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Neurosteroid regulation of CNS development

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Abstract

Neurosteroids are a relatively new class of neuroactive compounds, brought to prominence in the past two decades. Despite knowing of their presence in the nervous system of various species for over twenty years and knowing of their functions as $GABA_A$ and NMDA ligands, new and unexpected functions of these compounds are continuously being identified. Absence or reduced concentrations of neurosteroids during development and in adults may be associated with neurodevelopmental, psychiatric, or behavioral disorders. Treatment with physiologic or pharmacologic concentrations of these compounds may also promote neurogenesis, neuronal survival, myelination, increased memory, and reduced neurotoxicity. This review highlights what is currently known about the neurodevelopmental functions and mechanisms of action of four distinct neurosteroids – pregnenolone, progesterone, allopregnanolone and dehydroepiandrosterone.

Keywords

Pregnenolone; Progesterone; Allopregnanolone; Dehydroepiandrosterone; neurogenesis; neurotoxicity; neurodegeneration; Niemann Pick Type C

1. Introduction

The first steroids identified in large concentrations in the rat brain were dehydroepiandrosterone and its sulfated ester (Corpechot et al., 1981). Shortly after this first publication, the immediate precursors of DHEA and DHEAS, pregnenolone and pregnenolone sulfate, were also identified in rat brains (Corpechot et al., 1983). The brain concentrations of all of these steroids were far greater than those found in the circulation. Furthermore, their concentrations in brain remained high after adrenalectomy and orchiectomy, suggesting that these steroids did not originate from steroidogenic tissue but rather that they originated through local brain synthesis. Hence, the concept of endogenous steroid synthesis in the brain, *neurosteroidogenesis*, was formed. Since that time, the function for these compounds began to be uncovered, and the mechanisms and receptors through which these compounds mediated their action also began to be studied (Baulieu et al., 2001; Belelli and Lambert, 2005; Compagnone and Mellon, 2000; Costa and Paul, 1991; Mensah-Nyagan et al., 1999; Puia et al., 2003; Rupprecht, 2003; Rupprecht and Holsboer, 1999a) and functions for these compounds in human diseases was also proposed (Backstrom et al., 2003; Barbaccia, 2004; Belelli et al., 2006; Bernardi et al., 2004; Brinton and Wang, 2006; Campbell, 2006; Charalampopoulos et al., 2006; Guarneri et al., 2003; Matsumoto et al., 2005; Mellon and

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Griffin, 2002; Morrow et al., 2001; Reddy, 2002; Rogawski and Reddy, 2002; Rupprecht and Holsboer, 1999b; Stoffel-Wagner, 2003; Uzunova et al., 2006). These references are merely a partial list of review articles written about neurosteroids and their functions, indicating the breadth of research on this topic. Among their multitude of functions, the various neurosteroids are modulatory ligands for a variety of neurotransmitter and nuclear steroid hormone receptors. As they are not unique or requisite ligands for many of these receptors, their functions as endogenous ligands for these receptors may be redundant with other endogenous ligands. Hence, loss of a particular neurosteroid may not result in devastating neurological consequences. However, as these compounds may be modulatory ligands for a variety of membrane and intracellular receptors, as well as for other as yet unidentified receptors, they may play a different type of modulatory role during development and in the adult. Furthermore, treatment of animal or human model systems with these compounds may reveal additional, and unexpected, functions for these compounds. This review highlights the novel functions and mechanisms of action of four neurosteroids, pregnenolone, progesterone, allopregnanolone and dehydroepiandrosterone, and their potential roles in the development and maintenance of the nervous system.

