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Chapter X. Dynamic Adaptation in Neurosteroid Networks in Response to Alcohol

Deborah A. Finn^{1,2,*} and Vanessa A. Jimenez^{1,3}

¹Department of Behavioral Neuroscience, Oregon Health & Science University, Portland OR

²VA Portland Health Care System, Portland OR

³Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Portland OR

Abstract

The term neurosteroid refers to rapid membrane actions of steroid hormones and their derivatives that can modulate physiological functions and behavior via their interactions with ligand gated ion channels. This chapter will highlight recent advances pertaining to the modulatory effects of a select group of neurosteroids that are primarily potent positive allosteric modulators of γ -aminobutyric acid_A receptors (GABA_ARs). Nanomolar concentrations of neurosteroids, which occur *in vivo*, potentiate phasic and tonic forms of GABA_AR-mediated inhibition, indicating that both synaptic and extrasynaptic GABA_ARs possess sensitivity to neurosteroids and contribute to the overall ability of neurosteroids to modulate central nervous system excitability. Common effects of alcohol and neurosteroids at GABA_ARs have stimulated research on the ability of neurosteroids to modulate alcohol's acute and chronic effects. Background on neurosteroid pharmacology and biosynthetic enzymes will be provided as it relates to experimental findings. Data will be summarized on alcohol and neurosteroid interactions across neuroanatomical regions and models of intoxication, consumption, dependence, and withdrawal. Evidence supports independent regulation of neurosteroid synthesis between periphery and brain as well as across brain regions following acute alcohol administration and during withdrawal. Local mechanisms for fine tuning neuronal excitability via manipulation of neurosteroid synthesis exert predicted behavioral and electrophysiological responses on GABA_AR-mediated inhibition. Collectively, targeting neurosteroidogenesis may be a beneficial treatment strategy for alcohol use disorders.

Keywords

Allopregnanolone; GABA_A receptors; Ethanol; Consumption; Withdrawal

1. Introduction

Steroid hormones and their derivatives can influence brain function and behavior via classical genomic actions and rapid membrane effects (see Figure 1 for biosynthetic pathway). Pioneering studies of Hans Selye (1942) reported the sedative-anesthetic activity

*Corresponding author (Deborah Finn; finnd@ohsu.edu); Co-author (Vanessa Jimenez; wakeling@ohsu.edu).

of several steroidal compounds. Seminal studies by Margarethe Holzbauer and her colleagues isolated and identified many steroidal compounds from the ovarian venous blood of the rat (reviewed by Holzbauer 1976) and demonstrated the *in vivo* secretion of pregnenolone, progesterone and allopregnanolone (ALLO; 3 α ,5 α -THP or tetrahydroprogesterone) by the adrenal gland of the rat in quantities similar to those secreted by the ovary in estrus (Holzbauer et al. 1985). Then, a mechanism underlying rapid steroid actions was provided by the demonstration that the synthetic steroid alphaxalone potentiated γ -aminobutyric acid_A receptor (GABA_AR)-mediated chloride currents (Harrison and Simmonds 1984). Subsequently, evidence accumulated that alphaxalone and steroid derivatives have rapid membrane actions via an interaction with ligand gated ion channels (e.g., Belelli and Lambert 2005; Belelli et al. 1990; Paul and Purdy 1992; Purdy et al. 1990; Rupprecht and Holsboer 1999; Veleiro and Burton 2009). These findings gave rise to the terms “neuroactive steroids” and “neurosteroids” to refer to the rapid membrane actions and prompted interest in the ability of endogenous neurosteroids to modulate physiological functions and behavior (e.g., Belelli and Lambert 2005; Finn and Purdy 2007; Porcu et al. 2016; Zorunski et al. 2013).

Alcohol administration affects multiple neurotransmitter systems, and common effects of alcohol and neurosteroids at GABA_ARs have stimulated research on the ability of neurosteroids to modulate alcohol's acute and chronic effects (e.g., Finn et al. 2004, 2010; Helms et al. 2012; Morrow et al. 2001, 2006, 2009; Porcu and Morrow 2014). This chapter highlights recent advances pertaining to the modulatory effects of neurosteroids that are potent positive allosteric modulators of GABA_ARs. Background on neurosteroid pharmacology and biosynthetic enzymes is provided as it relates to experimental findings. Data are summarized on alcohol and neurosteroid interactions across neuroanatomical regions and models of alcohol intoxication, consumption, dependence, and withdrawal.

2. Neurosteroid Chemistry and Pharmacology

a. Actions on GABA_A Receptors (GABA_ARs)

The progesterone metabolites, ALLO and pregnanolone (3 α ,5 β -THP), and the deoxycorticosterone (DOC) metabolite, 3 α ,5 α -tetrahydrodeoxycorticosterone (5 α -THDOC), are the three most potent positive modulators of GABA_ARs characterized to date (Table 1), as they enhance GABA_AR-mediated inhibition with nanomolar potencies, directly activate GABA_ARs with micromolar potencies, and interact with known modulatory sites on GABA_ARs in a noncompetitive manner (Belelli and Lambert 2005; Belelli et al. 1990; Carver and Reddy 2013, 2016; Gee et al. 1988; Morrow et al. 1987; Paul and Purdy 1992; Purdy et al. 1990; Veleiro and Burton 2009). The testosterone metabolite 3 α ,5 α -androstenediol and the dehydroepiandrosterone (DHEA) metabolite 3 α ,5 α -androsterone potentiate GABA_ARs (Table 1), but with lower potency than ALLO and 5 α -THDOC (Carver and Reddy 2013, 2016; Porcu et al. 2016). Notably, the interaction of these neurosteroids with GABA_ARs is stereospecific, in that the two key features necessary for activity are a 5 α - or 5 β -reduced steroid A-ring and a 3 α -hydroxyl group. The 3 β -hydroxy analogues are devoid of activity or exhibit a partial inverse agonist profile. In addition to this structural specificity, elegant work by Hosie and colleagues determined that specific amino

acid residues within the GABA_AR α subunits are critical for neurosteroid potentiation and that distinct residues within the α - β subunit interface are important for direct activation (Hosie et al. 2006, 2009), providing unequivocal confirmation of neurosteroid binding sites on GABA_ARs. Importantly, the positive modulatory effect of neurosteroids at GABA_ARs is relatively specific, in that these steroids do not interact with any other neurotransmitter receptor in the nanomolar to low micromolar concentration range. Interactions of the pregnane neurosteroids at ionotropic nicotinic acetylcholine, serotonin type 3, *N*-methyl-D-aspartate (NMDA), and metabotropic sigma 1 receptors occur within the 10–100 μ M range (see Finn and Purdy 2007; Rupprecht and Holsboer 1999) and will not be discussed, because they are unlikely to have physiological relevance even under challenge conditions (i.e., stress or pregnancy, see section 2c).

