influences GABAergic inhibition in epileptic animals, however the rodent model of TLE provides indication of compensatory shifts in subunit composition that influences tonic current (Mody, 2012). Nevertheless, pharmacological shifts in receptor sensitivity are concomitant with changes in receptor subunit composition.

In epileptic CA1 pyramidal cells, α_5 subunit was down-regulated (Houser and Esclapez, 2003), but tonic current was increased, providing evidence that compensation of other subunit types mediates tonic current in epileptic tissue instead of α_5-containing receptors (Scimemi et al., 2005). The α_5 subunit has been implicated in modulating hippocampal excitability (Bonin et al., 2007), but the extent and degree of their inhibitory function in the context of epileptogenesis have yet to be fully explored. The down-regulation of key subunits involved in tonic inhibition demonstrates a mechanism of receptor plasticity, but it is not known why increasing excitation within the hippocampus results in dampened expression of receptors that control the excitability. Status epilepticus rats have a more positive reversal potential for IPSC than control counterparts due to an increase in NKCC1 chloride ion cotransporters (Barmashenko et al., 2011). Maintaining inhibitory tonic current through extrasynaptic receptors appears to be integral in preventing hyperexcitable states. The retention of neurosteroid ability to counteract epileptic effects within the brain illustrates the capability of neurosteroids beyond their typified GABA$_A$ receptor subunit specificity. Further insight into neurosteroid treatment of epileptic animals will yield a more precise understanding of the efficacy of neurosteroids to control seizures. Additional investigation into the bioavailability and efficacy of neurosteroids in conditions that reflect seizure susceptibility holds potential in devising therapies for epilepsy.

Mechanisms of plasticity

There is strong support that the potentiating effects of neurosteroids are incongruent with classical steroid hormone receptor interaction. Nuclear receptors do not play a role in GABA$_A$ subunit plasticity or inhibitory current properties observed during ovarian-cycle related fluctuations. Therefore, the presence of neurosteroid to drive subunit expression may act through a mechanism disparate from classical steroid gene transcription, but this mechanism is largely unknown. Conversely, it remains a possibility that neurosteroid biotransformation into steroidal precursors could genomically alter expression of subunits. While various different conditions can convey an underlying ability of neurosteroids to modulate GABA$_A$ receptor inhibition, regulation of the receptor structures and subunit assembly remains elusive. The early growth response factor-3 (Egr3) pathway has been implicated in mediating α_4 subunit up-regulation induced by neurosteroid withdrawal, and this has found to occur independently of any progesterone receptor interaction (Gangisetty and Reddy, 2010). The extent of transcription factor-based mediation of subunit regulation remains to be clarified. Expressional changes in transcriptional control of mRNA, based on Egr3 activation data, shed some light on the uncertainty of subunit regulation. Protein kinases or cellular machinery involved in protein and receptor trafficking may have an important role in the mechanistic changes observed in subunit composition as well (Jacob et al., 2008).

Sex-specific differences

Since endocrine fluctuations in plasma levels of progesterone and other steroids can mediate neurosteroid availability, there are apparent differences between males and females concerning concentrations of neurosteroids in the brain. In addition, brain development greatly differs between genders and likely contributes to neurosteroid function (Reddy,

2009b). While neurosteroids are able to shape inhibition and produce behavioral effects in both genders, regulation of neurosteroid activity may be sex-specific (Gulinello and Smith, 2003). Differences in maximal GABA$_A$ receptor potentiation are observed between male and female rats for THDOC, but not for allopregnanolone or androgenic neurosteroids (Wilson and Biscardi, 1997). Gender differences in expression of 3 -hydroxysteroid dehydrogenase are evident during puberty, but these differences subside in the brain as it matures into adulthood; sex-specific gonadal and adrenal endocrine activity have a significant effect on the ability of allopregnanolone to modify anxiolytic actions based on variations in biosynthesis of steroid hormones (Mitev et al., 2003).

In early stages of nervous system development, GABA$_A$ receptors possess excitatory functions on neurons by chloride channel-mediated depolarization, and this switches to inhibitory function as the brain matures (Cherubini et al., 1991; Rivera et al., 1999). Developmental disparities in neurosteroid activity may be exemplified by evidence that the hippocampus in newborn males undergoes a longer period of this GABA-directed excitatory function than in females (Nuñez and McCarthy, 2007; Galanopoulou 2008a). Fluctuations in intracellular chloride concentrations have been shown to modulate α_1, α_3, and δ subunit expression as well as influence tonic inhibition (Succol et al., 2012). Such modifications within the brain have implications on seizure susceptibility within the developing hippocampus and changing neuronal network environment. In addition, allopregnanolone and other neurosteroids could have differential neuroprotective effects in male and females experiencing potential excitotoxic or ischemic events that could damage neurons (Kelley et al., 2011).

Post-translational modification of δ-containing receptors

Phosphorylation

Post-translational modifications to GABA-gated receptors may possess a significant role in the allocation of functioning receptors in the membrane. GABA$_A$ receptor trafficking, assembly, and recycling often occur in concert with intracellular phosphorylation. The phosphorylation state of the receptors within the membrane could be influential in adjusting their interaction with neurosteroids. Inhibition of either protein kinase A or protein kinase C greatly reduces sensitivity of CA1 synaptic GABA$_A$ receptors to pregnenolone, but not to pentobarbitone, whereas stimulation of PKC has no effect on steroid sensitivity of CA1, but increases steroid sensitivity on DGGCs (Harney et al., 2003). Specific subunit targets of phosphorylation may have selective influence over neurosteroid sensitivity in conjunction with preferential modification of receptor conformations. PKA-directed activation produced greater spontaneous currents in $\alpha_4\beta_3$ than in $\alpha_4\beta_3\gamma_{2L}$ receptors due to greater channel open frequency, however it did not alter total tonic current (Tang et al., 2010). PKA may possibly aid in regulation of tonic currents in a GABA concentration-dependent manner when other expressional changes occur. This phosphorylation of extrasynaptic receptors reiterates the low-efficacy properties that may be adjusted to produce tonic levels of chloride ion conductance.