2. Pregnenolone and pregnenolone sulfate

2.1 General overview

Two of the original neurosteroids identified in brains of rats was pregnenolone and pregnenolone sulfate (Corpechot et al., 1983). In those studies, it was noted that concentrations of pregnenolone exceeded those of pregnenolone sulfate, and brain concentrations of both of these steroids were greater than that found in the circulation, even after removal of steroidogenic organs. More recent data have suggested that the original identification of pregnenolone sulfate in rodent brains by radioimmunoassay or by gas chromatography/mass spectrometry was in error as a consequence of indirect methods of measure. Direct methods such as high performance liquid chromatography-electrospray ionization tandem mass spectrometry, capillary column HPLC-nanoelectrospray ionization MS/MS and ELISAs for the quantification of sulfated steroids have failed to demonstrate the existence of pregnenolone sulfate in the rodent brain (Higashi et al., 2003a; Higashi et al., 2001; Higashi et al., 2003b; Liere et al., 2000; Liere et al., 2004; Liu et al., 2003). However, the presence of pregnenolone sulfate in human brain samples has been confirmed (Weill-Engerer et al., 2002).

2.2 Action at ligand-gated ion-channel receptors

Studies identifying receptors that are activated by pregnenolone or pregnenolone sulfate have used pregnenolone sulfate. Pregnenolone sulfate acts at many different ligand-gated ion-channel receptors. It is a negative modulator of $GABA_A$ receptors (Majewska et al., 1985; Majewska and Schwartz, 1987; Rabow et al., 1995), kainate and AMPA receptors (Mameli et al., 2005; Shirakawa et al., 2005; Spivak et al., 2004; Wu and Chen, 1997), and it is a positive modulator of NMDA receptors (Bowlby, 1993; Horak et al., 2004; Irwin et al., 1994; Park-Chung et al., 1997; Wu et al., 1991). Hence, pregnenolone and pregnenolone sulfate are excitatory neurosteroids. At NMDA receptors, pregnenolone sulfate mediates its effects at a site distinct from the glycine modulatory site (Wu et al., 1991). Pregnenolone sulfate has been shown to augment responses from recombinant NMDA receptors in heterologous cells. The NR1 subunit can combine with NR2A, NR2B, NR2C, or NR2D subunits to form functional NMDA receptors. Pregnenolone sulfate potentiates NMDA-, glutamate-, and glycine-induced currents of NR1/NR2A and NR1/NR2B receptors (Yaghoubi et al., 1998) and is inhibitory at NR1/NR2C and NR1/NR2D receptors. (Malayev et al., 2002). NR2 subunit expression differs regionally and during development (Laurie et al., 1997; Monyer et al., 1994). Thus, pregnenolone sulfate may have different effects in different regions and at different times during development. The modulatory effects of pregnenolone sulfate on dose-response curves

for NMDA, glutamate, and glycine are consistent with a model in which pregnenolone sulfate either stabilizes or destabilizes the active state of the receptor, depending upon which NR2 subunit is present. (Malayev et al., 2002).

In addition to pregnenolone sulfate activating NMDA receptors, other pregn-5-ene steroids also potentiate NMDA responses (Irwin et al., 1994; Park-Chung et al., 1997). Reduction of the C21 ketone or C17 hydroxylation reduces activity at NMDA receptors, and removal of the C19 side chain to yield dehydroepiandrosterone sulfate reduces activity even further. Reduction of the C5,6 double bond, C7 hydroxylation/oxidation, C11 hydroxylation, or replacement of C3 sulfate with glucuronate, but not hemisuccinate, reduces activity further. Dicarboxylic acid hemiesters of various lengths can substitute for the sulfate group of pregnenolone sulfate (Weaver et al., 2000). Hence, the nature of or requirement for a charged group at C3 is not clear. However, reduction of the charged residue at C3 results in ablation of activity (Park-Chung et al., 1997). Studies using pregnenolone, in which a hydroxyl group is at C3, have not been reported. Compounds that act as positive or negative modulators of the NMDA receptor likely mediate these effects through distinct sites, since their effects are not altered by increased concentrations of one another (Park-Chung et al., 1997).