Nanomolar concentrations of neurosteroids potentiate phasic and tonic forms of GABA_AR-mediated inhibition (e.g., Belelli and Lambert 2005; Carter and Reddy 2016; Helms et al. 2012; Herd et al. 2007; Zorumski et al. 2013), indicating that both synaptic and extrasynaptic GABA_ARs possess sensitivity to neurosteroids and contribute to the overall ability of neurosteroids to modulate central nervous system (CNS) excitability. Moreover, physiologically relevant concentrations of ALLO affect presynaptic GABA_ARs that are located on GABAergic or glutamatergic terminals to increase GABA (e.g., Park et al. 2011; also reviewed in Herd et al. 2007) or glutamate (e.g., Iwata et al. 2013) release, respectively, but it is not known whether all GABA_AR-active neurosteroids exert similar influences on presynaptic GABA and glutamate release. Thus, brain regional differences in the anatomical localization of presynaptic and postsynaptic GABA_ARs could produce mixed effects of neurosteroids on CNS excitability.

Steroids with GABA-negative actions also have been reported [e.g., pregnenolone sulfate (PS) and DHEAS as the prototypical steroids with a sulfate at C-3], with the demonstration that PS and DHEAS antagonized GABA-gated chloride uptake and conductance in a noncompetitive manner (Table 1; discussed in detail in Finn and Purdy 2007). It is interesting that sulfated and unsulfated pregnane neurosteroids (e.g., ALLO) have opposing effects on GABA_AR function (Park-Chung et al. 1999). Thus, even though sulfation of steroids is a major enzymatic reaction to facilitate steroid excretion, it also can change the pharmacological activity of steroids (Mellon and Vaudry 2001). While the presence of sulfated steroids in the mammalian brain is still a matter of controversy (Do Rego et al. 2009; Finn and Purdy 2007), it is possible that the addition and removal of the sulfate group could be a critical control point for neurosteroid modulation of neurotransmitter receptors (see Gibbs and Farb 2000).

b. Neurosteroid Synthesis and Enzyme Distribution

Most of the enzymes present in the adrenals, gonads, and placenta have been found in brain, and steroid synthesis is dependent on the tissue-, cell-, and developmentally specific expression of these enzymes (reviewed in Mellon and Vaudry 2001). As depicted in Figure 1, the first rate-limiting step in steroid synthesis is the conversion of cholesterol to pregnenolone via the P450 side chain cleavage (P450_{scc} or *CYP11A1*) enzyme upon the translocation of cholesterol from the outer to the inner mitochondrial membrane by the

chaperone proteins steroidogenic acute regulatory protein (StAR; Stocco 2000) and translocator protein 18 kDa (TSPO; formerly peripheral or mitochondrial benzodiazepine receptor; Papadopoulos et al. 2006). And evidence confirms brain regional expression of StAR and P450scc (Kimoto et al. 2001; King et al. 2002). Then, through sequential steps, pregnenolone is converted to ALLO via 3 β -hydroxysteroid dehydrogenase (HSD), 5 α -reductase, and 3 α -HSD, with progesterone and 5 α -dihydroprogesterone as intermediates (Figure 1). The reduction of progesterone, testosterone, and DOC via 5 α -reductase is another rate-limiting step for neurosteroid production (Celotti et al. 1997). Within the CNS, 5 α -reductase has been detected in neurons, astrocytes, and glia, and the predominant isoform is type 1 (see Mellon and Vaudry 2001). We found that 5 α -reductase expression is widely distributed throughout mouse brain, with highest expression in specific regions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala (Roselli et al. 2011). Agis-Balboa et al. (2006) demonstrated that 5 α -reductase and 3 α -HSD are co-localized in cortical, hippocampal, and olfactory bulb glutamatergic principal neurons and in some output neurons of the amygdala and thalamus as well as in principal GABAergic output neurons such as striatal medium spiny, reticular thalamic nucleus, and cerebellar Purkinje neurons, but not in cortical and hippocampal GABAergic interneurons. Thus, GABA_AR-active neurosteroids likely have important paracrine and autocrine effects on neuronal activity (discussed in Agis-Balboa et al. 2006).

c. Brain and Peripheral Sources

GABA_AR-active neurosteroids are synthesized from the metabolism of progesterone, DOC, testosterone, and DHEA (Figure 1; Table 1), and a number of studies have established that the enzymes identified in classic steroidogenic tissues are also found in the nervous system (see Do Rego et al. 2009; Mellon and Vaudry 2001) and are maintained in the brain after gonadectomy (GDX) and adrenalectomy (ADX) in male and female rats (Kim et al. 2003). Thus, it is generally accepted that brain neurosteroid levels reflect a combination of neuroactive compounds produced *de novo* as well as peripherally derived precursor steroids that are metabolized to neurosteroids in the brain. For this reason, it has been proposed that the definition of the term “neurosteroid” be broadened to include both sources of neuroactive steroids (Mellon and Vaudry 2001). So, we will use the term “neurosteroid” throughout this chapter.

Concentrations of the progesterone derivative ALLO, the most potent positive allosteric modulator of GABA_ARs (e.g., Belelli and Lambert 2005), are detected in brain or plasma/serum of the rat, mouse, dog, monkey and human (e.g., see Finn et al. 2004; Porcu and Morrow 2014 and references therein; also Caruso et al. 2013; Cozzoli et al. 2014; Hill et al. 2005; Jensen et al. 2017; Porcu et al. 2009, 2010; Romeo et al. 1996; Snelling et al. 2014). In addition, brain ALLO level is detectable in ADX animals and is higher than plasma level in intact animals (see Finn and Purdy 2007).