Furthermore, homeostatic stability of endogenous phosphatase and PKC activity may exert an influence over the sensitivity of the GABA$_A$ receptor to allopregnanolone (Brussaard and Koksma, 2003). PKC has been found to have an active role in phosphorylating residues on β_4 and β_3 subunits, but not δ. In addition, PKC-dependent phosphorylation of β_4 has a post-translational task in increasing the availability and rate of membrane insertion of β_4-containing receptors (Abramian et al., 2010). Different PKC isoforms exist for phosphorylation of GABA$_A$ receptors responsible for phasic and tonic currents, however this recent finding is not yet comprehensively confirmed across various neuronal types known to be involved in tonic inhibition (Kia et al., 2011).

Receptor trafficking, scaffolding, and clustering

GABA$_A$ receptor associated protein (GABARAP) is involved in receptor trafficking to the plasma membrane. It associates with   subunits as well as cytoskeletal microtubules. GABARAP and other similar proteins are highly available at Golgi, but are not present at synapses (Luscher et al., 2011). The over-expression of GABARAP results in transport of GABA$_A$ receptors in hippocampal neurons to the neuronal membrane (Leil et al., 2004). In addition, GABARAP mediated exocytosis of receptors onto the membrane has significance in regulating inhibitory, activity-dependent function of GABA$_A$ receptors (Vithlani et al., 2011). A recent studied has detailed   subunit types to have a direct role in targeting of receptors to extrasynaptic or synaptic sites (Wu et al., 2012).

Gephyrin is an important postsynaptic clustering protein, closely associated with $_2$- and $_2$-containing receptors. Clustering of gephyrin is essential for synaptic formation and maintenance of GABAergic receptors (Yu et al., 2007). Hippocampal immunohistochemistry reveals that colocalization of gephyrin and GAD65 with $_1$ or $_2$ is significantly higher than with $_4$ and   subunits (Sun et al., 2004). The fact that $_4$ and   subunits are not closely associated with synaptic markers suggests that there may be separate coordinated expression of structural proteins located on the dendritic spines of neurons. It is unclear what scaffolding or structural proteins are necessary in directing non-synaptic receptor associations with the membrane. The interaction of gephyrin and the $_1$ subunit modulates the accumulation of receptors at the synapse and influences amplitude of mIPSCs (Mukherjee et al., 2011). Immunostaining confirms gephyrin colocalization in high levels with $_1$-containing receptors. In $_1$ knockout mice, cerebellar gephyrin clusters are decreased, but gephyrin clusters that are colocalized with $_3$-containing receptors increase (Kralic et al., 2006). Gephyrin, in conjunction with phosphorylated residues on GABA$_A$ receptors, is important for the localization of receptors at synaptic regions and the modulation of GABAergic inhibition. Collybistin is coexpressed with gephyrin, and is important for clustering at inhibitory synapses (Luscher et al., 2011). It is unclear how neurosteroid modulation affects the trafficking and targeting of receptors to synaptic or extrasynaptic loci within neurons.

Conclusions and future directions

New questions raised by recent research on the interaction between neurosteroids and GABA$_A$ receptors provide opportunities to further explore inhibitory function in the brain. The specific physiology of extrasynaptic receptors remains to be clarified, although neurosteroid specificity provides indication of an important modulatory role in network inhibition. This modulation is observed to be highly plastic under a variety of physiological and pathophysiological conditions. More precise experimental and conditional manipulation of   subunit expression would aid in explaining compensational functions of receptor plasticity. Thus far, global knockout mice have implicated expressional compensation of receptor subunits in response to lack of a particular isoform present in neuronal membrane. Regional knockout of these receptor profiles would serve to clarify the role that the   subunit plays in tonic inhibition and compensation adjustment could be mapped to specific neural tissue. The true nature of hippocampal receptor plasticity could be attributed to specific changes in the hippocampus physiology. Temporal control of   subunit gene expression in animal models would also substantiate claims that   incorporation within GABA$_A$ receptors is a developmentally regulated function. In addition, precise changes in subunit expression at an investigator-determined instance could establish functional significance of this particular receptor isoform in epilepsy, ethanol dependence, stress, and other behavioral paradigms in which neurosteroid interaction plays a significant role.

As exploration of neurosteroid pharmacology continues, new agents that are more specific in binding to extrasynaptic GABA$_A$ receptors could help to further identify the allosteric and direct binding of neurosteroids. The current perception is that subunit incorporation involves indirect modulation by pharmacological agents at altogether separate binding sites on the GABA$_A$ receptor. The lipophilic nature of neurosteroids denotes more complex lateral binding on the receptor structure. Various physiological and pathological conditions cause plastic changes in GABA$_A$ receptors that reflect modulatory control of inhibition. Studies in epilepsy provide distinctive perspectives that suggest fine-adjustment to excitability within the hippocampus and other limbic regions. While there are a myriad of agents that operate in modulation of brain inhibition, neurosteroids possess a unique ability to alter receptors with a specificity and efficacy unlike any other endogenous compounds synthesized within the brain. The diversity of their function in various physiological conditions and diseases indicates a wide influence on regulation of neural network homeostasis.

Extrasynaptic GABA$_A$ receptors that mediate tonic inhibition are emerging as important targets for novel neurological drugs (Houston et al., 2012). During the past two decades, a number of synthetic neurosteroid analogs have been developed that target GABA$_A$ receptors. In animal models, neurosteroid analogs display pharmacological profile that is much superior to benzodiazepines. However, the success in identifying a clinically viable analog is limited. Typical limitations of synthetic analogs include short half-life, water-insolubility, and non-selective activation of GABA$_A$ receptors. The example of ganaxolone a steroid that has been undergoing clinical evaluation for epilepsy indications demonstrates that more efforts are needed to identify compounds with improved pharmacokinetics, subtype-specificity and efficacy. Further studies are required to determine if neurosteroid therapy is a useful therapeutic approach for seizure exacerbations related to endogenous neurosteroid fluctuations, such as in catamenial epilepsy and stress (Reddy and Rogawski, 2009). In the future, agents that influence the endogenous synthesis of neurosteroids, such as TSPO ligands, may find utility as an alternative to neurosteroids themselves in the treatment of insomnia, anxiety, epilepsy and other neurological conditions.