Pregnenolone sulfate may mediate its actions at NMDA receptors through decreasing agonist unbinding and by affecting deactivation kinetics and macroscopic desensitization of NMDA responses (Bowlby, 1993; Ceccon et al., 2001). NMDA receptor function is modulated by several endogenous molecules, including zinc, polyamines, and protons. Whereas zinc and polyamines exert their modulatory effects by increasing or relieving tonic proton inhibition, the effect of pregnenolone sulfate is independent of the proton sensor. One steroid modulatory domain, called SMD1, on the NMDA receptor NR2B subunit, may be critical both for pregnenolone sulfate enhancement and for proton sensitivity. This domain includes the J/K helices in the S2 region of the glutamate recognition site and the fourth membrane transmembrane region, and may contribute residues to a hydrophobic pocket enabling binding of pregnenolone sulfate (Jang et al., 2004). In addition to mediating effects at NMDA receptors, pregnenolone sulfate also reduces the magnitude of GABA and glycine currents (Majewska and Schwartz, 1987; Mienville and Vicini, 1989; Wu et al., 1990) and inhibits voltage-activated Ca^{+2} channels (ffrench-Mullen and Spence, 1991; Spence et al., 1991).

Pregnenolone sulfate also induces long-term potentiation of post-synaptic currents mediated by AMPA receptors (Mameli et al., 2005). Using cell-patch clamp analysis of CA1 pyramidal neurons, investigators found that a brief, 5 minute exposure to pregnenolone sulfate induced long-term potentiation in hippocampal cultures from postnatal day 3-5 rats, but not from postnatal day 6 rats. Other experiments showed that pregnenolone sulfate induced a transient increase in glutamate release at the presynaptic level that then triggered long term potentiation of postsynaptic AMPA receptor function. These latter post-synaptic effects were mediated by NMDA receptors containing NR2B subunits. The presynaptic mechanism of pregnenolone sulfate action involves an elevation in intracellular calcium which is mediated by NMDA receptors containing NR2D subunits, whose expression may also be regulated postnatally. Pregnenolone/sulfate produced endogenously in hippocampal neurons may also mediate similar effects. Depolarization of hippocampal slices resulted in long-lasting increases of miniature excitatory postsynaptic current frequencies; this effect was seen only in hippocampal sections from postnatal day 3-4, but not postnatal day 6-10 rats. Furthermore, preincubation of slice cultures with an antibody against pregnenolone sulfate blocked this effect, suggesting that locally produced pregnenolone sulfate released after depolarization could be released from hippocampal neurons to affect the maturation of synapses.

Do pregnenolone or pregnenolone sulfate act as endogenous ligands of NMDA receptors? The highest concentrations of pregnenolone and pregnenolone sulfate in the central nervous system

are less than 1 μM (Corpechot et al., 1983; Lanthier and Patwardhan, 1986), the concentration at which neuromodulation is observed. It is possible that these neuromodulatory concentrations are obtained locally at sites of pregnenolone sulfate synthesis (Compagnone et al., 1995a; Hu et al., 1987; Jung-Testas et al., 1989), and may be increased, for example, after stress (Barbaccia et al., 1996; Purdy et al., 1991).

P450scc, the enzyme that converts cholesterol into pregnenolone, is found in rodent brains early in development (Compagnone et al., 1995a; Hammer et al., 2004). However, NMDA receptor expression peaks late in development (Monyer et al., 1994). Hence, endogenous synthesis of pregnenolone and pregnenolone sulfate may affect NMDA, $GABA_A$, kainate and AMPA receptor function late in development (reviewed in Herlenius and Lagercrantz, 2004; Takai et al., 2003). $GABA_A$ receptors are found during development and in the early neonatal period (Beattie and Siegel, 1993; Cobas et al., 1991; Liu et al., 1997; Maric et al., 2001; Tobet et al., 1999) and the subunit expression changes over time and region (Laurie et al., 1992b).