Evidence indicates that endogenous GABA_AR-active neurosteroids such as ALLO and 5 α -THDOC can reach levels that are within the range of concentrations previously shown to potentiate the *in vitro* action of GABA at GABA_ARs. ALLO and 5 α -THDOC levels fluctuate in response to acute stress in rodents, with significant increases following ambient

temperature swim, foot shock or CO₂ inhalation in male rats to the equivalent of 10 – 30 nM (Barbaccia et al. 2001; Purdy et al. 1991; Reddy and Rogawski 2002) and significant increases following restraint, tail suspension, or predator odor exposure in male and female mice to the equivalent of 10 – 20 nM (Cozzoli et al. 2014). Notably, the swim stress-induced increase in 5 α -THDOC exerted an anticonvulsant effect (Reddy and Rogawski 2002). Plasma ALLO levels also were increased significantly during PhD examination stress in males and females (Droogleever Fortuyn et al. 2004). In the female rodent, brain and plasma levels of ALLO temporally follow those of progesterone, with levels in the range of 10 – 30 nM during estrus and increasing to 100 nM during pregnancy (e.g., Concas et al. 1998; Finn and Gee 1994; Paul and Purdy 1992). Taken in conjunction with the finding that manipulation of local ALLO levels within the hippocampus and thalamus significantly altered GABA_AR-mediated inhibition (Belelli and Herd 2003; Brown et al. 2015), the results suggest that fluctuations in endogenous neurosteroid levels are physiologically relevant (e.g., Belelli and Lambert 2005).

3. Physiological Significance of Neurosteroid Fluctuations and Interaction with Alcohol

Neurosteroids that are positive modulators of GABA_ARs possess anesthetic, hypnotic, anticonvulsant, anxiolytic, antidepressant, analgesic, and amnesic effects (see reviews by Finn and Purdy 2007; Gasior et al. 1999; Porcu et al. 2016). These behavioral responses are consistent with *in vitro* evidence and suggest that GABAergic steroids modify the functioning of central GABA_ARs *in vivo*. Thus, if the findings with exogenous administration of GABA_AR-active neurosteroids are indicative of GABA_AR sensitivity to endogenous concentration, then endogenous neurosteroids may participate in the physiological control of CNS excitability. Consistent with this idea, use of a 5 α -reductase inhibitor to decrease endogenous ALLO levels was proconvulsant (Gililand-Kaufman et al. 2008) and blocked the anticonvulsant effect produced by a stress-induced increase in 5 α -THDOC levels (Reddy and Rogawski 2002).

Based on the similar pharmacological profile of GABA_AR-active neurosteroids and alcohol, there is interest in the ability of neurosteroid fluctuations to influence alcohol sensitivity. Acute administration of alcohol (1 – 2.5 g/kg) produces a steroidogenic effect, measured by a significant increase in levels of ALLO and 5 α -THDOC and their precursors in brain and plasma of rodents and in plasma ALLO levels in humans, although some conflicting results in mice and humans have been reported (see reviews by Finn et al. 2004; Kumar et al. 2009; Morrow et al. 2006; Porcu and Morrow 2014), and increases have not been detected in monkeys (reviewed in Helms et al. 2012). Notably, these alcohol-induced elevations in GABA_AR-active neurosteroids reach concentrations that enhance GABA_AR inhibition and influence several behavioral effects of alcohol (Finn et al. 2004; Kumar et al. 2009; Morrow et al. 2006; Porcu and Morrow 2014), indicating that the alcohol-induced increases in GABA_AR-active neurosteroid levels are physiologically relevant. For example, a reduction in GABA_AR-active neurosteroid levels via pretreatment with a 5 α -reductase inhibitor or prior ADX significantly reduced alcohol's anticonvulsant, sedative, amnesic, anxiolytic, antidepressant-like, and pro-aggressive effects. Moreover, alcohol's steroidogenic effect in

the rat was demonstrated in hippocampal slices *in vitro* (Sanna et al. 2004), was enhanced in animals with a chronic stress-induced decrease in endogenous ALLO levels (Serra et al. 2003), and was associated with increased StAR expression in cortex, hypothalamus, and hippocampus (Kim et al. 2003; Serra et al. 2006). Subsequent studies identified two independent mechanisms contributing to alcohol's steroidogenic effect in the rat: pituitary activation to release adrenocorticotrophic hormone (ACTH) and *de novo* adrenal StAR formation (Boyd et al. 2010a). Collectively, these data indicate that fluctuations in GABA_AR-active neurosteroids influence sensitivity to many behavioral effects of alcohol.

Neurosteroids also possess rewarding properties in rodents and monkeys. Rodents exhibit conditioned place preference to ALLO, display preference for ALLO solutions over water, and consume anxiolytic doses of ALLO (Finn et al. 1997, 2003; Sinnott et al. 2002). And one study in monkeys determined that pregnanolone functioned as a reinforcer in animals trained to administer this neurosteroid intravenously (Rowlett et al. 1999). However, in contrast to the ability of neurosteroids to contribute to several behavioral effects of alcohol, as described above, ALLO levels did not influence alcohol-induced conditioned place preference in mice (Gabriel et al. 2004; Murphy et al. 2006).