Acknowledgments

Abbreviations

3 -HSOR	3 -hydroxysteroid oxidoreductase
17PA	(3 ,5)-17-phenylandrost-16-en-3-ol
AP	allopregnanolone (3 -hydroxy-5 -pregnan-20-one)
CGC	cerebellar granule cell
CNS	central nervous system
DGGC	dentate gyrus granule cell
DHEAS	dehydroepiandrosterone sulfate
GABARAP	GABA$_A$ receptor associated protein
IPSC	inhibitory postsynaptic current
NMDA	N-methyl-D-Aspartate

P	progesterone
PS	pregnenolone sulfate
TBI	traumatic brain injury
TBPS	*tert*-butylbicyclophosphorothionate
THDOC	allotetrahydrodeoxycorticosterone (3 ,21-dihydroxy-5 -pregnan-20-one)
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol
TLE	temporal lobe epilepsy
TSPO	translocator protein

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Allopregnanolone   THDOC   Androstanediol
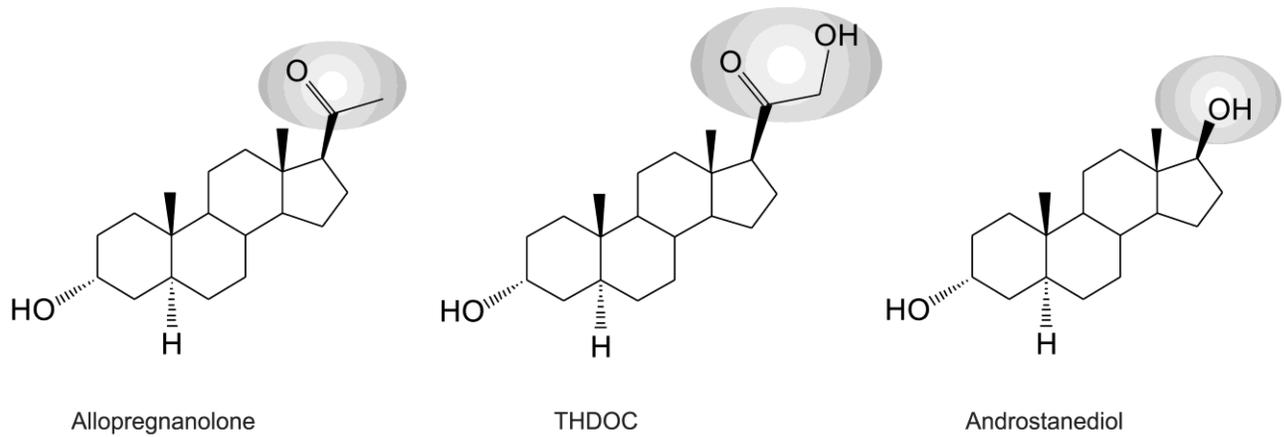
Fig. 1. Chemical structures of three prototype endogenous neurosteroids.
THDOC differs from allopregnanolone by a 21 -hydroxyl group, while androstane differs
from allopregnanolone by a 17 -hydroxyl group instead of 17 -methyl-carbonyl group.
Synthetic analogs of neurosteroids are prepared by additional moieties at C3-position
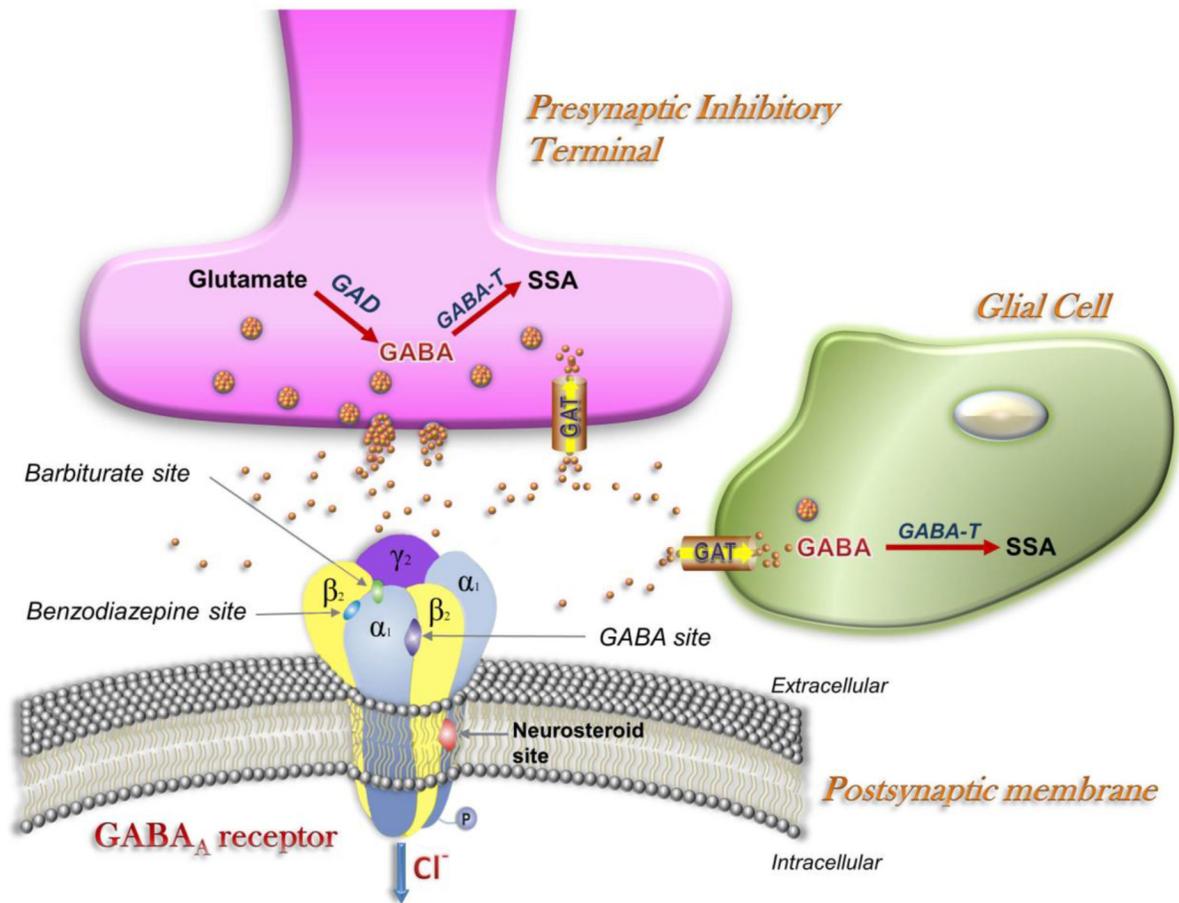(ganaxolone), C11-position (alphaxolone), and C2- and C11-positions (minaxolone).