2.3 Functions of pregnenolone/pregnenolone sulfate in vivo

Various functions for pregnenolone sulfate modulating nervous system function have been proposed, based upon extrapolation from mechanistic data as well as from *in vivo* animal and human studies. Behavioral studies have shown that pregnenolone sulfate increases the convulsant potency of NMDA (Maione et al., 1992) and enhances memory retention in mice (Flood et al., 1992; Flood et al., 1995) and memory performance in the rat when injected directly into the nucleus basalis magnocellularis (Mayo et al., 1993). Pregnenolone sulfate also prevents NMDA receptor antagonist-induced deficits in a passive avoidance memory task (Mathis et al., 1994) and antagonizes dizocilpine-induced amnesia in rats (Cheney et al., 1995). The necessity of a sulfated moiety is suggested by studies in which steroid sulfatase activity is chemically inhibited. In that study, chronic inhibition of steroid sulfatase activity by estrone sulfamate enhanced passive avoidance memory (Li et al., 1997; Li et al., 1995). The studies injecting pregnenolone sulfate and inhibiting steroid sulfatase activity suggest that the behavior of animals correlates with positive modulation of NMDA receptor function.

To determine whether pregnenolone or pregnenolone sulfate has crucial functions in nervous system development, endogenous production of pregnenolone needs to be ablated. This was achieved in P450scc knockout mice (Hu et al., 2002). Remarkably, the homozygous knockout mice were born without embryonic lethality suggesting that P450scc is not essential for survival of mouse embryos. However, most homozygous knockout null mice died within 1–2 days after birth and few survived to 7 days, due to a lack of glucocorticoids and mineralocorticoids. During development, the fetal concentration of corticosterone was normal, probably due to the supply of maternal corticosteroids through the placenta. While corticosterone concentrations were normal, it is unknown if steroid precursors such as pregnenolone were similarly normal. In addition, late in development (E18.5), ACTH was elevated, suggesting that the maternal steroid supply of corticosterone may have been insufficient to inhibit fetal ACTH production. Furthermore, maternal supply of androgens or its precursors were also insufficient to yield masculinizing concentrations of testosterone, as XY mice are phenotypically female, have external female genitalia, and their internal genitalia are underdeveloped, due to androgen deficiency. Brain development was not noted as abnormal, and behavioral abnormalities were also not noted. Hence, these results suggest that pregnenolone may not be crucial for nervous system development or for nervous system function after birth.

Pregnenolone may have functions unrelated to acting at $GABA_A$ or NMDA or other neurotransmitter receptors. Baulieu's group reported a high affinity (Kd in the nanomolar range), low capacity pregnenolone binding protein in rodent fetal and adult brains (Fontaine-Lenoir et al., 2006; Murakami et al., 2000). This protein was identified as microtubule-associated protein 2 (MAP2). Pregnenolone binding to MAP2 was shown to be saturable, high

capacity, and increased by association with tubulin. Pregnenolone sulfate, by contrast, does not bind to microtubules. Progesterone, however, could bind to microtubules with an affinity similar to that of pregnenolone, but did not increase MAP2 binding to tubulin. Tubule polymerization was also induced by pregnenolone in neuronal cultures, suggesting that pregnenolone may control microtubule formation and stabilization in neurons. Pregnenolone and a 3 methoxy derivative of pregnenolone also increased neurite growth in PC12 cells treated with NGF, consistent with an increased formation and stability of tubules. Since the association of pregnenolone or 3methoxy pregnenolone with MAP2 was found in both fetal and adult rat brains, the results therefore suggested that pregnenolone may affect both neuronal development and plasticity of neurons during aging.