Drug discrimination procedures indicate that neurosteroids that are positive modulators of GABA_ARs have alcohol-like discriminative stimulus properties in rodents and non-human primates, whereas neurosteroids that are negative modulators of GABA_ARs do not substitute for alcohol (reviewed in Morrow et al. 2006). In female macaques, lower doses of ALLO substituted for alcohol during the luteal versus follicular phase of the menstrual cycle (Grant et al. 1997), suggesting that females have enhanced sensitivity to alcohol's subjective effects when progesterone and ALLO levels are high. In male rodents, ALLO promoted reinstatement of extinguished alcohol self-administration (Finn et al. 2008; Nie and Janak 2003), and similar results were found with ganaxolone (GAN; Ramaker et al. 2014), the 3 β -methylated analog of ALLO that has a similar pharmacological profile as ALLO but a half-life about 3 – 4 times longer when given systemically (Carter et al. 1997). And consistent with the importance of the GABAergic system in regulating alcohol consumption, systemic administration of ALLO and GAN produced dose-dependent and biphasic changes in alcohol intake in a variety of procedures in male rodents (see Morrow et al. 2006; Ramaker et al. 2015), with low doses enhancing, and higher doses reducing, alcohol self-administration. The 5 α -reductase inhibitor finasteride (FIN) also suppressed alcohol consumption in male mice via different effects on the microarchitecture of alcohol drinking than ALLO, and female mice exhibited a lower sensitivity to the modulatory effects of ALLO and FIN on alcohol drinking (reviewed in Finn et al. 2010). Interestingly, FIN reduced the subjective effects of alcohol in humans, an effect that was dependent on GABA_AR α 2 subunit genotype (Pierucci-Lagha et al. 2005), and the 5 α -reductase inhibitor dutasteride reduced alcohol consumption in male subjects classified as heavy drinkers (Covault et al. 2014). Collectively, there is a strong relationship between neurosteroid levels and alcohol consumption, subjective effects, and measures of relapse, but additional studies are necessary to better understand the genetic factors and mechanisms underlying sex differences in different species.

Following the induction of physical dependence in rodents, differences in sensitivity to the anticonvulsant effect of GABA_AR-active neurosteroids or synthetic neurosteroids have been identified (see Finn et al. 2010 and references therein; also Cagetti et al. 2004; Devaud et al. 1995). Specifically, rodents with a low withdrawal convulsive profile (e.g., rats, C57BL/6J mice, Withdrawal Seizure-Resistant (WSR) selected line) exhibit increased sensitivity to the anticonvulsant effect of ALLO and alphaxalone versus controls. In contrast, mice with a high withdrawal convulsive profile (e.g., DBA/2J mice, Withdrawal Seizure-Prone (WSP) selected line) exhibited tolerance to ALLO's anticonvulsant effect during withdrawal when compared to sensitivity in controls. Notably, these changes in sensitivity corresponded to leftward (rats) and rightward (WSP mice) shifts in functional sensitivity of GABA_ARs to ALLO, and similar behavioral results were found in males and females. These findings suggest that the plasticity of GABA_ARs during alcohol withdrawal may differ between alcohol withdrawal seizure-prone and -resistant genotypes, particularly with regard to ALLO sensitivity.

4. Alcohol and Neurosteroid Interactions Across Neuroanatomical Regions

As described above, acute stress (section 2c) and acute alcohol administration (section 3) can significantly increase GABA_AR-active neurosteroid levels and influence behavior and alcohol sensitivity. In contrast, chronic stress (e.g., social isolation) produces a consistent decrease in endogenous ALLO levels that is associated with an increase in anxiety-related behavior and contextual fear responses, decreased sensitivity to the hypnotic effects of GABA_AR-active compounds, and an enhanced steroidogenic effect to acute alcohol administration and acute stress exposure (see Biggio et al. 2014; Finn and Purdy 2007; Pibiri et al. 2008; Serra et al. 2003). Thus, the period of exposure to stress may produce opposite effects on endogenous neurosteroid levels and subsequent physiological responses.

Endogenous neurosteroid levels influence the rebound neuronal hyperexcitability seen during withdrawal from an hypnotic alcohol dose (i.e., acute withdrawal response). Specifically, ADX/GDX to decrease endogenous neurosteroid levels increased acute withdrawal-induced convulsive behavior, which was reversed by replacement with GABA_AR-active steroid precursors and metabolism to GABAergic neurosteroids (Kaufman et al. 2010). Additionally, chronic alcohol exposure and withdrawal is associated with a decrease in GABA_AR inhibition mediated by a variety of factors that includes a reduction in the steroidogenic effect of acute alcohol administration (Boyd et al. 2010b), functional changes in GABA_AR properties, and a decrease in endogenous ALLO levels (see Finn et al. 2004; Kumar et al. 2009). In rodents, monkeys, and humans, withdrawal decreases ALLO levels in plasma and several brain regions (Beattie et al. 2017; Cagetti et al. 2004; Hill et al. 2005; Jensen et al. 2017; Maldonado-Devincci et al. 2014; Romeo et al. 1996; Snelling et al. 2014). For example, a withdrawal-induced decrease in hippocampal ALLO levels was associated with a significant increase in anxiety and impairment in hippocampal-dependent memory function in male rats (Cagetti et al. 2003, 2004). In small cohorts of male and female alcoholics, the decrease in ALLO and 5 α -THDOC levels corresponded to an increase in the subjective ratings of anxiety and depression during days 4 – 5 of withdrawal, versus controls (Hill et al. 2005; Romeo et al. 1996). Furthermore, an examination of the pattern of changes in several GABA_AR-active neurosteroid levels in mouse brain and plasma

during withdrawal revealed a broad and complex dysregulation in neurosteroid biosynthesis (Jensen et al. 2017; Snelling et al. 2014). The brain versus plasma differences in the withdrawal-induced changes is consistent with the findings that basal neurosteroid levels in plasma do not simply reflect levels in cortex and hippocampus (Caruso et al. 2013), and argues for independent regulation of neurosteroid synthesis in periphery and brain during withdrawal. Thus, chronic alcohol withdrawal produces a consistent reduction in endogenous ALLO levels and dysregulation in neurosteroid synthesis that may be associated with increased cellular excitability and increased aversive behavioral effects (e.g., anxiety, depression, convulsive activity).

a. Hypothalamic-Pituitary-Adrenal (HPA) Axis Stress Circuit

Acute stress stimulates the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN), ACTH from the pituitary, and glucocorticoids from the adrenal (cortisol in primates and corticosterone in rodents). And as mentioned in section 2c, acute stressors also increase levels of the GABA_AR-active neurosteroids ALLO and 5 α -THDOC (see Finn and Purdy 2007). Administration of ALLO reduced anxiety that was induced by CRH (Patchev et al. 1994) in addition to exerting actions within the hypothalamus to dampen the activity of the HPA axis (Patchev et al. 1994, 1996). These data demonstrate that GABAergic neurosteroids participate in the activity of the HPA axis.