Fig. 2. The GABAergic synapse
GABA, which is released from presynaptic vesicles, serves as the main fast inhibitory neurotransmitter in the brain by activation of postsynaptic GABA$_A$ receptors. GABA$_A$ receptors are pentameric in structure, with the five subunits arranged around a central Cl selective pore. A variety of chemical compounds are capable of acting on GABA$_A$ receptors to modulate its channel function. Apart from GABA sites, the receptor has specific sites for benzodiazepines, barbiturates, and neurosteroids, which are allosteric sites for modulation of GABA currents or Cl conductance. GABA transporters in neurons (GAT-1) and glia (GAT-2/3) remove synaptically released GABA, thereby limiting or terminating its inhibitory action. Reuptake into terminals permits immediate recycling by vesicular uptake, whereas reuptake into astrocytes leads to metabolism via GABA-transaminase (GABA-T) and succinic semialdehyde (SSA) and glutamate. Tiagabine, which is highly selective for GAT-1, inhibits GABA reuptake. Binding of neurosteroids to their binding site(s) enhances the effect of GABA by increasing the frequency and duration of channel opening.
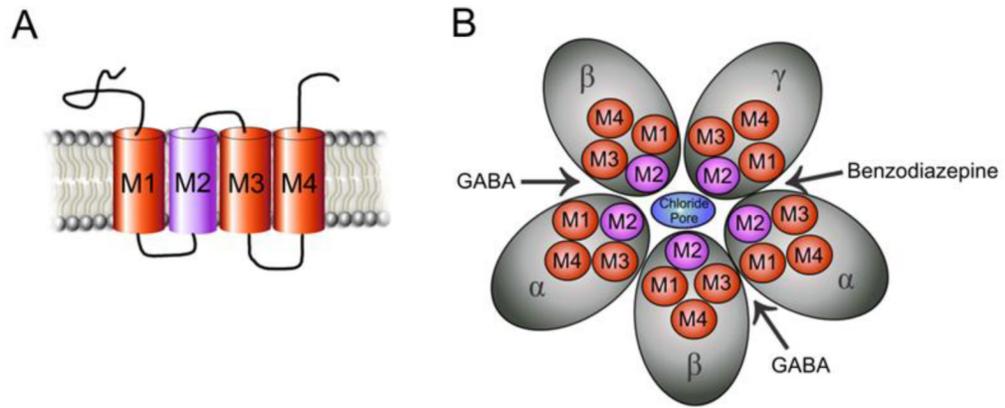
Fig. 3. GABA_A receptor structure and subunit families

GABA_A receptors are heteropentamers made up from 19 known subunits (1-6, 1-3, 1-3, , , , and 1–3) with an integral channel that is permeable to Cl^- ions. (A) A cross-section of the intramembrane structure shows the Cl^- channel pore formed by M2 helical elements. Each subunit has four transmembrane segments, with both the amino and carboxy terminal located extracellularly. (B) A top view of GABA_A receptor channel, illustrated as five cylinders arranged to form the Cl^- ionophore. The receptor is pentameric, being composed of two , two , and one subunit. The subunit may be assembled instead of . Binding of GABA in the two binding sites at the interface between and subunits open the receptor-associated Cl^- channel.
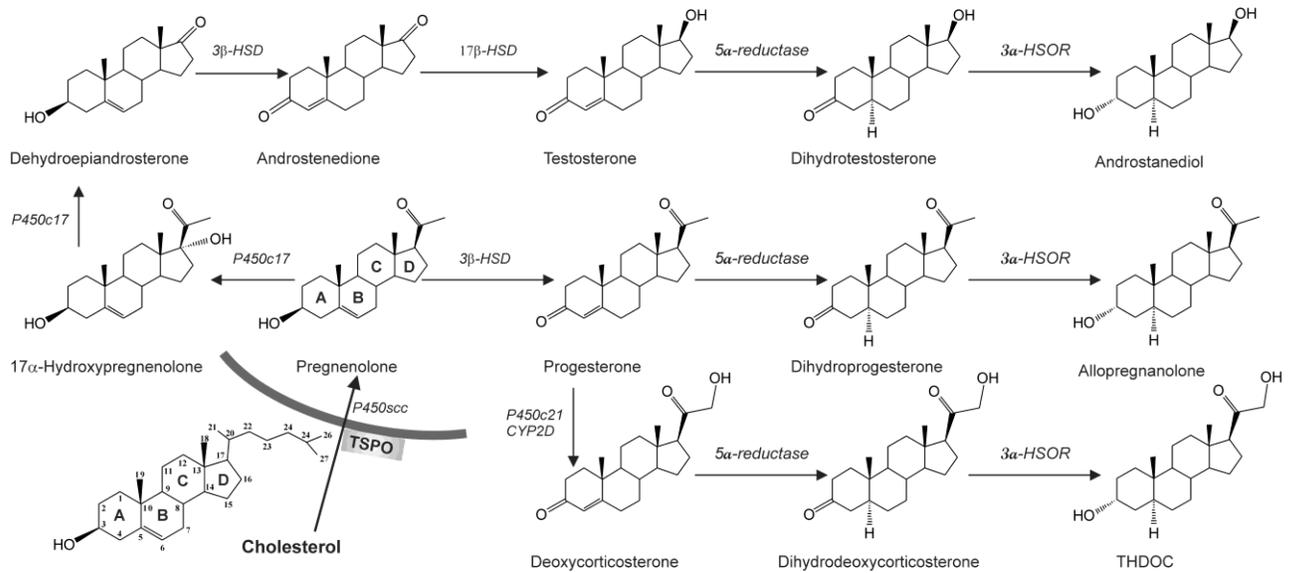
Fig. 4. Biochemical pathways of neurosteroid biosynthesis
Enzymatic pathways for the production of three prototype neurosteroids allopregnanolone, THDOC, and androstanediol are illustrated from cholesterol and intermediate steroid precursors. Cholesterol is converted to pregnenolone by P450scc in the inner mitochondrial membrane. Pregnenolone is the precursor for progesterone and other neurosteroids. Progesterone, deoxycorticosterone and testosterone undergo two sequential A-ring reduction steps catalyzed by 5 -reductase and 3 -HSOR to form the 5 , 3 -reduced neurosteroids. The conversion of progesterone, deoxycorticosterone or testosterone into neurosteroids occurs in several regions within the brain. The 5 -reductase, 3 -HSOR and other enzymes are present in the brain. Legend: P450scc, cholesterol side-chain cleavage enzyme; 3 HSD, 3 -hydroxysteroid dehydrogenase; 3 -HSOR, 3 -hydroxysteroid oxidoreductase; 17 -HSD, 17 -hydroxysteroid dehydrogenase; P450c21, cytochrome P450 21-hydroxylase.