3. Progesterone

3.1 General overview

Progesterone is synthesized from pregnenolone by 3ß hydroxysteroid dehydrogenase (3ßHSD). This enzyme is found in neurons and in glia rat brains (Carre et al., 2001; Guennoun et al., 1995; Ibanez et al., 2003; Kohchi et al., 1998; Ukena et al., 1999b). While this enzyme is found from birth to adulthood, whole brain expression of 3ßHSD mRNA decreases throughout life. Studies demonstrating expression of this enzyme in the hippocampus show similar declines throughout life. Consistent with this decline in 3ß HSD mRNA, concentrations of hippocampal pregnenolone and progesterone are highest on the day of birth and lower thereafter. 3ßHSD is associated with white matter (Sanne and Krueger, 1995) and gray matter (Dupont et al., 1994; Guennoun et al., 1995; Ukena et al., 1999a), and enzyme activity is found *in vitro* in glial cells (Akwa et al., 1993; Jung-Testas et al., 1989). Progesterone may also be metabolized further by the enzyme 5alpha reductase, to 5α-dihydroprogesterone, which can also be metabolized by the reversible enzyme 3α hydroxysteroid dehydrogenase, to the neurosteroid allopregnanolone (Compagnone and Mellon, 2000). In addition, progesterone can be metabolized by 3α hydroxysteroid dehydrogenase to yield 3α hydroxy-4-pregnen-20-one (Griffin and Mellon, 2001; Wiebe, 1982; Wiebe et al., 1997; Wiebe et al., 1994; Wiebe et al., 1980). The concentrations of progesterone, 5α dihydroprogesterone, 3α hydroxy-4-pregnen-20-one and allopregnanolone depend upon the presence of the metabolizing enzymes, which are expressed in a region-specific manner (Compagnone and Mellon, 2000; Dong et al., 2001; Griffin and Mellon, 2001; Korneyev et al., 1993; Uzunov et al., 1996). Progesterone and 5α-dihydroprogesterone can mediate effects through the nuclear progesterone receptor, while 3α DHP and allopregnanolone cannot; 5α-dihydroprogesterone and allopregnanolone can mediate effects through $GABA_A$ receptors, while progesterone cannot (Belelli and Lambert, 2005). Hence, the presence of the enzymes 5alpha reductase and 3α hydroxysteroid dehydrogenase in a cell may convert progesterone to additional neuroactive compounds. Thus, whether effects attributed to progesterone, as described in the sections below, are mediated by some other neurosteroid requires additional testing of those neurosteroids in similar experimental paradigms, and/or determining the receptors/mechanisms through which the particular effects are elicited.

3.2 Myelination

Progesterone has effects on myelination in the peripheral nervous system. Studies by Koenig et al (1995) first showed that myelinating Schwann cells of the peripheral nervous system are able to synthesize progesterone (Koenig et al., 1995). Furthermore, this group showed that locally synthesized progesterone promoted myelination after cryolesion injury. The steroid effect was mediated through progesterone receptors, rather than through conversion of progesterone to other neuroactive compounds. Some of the effect of progesterone is mediated through increased expression of myelin basic protein. In parallel studies, another group showed that progesterone, as well as dexamethasone, enhanced both the rate of myelin synthesis and

accelerated the time of initiation of myelin synthesis (Chan et al., 1998). Progesterone has also been shown to increase mRNAs for myelin-specific proteins, including protein 0 (P0) and the peripheral myelin protein-22 (PMP-22) (Desarnaud et al., 1998; Melcangi et al., 1998; Melcangi et al., 1999; Notterpek et al., 1999), as well as increasing the expression of Krox-20, a transcription factor that is crucial for myelination in the peripheral nervous system (Guennoun et al., 2001). In co-cultures of Schwann cells and neurons, progesterone increased the rate of myelin synthesis in a dose-dependent manner. P450scc and 3ßHSD mRNAs increased in Schwann cells during active myelin synthesis (Chan et al., 2000). In addition, progesterone increased expression of its receptor and of myelin proteins in co-cultured dorsal root ganglial neurons, but did not increase expression or nuclear localization of the progesterone receptor in Schwann cells (Chan et al., 2000). Neither progesterone nor RU-486 affected expression of progesterone receptor mRNA in Schwann cell cultures, suggesting that effects of progesterone on Schwann cells may be mediated through other receptors, or may be mediated through metabolism to other neurosteroids such as allopregnanolone (Magnaghi et al., 2006; Magnaghi et al., 2001). Thus, progesterone affects myelin synthesis directly in Schwann cells as well as indirectly through induction of gene expression in neurons. The effects in neurons are likely due to activation of the progesterone receptor, as these effects are blocked by RU-486.