Acute alcohol stimulates the HPA axis (e.g., Lee et al. 2004; Ogilvie et al. 1997; Rivier and Lee 1996) and synthesis of neurosteroids (see section 3). With repeated alcohol exposure, both the HPA axis and neurosteroid synthesis show tolerance (Boyd et al. 2010b; Richardson et al. 2008). In cynomolgus macaques, disinhibition of the PVN (following naloxone, a μ -opioid receptor antagonist), but not stimulation of the pituitary (ovine-CRH) or adrenal gland (exogenous ACTH), increased pregnenolone levels (see Figure 1; Porcu et al. 2006), indicating the PVN plays a role in regulation of neurosteroid synthesis. Interestingly, DOC secretion was increased following pituitary stimulation (ovine-CRH), but not disinhibition of the PVN (naloxone; Jimenez et al. 2017), suggesting possible differential regulation of neurosteroid precursors by activation of the HPA axis. Regulation of DOC by the HPA axis was altered during the induction of alcohol drinking using schedule-induced polydipsia, where the response to ovine-CRH was blunted and the response to naloxone was potentiated (Jimenez et al. 2017), hinting that an additive effect of schedule-induced stress and alcohol consumption may influence the relationship between the HPA axis and neurosteroid synthesis. And, *de novo* synthesis has been demonstrated following alcohol exposure in the PVN (Figure 2). Acute alcohol administration (2 g/kg) significantly increased ALLO immunohistochemistry (IHC) in rats, and this effect was independent of the adrenal glands (Cook et al. 2014a, 2014b).

Although alcohol interacts with several components of the HPA axis, the increase in HPA axis activity relies on activation of the PVN (Lee et al. 2004). The majority of synaptic connections within the PVN are GABAergic and glutamatergic (Miklós and Kovács 2002; van den Pol et al. 1990). Tonic inhibition of the PVN likely occurs via glutamatergic forebrain afferents that increase GABA release in the PVN or activation of the PVN via

inhibition by upstream GABAergic projection neurons (Figure 2; Cullinan et al. 2008). Thus, GABA_AR-active neurosteroids are particularly well suited to modulate activity in the PVN, since physiological concentrations of ALLO (10–100 nM) inhibit the output of PVN neurons (i.e., CRH release) via a potentiation of GABA_ARs (Gunn et al. 2011). This ability of GABA_AR-active neurosteroids to inhibit CRH release could contribute to a termination of the stress response.

b. Extra-Hypothalamic Stress Circuit

Amygdala—The amygdala contributes to fear- and anxiety-like behavior as well as HPA axis activity. Its role in alcohol use disorders (AUDs) is rapidly gaining attention (see Gilpin et al. 2015). The amygdala plays a pivotal role in the assessment of and response to danger, with connections to the cortex and locus coeruleus and projections to the striatum, hypothalamus, midbrain, and brainstem (simplified circuit in Figure 2; see Gilpin et al. 2015 for details on amygdala microcircuitry and projection neurons). Microinfusion of ALLO into the central nucleus of the amygdala (CeA) decreased anxiety-like behavior in rodents (Akwa et al. 1999; Engin and Treit 2007). Electrophysiologically, ALLO's effect on evoked GABA_AR-mediated currents appeared to depend on network activity and involved NMDA-mediated currents (Wang et al. 2007). Recent imaging results indicate that administration of pregnenolone, which increased ALLO levels, was associated with reduced activity in the amygdala, increased activity in the dorsal medial prefrontal cortex (mPFC), enhanced connectivity between the two regions, and less self-reported anxiety (Sripada et al. 2013). These data suggest that ALLO modulates emotion neurocircuits.

In response to an acute alcohol injection (2 g/kg), ALLO IHC within the CeA was significantly decreased in rats, independent of ADX (Cook et al. 2014a, 2014b). Similarly, mice exposed to chronic intermittent alcohol (CIE) had reduced ALLO in the CeA at 8-h but not 72-h withdrawal when compared to controls (Maldonado-Devincci et al. 2014). In male cynomolgus monkeys that had been consuming alcohol daily for over 12 months, there was a significant decrease in plasma ALLO levels and in ALLO IHC in the lateral and basolateral amygdala versus controls (Beattie et al. 2017). A significant negative correlation between ALLO IHC in the lateral and basolateral amygdala and average daily ethanol consumption suggested that long-term high alcohol consumption dampens ALLO IHC. Collectively, alcohol exposure reveals a consistent reduction in endogenous ALLO levels in the amygdala (Figure 2) that may be associated with increased cellular excitability and high prior alcohol consumption or exposure.

Bed nucleus of the stria terminalis (BNST)—The BNST is important for fear- and anxiety-like behavior, serves as a relay from the amygdala, cortex and hippocampus to the PVN (Lebow and Chen 2016), and has a well-established role in AUDs (Kash 2012). Both the BNST and CeA have a high density of GABA_ARs, and GABA is the predominant co-transmitter in CRH⁺ neurons in these brain regions (Partridge et al. 2016). ALLO IHC increased in the BNST of rats following acute alcohol administration (Figure 2), and this effect was independent of adrenal sources (Cook et al. 2014a, 2014b). In contrast, withdrawal from CIE exposure did not alter ALLO IHC in the BNST in mice (Figure 2; Maldonado-Devincci et al. 2014). As noted above, the response to acute alcohol and to CIE

both resulted in a decrease in ALLO IHC within the CeA, suggesting that the effect of acute and chronic alcohol exposure may be regionally-specific.

c. Mesocorticolimbic Circuit

As recently reviewed (Koob and Volkow 2010), drug addiction can be comprised of binge/intoxication, withdrawal/negative affect, and craving stages that recruit different neuroanatomical regions within the mesocorticolimbic circuit. An excellent review of synaptic and extra-synaptic GABA_AR isoforms important in the mesocorticolimbic reward circuitry also is available (Stephens et al. 2017). States of reward and aversion are encoded by the activity of GABAergic medium spiny neurons (MSNs) in the nucleus accumbens (NAc; see Stephens et al. 2017), which receives glutamatergic inputs from hippocampus, amygdala, and cortical areas. The receipt of important limbic information from the amygdala, frontal cortex and hippocampus is integrated in the NAc and converted to motivational action through outputs via the direct striato-nigral and the indirect striato-pallidal pathways (see Koob and Volkow 2010; Stephens et al. 2017). The location of both synaptic and extrasynaptic GABA_AR isoforms throughout this circuitry suggests the possibility of spatially controlled regulation of GABA_AR function by neurosteroids and alcohol in motivational and withdrawal effects.