Alphaxolone
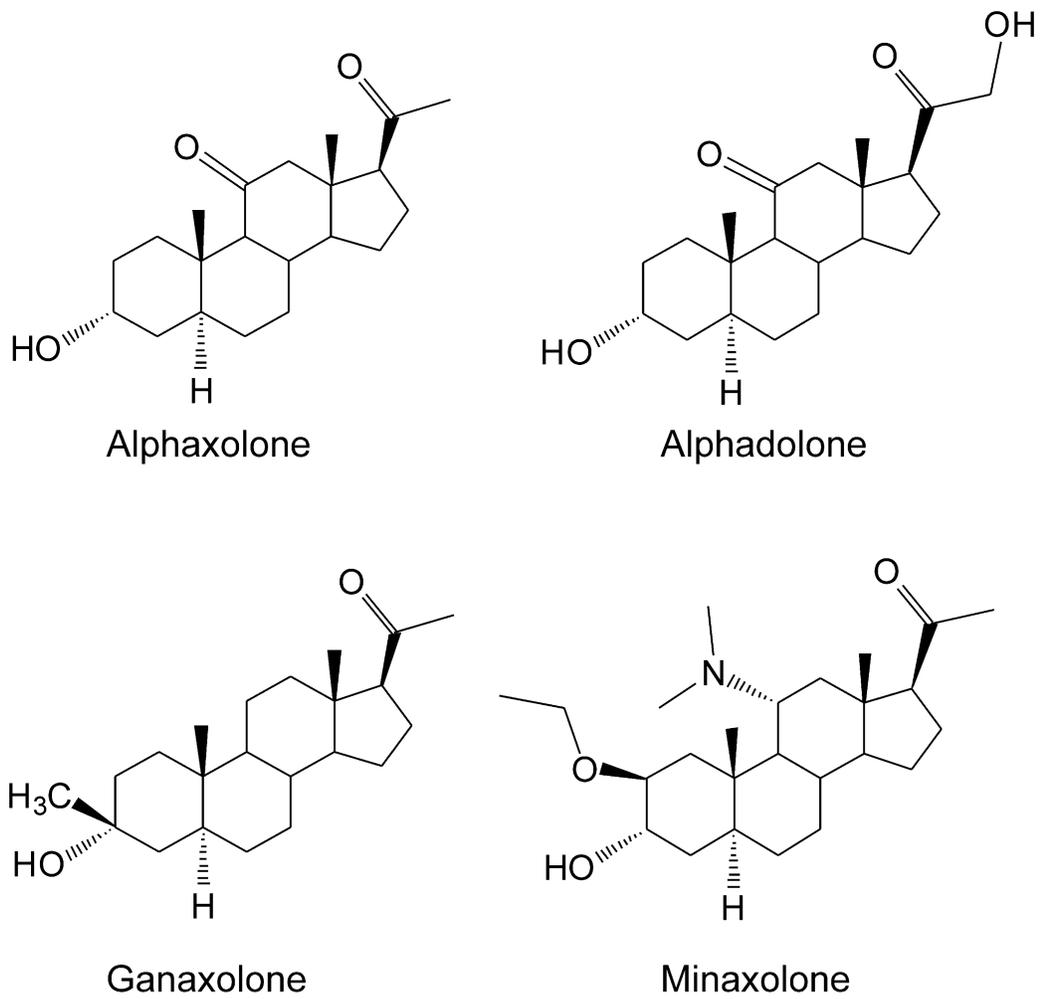
Alphadolone

Ganaxolone

Minaxolone

Fig. 5.
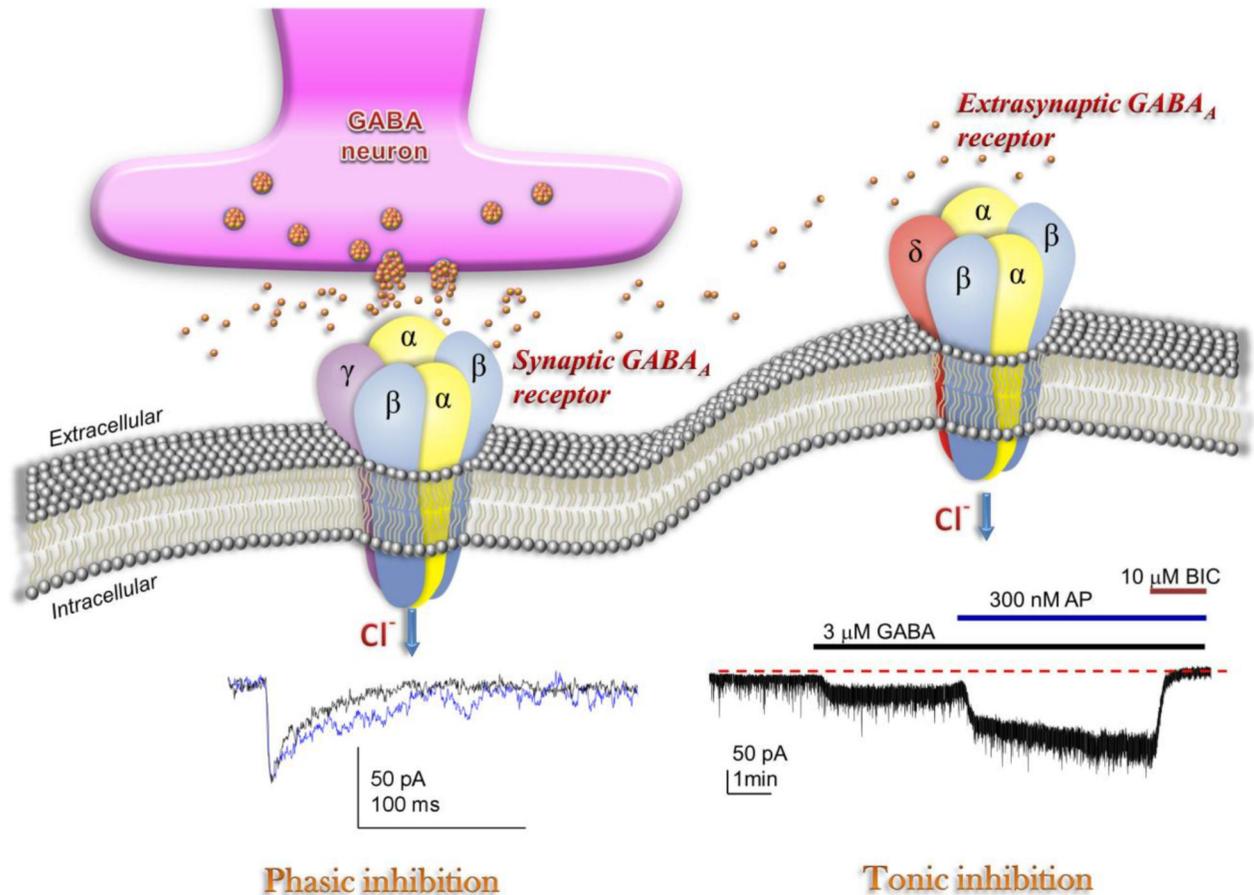Chemical structures of synthetic neurosteroids.

Fig. 6. Neurosteroid modulation of synaptic and extrasynaptic GABA$_A$ receptors
Postsynaptic GABA$_A$ receptors, which are pentameric chloride channels composed of 2 2
subunits, mediate the phasic portion of GABAergic inhibition, while extrasynaptic GABA$_A$
receptors, pentamers composed of 2 2   subunits, primarily contribute to tonic inhibition in
the dentate gyrus. Neurosteroids activate both synaptic and extrasynaptic receptors and