Progesterone also stimulates myelination in the central nervous system (Ghoumari et al., 2005; Ghoumari et al., 2003). In slice cultures of 7-day-old rat and mouse cerebella, high concentrations of progesterone (20-50 μM) increased expression of myelin basic protein about 4-fold. This effect may be mediated through progesterone receptors since the selective progesterone receptor agonist R5020 also increased expression of myelin basic protein while the progesterone receptor antagonist RU-486 abolished the effect of progesterone. The involvement of the progesterone receptor was confirmed using cerebellar slice cultures from progesterone receptor knockout mice. In those animals, progesterone had no significant effect on myelin basic protein expression. In addition to direct effects of progesterone on its nuclear receptor, some effects on expression of myelin basic protein were likely mediated through neurosteroid metabolites of progesterone (allopregnanolone). A 5α reductase inhibitor partially inhibited the effect of progesterone, and allopregnanolone significantly increased expression of myelin basic protein, although this stimulation was less than that found with progesterone treatment. In addition, the $GABA_A$ receptor antagonist bicuculline inhibited the effect of allopregnanolone on increasing expression of myelin basic protein. Thus, progesterone affects myelination not only in the peripheral nervous system, but in the central nervous system as well through mechanisms that involve both the progesterone receptor and the $GABA_A$ receptor.

3.3 Oligodendrocyte differentiation

Progesterone may also affect oligodendrocyte differentiation. Oligodendrocytes and their precursors differentially express enzymes needed for progesterone and other neurosteroid production, suggesting that these compounds may be involved in oligodendrocyte progenitor proliferation and differentiation during development (Gago et al., 2001; Gago et al., 2004; Ghoumari et al., 2005). Oligodendrocyte pre-progenitors, precursors, and fully differentiated oligodendrocytes differentially express 3ßHSD, 5α reductase and 3α hydroxysteroid dehydrogenase (3αHSD). Pre-progenitors have highest expression of 3ßHSD and 3α HSD and can convert pregnenolone to progesterone. 3α HSD activity is highest in oligodendrocyte pre-progenitors, but is also found in oligodendrocyte precursors and mature oligodendrocytes. By contrast, mature oligodendrocytes have the highest levels of expression of 5α reductase but are unable to convert pregnenolone to progesterone, suggesting a lack of 3ßHSD expression. These data are consistent with previous studies showing expression of 5α reductase activity in oligodendrocyte cultures (Melcangi et al., 1994), and in myelin sheaths (Melcangi et al., 1988; Poletti et al., 1997). The data demonstrating specific oligodendrocyte precursor

expression and induction of 3ßHSD further support the hypothesis that progesterone may have an effect during or contribute to oligodendrocyte differentiation.

3.4 Spinal Cord

Progesterone affects recovery of motoneuron function after spinal cord injury (De Nicola et al., 2006). Spinal cord injury results in increased pregnenolone and progesterone concentrations in the spinal cord and not plasma, without concomitant increases in the expression of steroidogenic enzymes P450scc and 3ßHSD, suggesting that injury induces local neurosteroidogenesis (Labombarda et al., 2006). In injured spinal motoneurons, progesterone treatment promotes functional recovery, due to increased choline acetyl transferase, Na,K-ATPase and GAP-43 mRNA (Labombarda et al., 2003) as well as due to increased myelin basic protein and increased production of oligodendrocyte precursors (De Nicola et al., 2003). These effects were hypothesized to be mediated by nuclear and membrane progesterone receptors, as both are found in spinal cord neurons and glia (Labombarda et al., 2003). Others have suggested that classic nuclear progesterone receptors do not play a role in mediating these effects since a transcriptionally-inactive ligand of the progesterone receptor, a progesterone enantiomer, mimicked the beneficial effects of progesterone in a similar spinal cord lesioned animal model (Vanlandingham et al., 2006). Progesterone metabolites such as allopregnanolone may also contribute to these beneficial effects, since both 5α reductase and 3α hydroxysteroid dehydrogenase are found in the spinal cord.