Nucleus accumbens (NAc)—Approximately 97% of NAc neurons (principal neurons, MSNs, and interneurons) utilize the neurotransmitter GABA, and infusion of GABA_AR agonists or antagonists into the NAc shell (e.g., Eiler and June 2007; Hyytiä and Koob 1995; Stratford and Wirtshafter 2011) as well as viral knockdown of GABA_AR δ or $\alpha 4$ subunits (Nie et al. 2011; Rewal et al. 2009, 2012), significantly decreased alcohol intake in rodents. Intra-NAc shell administration of the synthetic neurosteroid GAN also significantly decreased alcohol intake, an effect that was similar to that observed following intracerebroventricular administration of GAN or ALLO (Ford et al. 2007; Ramaker et al. 2015). Taken in conjunction with the finding that intra-NAc ALLO substituted for the discriminative stimulus effects of systemic alcohol (Hodge et al. 2001), these results provide evidence for the sufficiency of GABA_ARs in the NAc to influence alcohol's subjective, motivational, and consummatory effects (see also section 3).

Acute administration of alcohol reduced ALLO IHC in the NAc core-shell border (Figure 2), an effect that was independent of peripheral sources (Cook et al. 2014a, 2014b). Withdrawal (72-h) from CIE also decreased ALLO IHC in the NAc core (Figure 2; Maldonado-Devincci et al. 2014), suggesting that subregion differences may exist in the regulation of neurosteroid synthesis.

Ventral tegmental area (VTA)—Microinjection of a viral vector to overexpress P450_{ssc} significantly increased ALLO IHC in the VTA and decreased alcohol self-administration (Cook et al. 2014c). ALLO was localized in neurons, primarily in all tyrosine hydroxylase positive neurons, which could reduce activity in cells that project to the NAc, mPFC, or lateral habenula (Cook et al. 2014c). Acute alcohol injection did not alter ALLO levels (Cook et al. 2014b), but CIE produced a persistent reduction in ALLO IHC in the VTA (Figure 2) at 8-h and 72-h withdrawal (Maldonado-Devincci et al. 2014). Microinjection of

ALLO produced an anticonvulsant effect in naïve mice that was reduced during alcohol withdrawal in mice with a high withdrawal phenotype, and microinjection of FIN (5 α -reductase inhibitor) during the induction of physical dependence (to determine the effect of a decrease in local ALLO levels on the expression of withdrawal) enhanced alcohol withdrawal severity (Tanchuck et al. 2013). Microinjections in the withdrawal studies were localized to the posterior VTA, which also projects to the SN compacta and connects the striatum to the output nuclei of the basal ganglia via the indirect pathway (discussed in Tanchuck et al. 2013). Collectively, the results suggest that manipulation of GABA_AR-active neurosteroid levels in the VTA influences alcohol self-administration and convulsive activity during withdrawal.

Substantia nigra reticulata (SNR)—The SNR is important in the propagation of convulsive activity, as it is one of two major output nuclei of the basal ganglia, with GABAergic projections to superior colliculus, brainstem nuclei, and thalamus (see Tanchuck et al. 2013). ALLO infusion into SNR exerted a potent anticonvulsant effect in naïve mice, at lower doses than observed following microinjection into the VTA (Tanchuck et al. 2013). It is possible that the greater sensitivity to ALLO's anticonvulsant effect in the SNR than in the VTA reflects direct versus indirect effects, respectively, on GABA_AR-mediated output of the basal ganglia. Similar to what was observed in the VTA, there was a diminished anticonvulsant effect during alcohol withdrawal in mice with a high withdrawal phenotype (Tanchuck et al. 2013). Microinjection of FIN during the induction of physical dependence did not influence alcohol withdrawal severity, but produced a delayed proconvulsant effect in naïve mice (Tanchuck et al. 2013). Overall, these results provide support for the sufficiency of the SNR in mediating the anticonvulsant effect of ALLO in naïve mice and the behavioral tolerance to ALLO's anticonvulsant effect during withdrawal in mice with a high withdrawal phenotype.

Hippocampus—Acute alcohol administration increased ALLO IHC in the hippocampal CA1 pyramidal cell layer and dentate gyrus (DG) polymorphic layer (Figure 2), which was independent of the adrenals (Cook et al. 2014a, 2014b). In contrast, withdrawal from repeated CIE did not alter ALLO labeling in either of these subregions, but there was an increase in ALLO IHC in the CA3 pyramidal layer (Maldonado-Devincci et al. 2014). Studies with dissected hippocampal tissue reported decreased ALLO levels during withdrawal in rats (Figure 2; Cagetti et al. 2004) and divergent changes in several GABA_AR-active neurosteroid levels during withdrawal in mice that were unrelated to a convulsive phenotype (Jensen et al. 2017). However, microinjection of ALLO into CA1 produced a potent anticonvulsant effect in WSP mice (Gililand-Kaufman et al. 2008), with brain regional differences in sensitivity to the anticonvulsant effect in alcohol naïve mice (CA1 SNR > VTA). In contrast, infusion of FIN into CA1 was proconvulsant (Gililand-Kaufman et al. 2008). These behavioral findings demonstrate that bi-directional manipulation of hippocampal ALLO levels produce opposite behavioral consequences that are consistent with alterations in GABA_AR inhibitory tone in naïve mice. During withdrawal, WSP mice were tolerant to the anticonvulsant effect of intra-CA1 ALLO, consistent with results following systemic injection (Finn et al. 2006), and intra-CA1 FIN during the development of physical dependence significantly increased alcohol withdrawal severity (Gililand-

Kaufman et al. 2008). Thus, alcohol withdrawal rendered WSP mice less sensitive to ALLO's anticonvulsant effect and more sensitive to FIN's proconvulsant effect, suggesting an alteration in the sensitivity of hippocampal GABA_ARs in response to fluctuations in GABA_AR-active neurosteroids during withdrawal. Collectively, the microinjection results provide support for the sufficiency of the CA1 in mediating the anticonvulsant effect of ALLO in naïve mice and the behavioral tolerance to ALLO's anticonvulsant effect during withdrawal in WSP mice.