enhance the phasic and tonic inhibition, and thereby promote maximal net inhibition. The
trace illustrating phasic inhibition shows an IPSC produced by endogenous GABA release
(black) or in the presence of 300 nM allopregnanolone (blue). Neurosteroids enhance the
IPSCs by prolonging the deactivation/decay kinetics. The trace illustrating tonic inhibition
shows tonic conductance activated by GABA that was further enhanced by application of
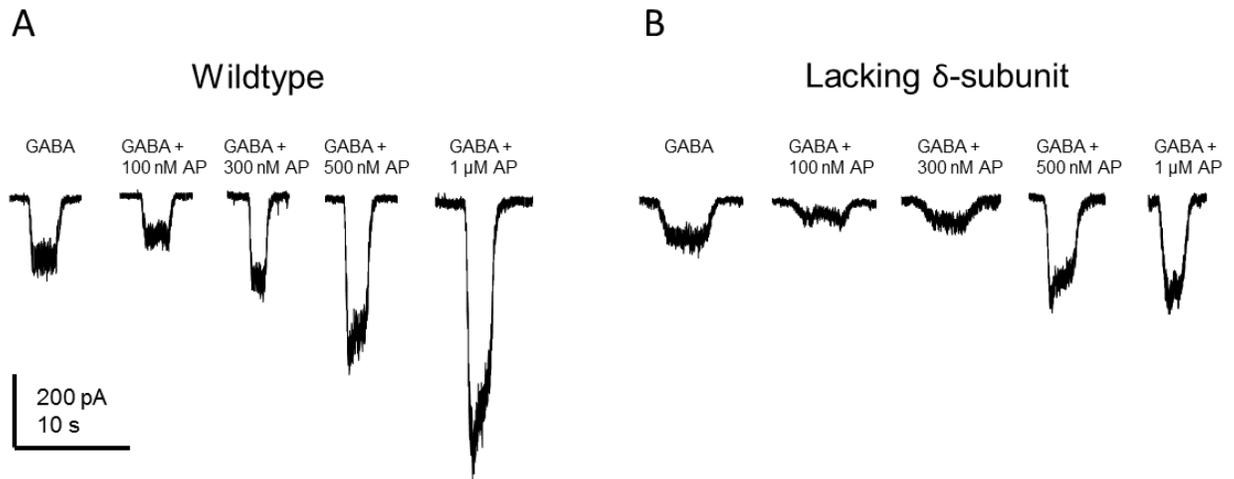allopregnanolone.

Fig. 7. Comparative efficacy of allopregnanolone on GABA-gated currents in wildtype (A) and -subunit knockout (B) mouse dentate gyrus granule cells

Concentration-dependent responses of allopregnanolone (AP) potentiation of inward chloride current in the presence of 3 μM GABA in DGGCs. Recordings were at -70 mV voltage-clamp, whole-cell patch of in acutely dissociated DGGCs. GABA was first locally applied to the bath solution for 5 seconds to establish a baseline level of gated, inward chloride current. Increasing concentrations of AP were applied for 10 seconds. After 5 seconds of application, GABA perfusion was initiated, resulting in concentration-dependent potentiation by AP. AP alone did not elicit a change in current, however, at 500 nM and 1 μM concentrations, AP application produced a slight inward current.
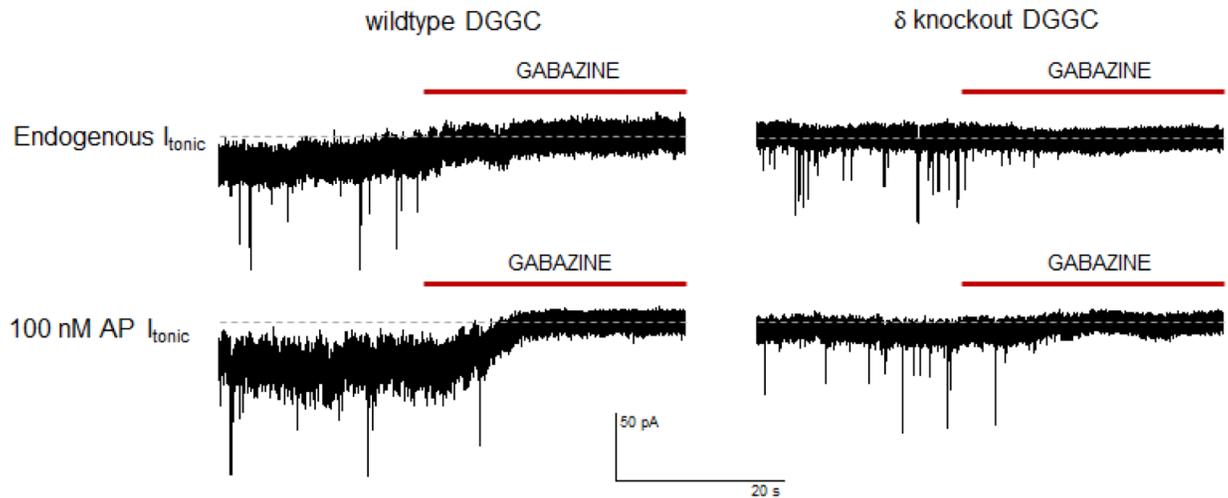
Fig. 8. Comparative analysis of allolpregnanolone potentiation of tonic currents in wildtype and -subunit knockout mouse hippocampus slices
Tonic current recordings were made from DGGCs in 300 μM slices at —65 mV voltage-clamp recording. ACSF perfusion contained tetrodotoxin, AP5 (2R-amino-5-phosphonopentanoate), and DNQX (6,7-dinitroquinoxaline-2,3-dione) to block voltage-gated Na^+ channels and glutamatergic currents to isolate GABAergic signal. No exogenous GABA or GABA transport blocker was included in this perfusion preparation. After holding current level was established in the presence of no endogenous GABAergic agent or 100 nM allopregnanolone, 50 μM gabazine (SR-95531) was applied to block all $GABA_A$ receptor currents. Root mean square noise is reduced and phasic IPSCs are blocked in the presence of gabazine in DGGCs. A noticeable shift in tonic conductance is observed in wildtype but not in knockout DGGCs. The gray dotted line indicates the mean holding level upon gabazine blockade of tonic current.
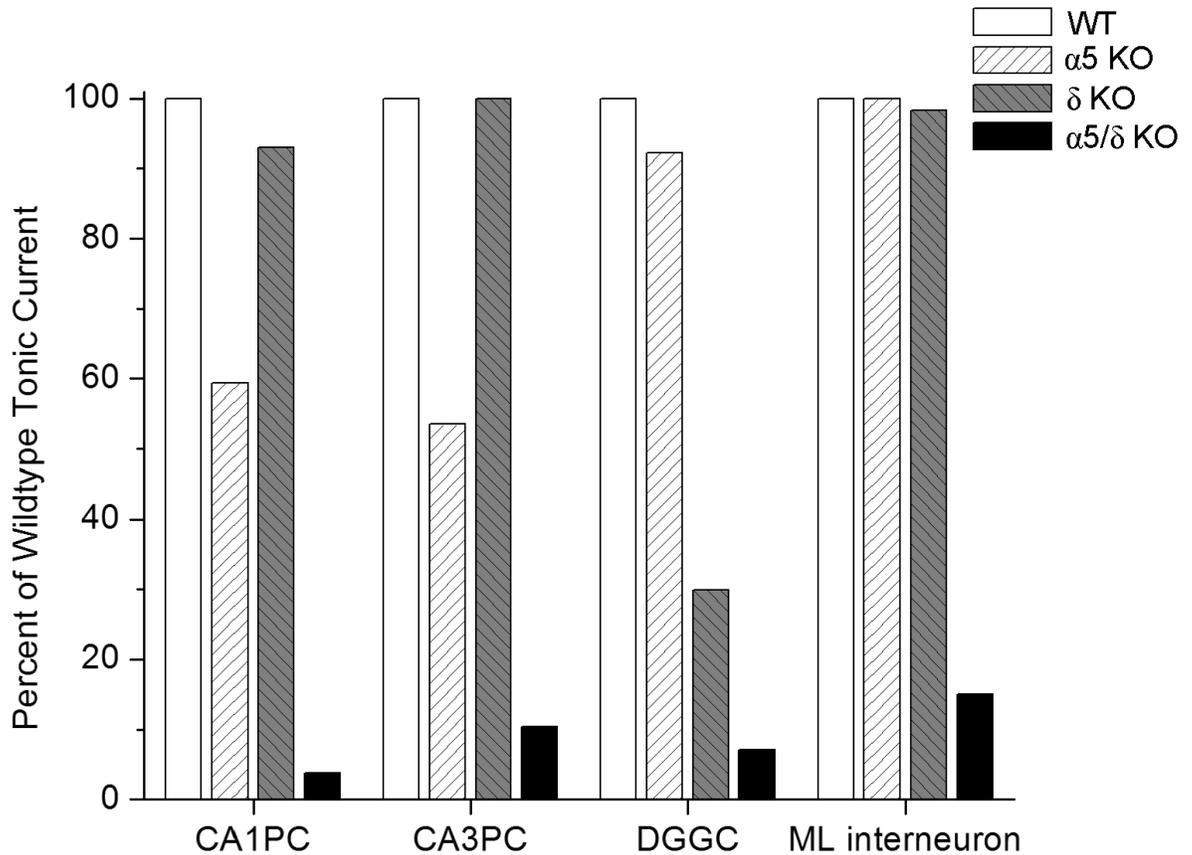
Fig 9. Relative contribution of and 5 subunits to tonic current in various hippocampus subfields as demonstrated using GABA$_A$ receptor subunit knockout mouse models
Graphical representation of relative contributions of and $_5$ subunits to tonic current in CA1 pyramidal cells (CA1PC), CA3 pyramidal cells (CA3PC), dentate gyrus granule cells (DGGC), and molecular layer interneurons (ML interneuron). Values are average percent of tonic current (pA) recorded in wildtype and knockout mouse models (Glykys et al., 2008).