Medial prefrontal cortex (mPFC)—The ability of an acute alcohol injection to increase ALLO levels in dissected mPFC of male rats has been well-documented (Figure 2; see reviews by Morrow et al. 2006; Porcu and Morrow 2014). More recent work confirmed an alcohol-induced elevation in ALLO IHC in mPFC (Cook et al. 2014a, 2014b), but determined that the increase was dependent on the adrenal glands (Cook et al. 2014a). This result differs from the independent regulation of neurosteroid synthesis after acute alcohol in other brain regions. In contrast, there was a sustained decrease in ALLO IHC in mPFC at 8-h and 72-h of withdrawal after CIE exposure (Figure 2; Maldonado-Devincci et al. 2014). Measurement of several GABA_AR-active neurosteroid levels in dissected tissue determined that cortical levels of ALLO and other GABA_AR-active neurosteroids were decreased at 8-h withdrawal only in mice with a low withdrawal convulsive phenotype (Jensen et al. 2017). Levels of cortical GABA_AR-active neurosteroids were unchanged or increased in mice with a high withdrawal convulsive phenotype, which contrasts with the suppression in plasma ALLO levels during withdrawal in these genotypes (Jensen et al. 2017; Snelling et al. 2014) and argues for independent adrenal versus brain regional regulation of neurosteroid synthesis.

5. Conclusions

GABA_AR-active neurosteroids exert behavioral and physiological responses that are consistent with their ability to enhance GABA_AR-mediated inhibition. Microinjection and electrophysiological studies provide evidence for brain regional differences in sensitivity, which may reflect differences in GABA_AR subunit composition or local synthesis and metabolism. Elegant studies indicate that locally produced neurosteroids in thalamocortical neurons enhanced GABA_AR-mediated inhibition (Brown et al. 2015) and that differences in neurosteroid metabolism in hippocampal DG versus CA1 produced predicted effects on GABAergic transmission (i.e., increased GABA_AR-mediated inhibition with increased neurosteroid level; Belelli and Herd 2003). Consistent with the idea that brain regional differences in neurosteroid synthesis and metabolism can influence effects of alcohol, use of a viral vector to overexpress P450scc in the VTA, but not in the NAc, significantly increased ALLO levels and decreased alcohol selfadministration (Cook et al. 2014c). Moreover, physiological concentrations of ALLO inhibit the output of PVN neurons via a potentiation of GABA_ARs (Gunn et al. 2011), representing another mechanism to terminate the stress response via an inhibition of CRH release. Thus, local brain regional mechanisms to fine tune neuronal excitability exist, and additional studies will be important to further understand the physiological significance of these brain regional differences (see Figure 2 for simplified circuitry). Then, strategies based on pharmacological agents or gene therapy

tools that can increase neurosteroid levels directly in discrete brain regions may represent a promising area of research.

Acute and chronic alcohol administration and withdrawal produced species and brain regional differences in neurosteroid levels (Figure 2), with many brain regional effects being independent of the adrenals, providing evidence for independent regulation between periphery and brain as well as across brain regions. The inverse relationship between levels of some neurosteroid enzymes and GABA (Do Rego et al. 2009) also may contribute to brain regional differences in regulation of neurosteroid synthesis as well as to sensitivity of GABA_ARs to neurosteroids, since potentiation of GABA_ARs with nanomolar concentrations of neurosteroids requires GABA. Additionally, the ability of alcohol to increase spontaneous and evoked GABA release in brain regions such as cerebellum, VTA, SN and amygdala but not in cortex, lateral septum, and thalamus (reviewed in Kelm et al. 2011) may indirectly influence brain regional differences in neurosteroid levels and GABA_AR sensitivity to neurosteroids. Finally, use of GAN, which has a similar pharmacological profile to ALLO but is resistant to oxidation at C-3, can help to distinguish whether reduced behavioral sensitivity to ALLO is due to enhanced metabolism or altered sensitivity of GABA_ARs to neurosteroids. For instance, WSP mice exhibit tolerance to the anticonvulsant effect of ALLO and GAN during alcohol withdrawal, consistent with a decrease in functional sensitivity of GABA_ARs during withdrawal (Finn et al. 2006; Nipper et al. 2017). In contrast, DBA/2J mice exhibited sensitivity to the anticonvulsant effect of GAN but not ALLO during alcohol withdrawal (Finn et al. 2000; Nipper et al. 2017), suggesting that a withdrawal-induced change in ALLO metabolism may play a larger role than decreased sensitivity of GABA_ARs to neurosteroids *per se*. As another example, sex differences in C57BL/6J mice in the ability of ALLO to decrease alcohol consumption may be due in part to enhanced ALLO metabolism in female mice, given that GAN was equally effective at decreasing alcohol intake in both male and female mice and that ALLO exerted a decrease in alcohol intake in female mice when the oxidation at C-3 was blocked (DA Finn & MM Ford, unpublished). Collectively, additional studies are necessary to further understand the interaction between alcohol's acute and chronic effects on neurosteroid levels and GABA_AR sensitivity to provide insight on whether pharmacological strategies targeting neurosteroid synthesis or using synthetic neurosteroid compounds that are resistant to metabolism (i.e., GAN) will be effective treatment approaches for AUD.

Administration of ALLO and GAN doses produce a fairly consistent suppression in alcohol consumption and self-administration (see section 3), and in conjunction with the pharmacological properties of GABA_AR-active neurosteroids (e.g., anxiolytic, anticonvulsant, antidepressant; see section 3), it has been hypothesized that elevations in neurosteroid levels may protect against the risk for alcohol dependence (see Morrow et al. 2006). Alcohol dependence and withdrawal are associated with a decrease in GABA_AR inhibition that is mediated by many factors, one of which is a fairly consistent reduction in endogenous ALLO levels (Figure 2) that is accompanied by increased anxiety in rodents and increased ratings of anxiety and depression in humans (see section 4). Given that altered neurosteroid synthesis or neurosteroid levels have been reported in patients with several mood disorders (see Finn and Purdy 2007; Porcu et al. 2016; Zorumski et al. 2013), it is possible that patients with comorbid AUD and mood disorders also exhibit a dysregulation

in neurosteroid synthesis and that this suppression in neurosteroid levels contributes to the withdrawal/negative affect stage of addiction. One strategy to reduce relapse risk would be to offset the potential negative affective state with a synthetic neurosteroid such as GAN, which is in clinical trials for treatment of various forms of depression and epilepsy (clinicaltrials.gov). Genetic diversity in enzyme levels also should be considered, given the finding that individuals with the minor C-allele of the *SRD5A1* gene, which encodes the enzyme 5 α -reductase-1, expressed both a higher ratio of dihydrotestosterone to testosterone and a decreased risk for alcohol dependence (Milivojevic et al. 2011), suggesting that a heightened level of GABA_AR-active neurosteroid production may be protective against the development of dependence. Certainly, alleles that decrease enzyme function and the biosynthesis of GABA_AR-active neurosteroids could exacerbate the risk of dependence. Thus, genetic differences in neurosteroid enzyme levels and biosynthesis are an important consideration for future studies examining the therapeutic potential of targeting neurosteroid biosynthesis.

Collectively, neurosteroids are extremely potent positive modulators of GABA_ARs that can exhibit exquisite neuroanatomical control of GABA_AR-mediated inhibition and physiological and behavioral responses. Additional research is necessary to better understand the physiological significance of the complex interactions with alcohol's acute and chronic effects across brain regions and in the periphery. Future studies also should determine the therapeutic potential of strategies to enhance neurosteroid synthesis or to administer synthetic neurosteroids for the treatment of AUD.

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Figure 1. Biosynthetic pathway of neurosteroids

Parent steroids and neurosteroids described in this chapter are depicted. Colored arrows refer to distinct enzymes, which are identified in the figure inset. Neurosteroids that are potent positive allosteric modulators of GABA_A receptors are highlighted with orange, with the most potent shown in bold text. Neurosteroids that are negative modulators of GABA_A receptors are highlighted with blue. This figure was modified from (Mellon and Vaudry 2001; Porcu et al. 2009; Snelling et al. 2014). ACTH: adrenocorticotrophic hormone; ALLO: allopregnanolone; CYP: cytochrome P450; DHEA: dehydroepiandrosterone; DOC: deoxycorticosterone; HSD: hydroxysteroid dehydrogenase; StAR: steroidogenic acute regulatory protein; THDOC: tetrahydrodeoxycorticosterone; THP: tetrahydroprogesterone

Figure 2. Simplified stress (blue) and mesocorticolimbic (yellow) circuitry and summary of the effects of acute and repeated alcohol administration and withdrawal

Glutamatergic, GABAergic, and dopaminergic projections are indicated by green, red, and blue arrows, respectively, in this simplified representation of the neuroanatomical regions described in the chapter. The black arrow from the paraventricular nucleus of the hypothalamus (PVN) to the adrenal cortex reflects a streamlined depiction of the hypothalamic-pituitary-adrenal axis. The tables in the figure summarize the overall effect(s) of acute and repeated alcohol administration and withdrawal on allopregnanolone (ALLO) levels, with increases (↑), decreases (↓), no change (=), or unknown (?) shown. Mixed results of chronic alcohol administration and withdrawal on hippocampal ALLO levels have been reported. An alcohol-induced increase in ALLO levels would enhance GABA_A receptor-mediated inhibition, whereas a decrease in ALLO levels would produce the opposite effect. BNST: bed nucleus of the stria terminalis; NAc: nucleus accumbens; mPFC: medial prefrontal cortex; PVN: paraventricular nucleus of the hypothalamus; SNR = substantia nigra reticulata; VTA: ventral tegmental area

Table 1**Neurosteroids and Actions on GABA_A Receptors**

Neurosteroid	GABA _A Receptor Action	Parent Steroid
ALLO (allopregnanolone; 3 α ,5 α -tetrahydroprogesterone; 3 α ,5 α -THP)	Positive allosteric agonist	Progesterone
Pregnanolone (3 α ,5 β -THP)	Positive allosteric agonist	Progesterone
5 α -THDOC (3 α ,5 α -tetrahydrodeoxycorticosterone)	Positive allosteric agonist	DOC (deoxycorticosterone)
3 α ,5 α -androstenediol	Positive allosteric agonist	Testosterone
3 α ,5 α -androsterone	Positive allosteric agonist	DHEA (dehydroepiandrosterone)
PS (pregnenolone sulfate)	Noncompetitive antagonist	Pregnenolone
DHEAS (dehydroepiandrosterone sulfate)	Noncompetitive antagonist	DHEA

Neurosteroids that are positive allosteric agonists enhance GABA_A receptor (GABA_AR)-mediated inhibition with nanomolar potencies, directly activate GABA_ARs with micromolar potencies, and interact with known modulatory sites on GABA_ARs in a noncompetitive manner. ALLO, pregnanolone, and 5 α -THDOC are the three most potent positive modulators of GABA_ARs characterized to date. The addition of a sulfate group at C-3 (e.g., to pregnenolone and DHEA) produces neurosteroids that antagonize GABA_AR-mediated inhibition in a noncompetitive manner, so these neurosteroids are noncompetitive antagonists.