TABLE 1

GABA$_A$ receptor subunit compositions and distribution in the brain.

Subunit Composition	Brain Location	GABA potency (EC$_{50}$)[*]	Specific Pharmacology Sensitivity[**]
Synaptic			
1 2 2	ubiquitous[1,3,9]	6.6 μM	Benzodiazepines[5,7]
1 3 2	ubiquitous	2.1 μM	Benzodiazepines[5]
2 3 2	hippocampus, amygdala, lateral septum, caudate putamen[1,3,9]	13.4 μM	Benzodiazepines[5]
3 2/3 2	cerebral cortex, thalamus	12.5 μM	Benzodiazepines[5]
4 2/3 2	hippocampus, thalamus[2]	2.1 μM	Furosemide, Ro15-4513[10]
6 3 2	cerebellum	0.17 μM	Furosemide, Ro15-4513[10]
Extrasynaptic			
4 2/3	dentate gyrus granule layer[8,11], hippocampus (limited)[8,11], thalamus[11]	0.91 – 1.7 μM	Furosemide[10], THIP[15]
1	dentate gyrus molecular layer[14,16], hippocampal interneurons[16]	3.7 μM	
5 3 2	cerebral cortex[12], CA1-3 hippocampus[6,12]	1.4 μM	L-655,708[6,12]
6 2/3	cerebellum granule layer[4]	0.17 μM	Furosemide[10], THIP[15]

Subunit source citations:

[1] Persohn et al., 1992;

[2] Wisden et al., 1991;

[3] Wisden et al., 1992;

[4] Korpi et al., 1995;

[5] Sieghart et al., 1995;

[6] Quirk et al., 1996;

[7] Wafford et al., 1996;

[8] Sperk et al., 1997;

[9] Pirker et al., 2000;

[10] Brown et al., 2002;

[11] Peng et al., 2002;

[12] Caraiscos et al., 2004;

[13] Pillai et al., 2004;

[14]Sun et al., 2004;

[15]Boehm et al., 2006;

[16]Glykys et al., 2007

[*] **potency data based on recombinantly expressed receptors in HEK293 cells (Mortensen et al., 2012)**

[**] **see text for pharmacology profiles**

TABLE 2

Neurosteroid and gaboxadol pharmacology for specific GABA$_A$ receptors.

Drug Interaction	Efficacy	Potency	References
Allopregnanolone & $\alpha_x\beta_1\gamma_2$	$\alpha 4 > \alpha 1 > \alpha 6 > \alpha 5 > \alpha 3 > \alpha 2$	$\alpha 3 > \alpha 1 > \alpha 2 > \alpha 6 > \alpha 5 > \alpha 4$	Belelli et al., 2002
Allopregnanolone & $\alpha_x\beta_s$	$\beta 6 > \beta 4$	$\beta 4 > \beta 6$	Brown et al., 2002; Pillai et al., 2004
THDOC	$\alpha 1\beta 2/3\gamma 2 > \alpha 5\beta 3\gamma 2$	$\alpha 5\beta 3\gamma 2 \approx \alpha 1\beta 3\gamma 2$	Caraiscos et al., 2004
Gaboxadol	$\alpha 6\beta 3\delta 2 > \alpha 1\beta 2/3\gamma 2 > \alpha 4\beta 3\delta 2 > \alpha 4\beta 3 > \alpha 6\beta 3$	$\alpha 6\beta 3\delta > \alpha 4\beta 3\delta > \alpha 6\beta 3\gamma 2 > \alpha 4\beta 3\gamma 2 > \alpha 1\beta 2/3\gamma 2$	Saarelainen et al., 2008; Mortensen et al., 2010; Meera et al., 2011

TABLE 3 Antiseizure potency (ED$_{50}$values) of neurosteroids in seizure models in mice.

Seizure Model	Allopregnanolone	THDOC	Androstanediol	References
Kindling models:				
Amygdala kindling	14 (8–23)	15 (10–30)	ND	Reddy et al., 2004
Hippocampus kindling	3.5	ND	50 (36-64)	Reddy and Jian, 2010
Electroshock models:				
Maximal electroshock model	29 (19–44)	48 (35–66)	ND	Kokate et al., 1994
6-Hz stimulation model	14 (10–19)	ND	ND	Kaminski et al., 2005
Chemoconvulsant models:				
Pentylenetetrazol	12 (10–15)	19 (77–122)	40 (27–60)	Kokate et al., 1994; Reddy, 2004ab
Bicuculline	12 (10–15)	12 (10–15)	44 (24–81)	Reddy, 2004a b
Picrotoxin	10 (5–19)	10 (5–19)	39 (21–74)	Reddy, 2004a b
N-methyl-D-aspartate	>40[**]	>40[**]	>200[**]	Kokate et al., 1994; Reddy, 2004ab
4-Aminopyridine	>40[**]	>40[**]	>200[**]	Kokate et al., 1994; Reddy, 2004ab
Status epilepticus models:				
Pilocarpine	7 (4–13)	7 (4–13)	81 (45–133)	Kokate et al., 1996; Reddy, 2004a
Kainic acid	>40[**]	>40[**]	>200[**]	Kokate et al., 1996; Reddy, 2004ab
Behavioral models:				
Sedation & ataxia	>30	>30	146 (96-226)	Kokate et al., 1994; Reddy, 2004ab
Anesthesia	30 (20-42)	>40	>200	Reddy and Apanites, 2005; Reddy and Zeng, 2007
Anxiolytic	(2-5)	(7.5-15)	ND	Reddy and Kulkarni, 2007; Reddy et al., 2005b

The potency of neurosteroids is expressed in terms of ED5, which is the dose in mg/kg producing seizure protection in 50% of animals. Values in parentheses are 95% confidence limits. ND, not determined.

[**] Considered as inactive because of such high (sedative or anesthetic) doses.