Some of the effects of progesterone may also be mediated through stimulation of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) (Begliuomini et al., 2007; De Nicola et al., 2006; Franklin and Perrot-Sinal, 2006; Gibbs, 1998; Gibbs, 1999; Gonzalez Deniselle et al., 2007; Gonzalez et al., 2004). The effects of BDNF on various markers of spinal cord injury and repair are similar to those elicited by treatment with progesterone. These include increased choline acetyl transferase in motoneurons (Yan et al., 1994), increased expression of GAP-43 (Kobayashi et al., 1997) and increased myelin-basic protein (Ikeda et al., 2002). Neurotrophic factors and their receptors are present in developing and in adult spinal cord neurons (Buck et al., 2000; Dreyfus et al., 1999; Schober et al., 1999) suggesting they may play a role in development and in neuronal survival and axonal regeneration (Sayer et al., 2002; Thoenen, 1995). Progesterone receptors, like neurotrophin receptors, are also found on motoneurons (Labombarda et al., 2000). Spinal cord injury results in decreased expression of BDNF mRNA and protein (Gonzalez et al., 2004). Progesterone treatment for 3 days had no effect on BDNF mRNA expression in motoneurons from control animals, but it increased BDNF mRNA and protein production 3-fold in motoneurons and neurons of the dorsal horn of injured spinal cords (De Nicola et al., 2006; Gonzalez et al., 2005; Gonzalez et al., 2004). However, progesterone did increase BDNF protein immunolabeling in motoneurons from normal animals, suggesting that progesterone may stimulate BNDF synthesis in regions outside the neuron. Thus, progesterone may act on injured or developing neurons by stimulating local BDNF production within the damaged neuron or within cells in proximity to the damaged neuron.

3.5 Cerebellum

Progesterone also stimulates cerebellar development (Tsutsui and Mellon, 2006). Purkinje neurons express P450scc, 3ßHSD, 5α reductase and 3α HSD and produces pregnenolone, pregnenolone sulfate, progesterone and allopregnanolone (Griffin et al., 2004; Ukena et al., 1999b; Ukena et al., 1998). Purkinje neurons synthesize progesterone and allopregnanolone during the neonatal period; during this time, 3ßHSD expression and enzymatic activities increase (Ukena et al., 1999a). In rodents, the cerebellum differentiates in the neonatal period, as rat Purkinje neurons differentiate just after birth, and the formation of the cerebellar cortex becomes complete in the neonate through migration of external granular cells, neuronal and

glial growth and synaptogenesis (Altman, 1972a; Altman, 1972b). Hence, this burst of growth and development coincides with the burst of neurosteroidogenesis that occurs concurrently. In newborn rat cultured cerebellar slices or *in vivo* in rats, progesterone promotes dendritic growth, dendritic spine formation, and synaptic density in Purkinje neurons (Sakamoto et al., 2001; Sakamoto et al., 2002). These effects are likely to be mediated through progesterone's action on the nuclear progesterone receptor, since the antiprogestin RU-486 blocks these effects, and the progesterone metabolite allopregnanolone does not affect these parameters of Purkinje cell development. The membrane 25-Dx progesterone receptor, which is a putative membrane progesterone receptor (Gerdes et al., 1998; Krebs et al., 2000), may also mediate some of these effects (Sakamoto et al., 2004). This receptor is now referred to as progesterone membrane receptor component 1 (PGRMC1) (Crudden et al., 2006; Engmann et al., 2006; Peluso et al., 2006; Viero et al., 2006).

Thus, progesterone has direct effects on oligodendrocyte precursor differentiation, Schwann cell function, myelination, Purkinje cell dendritic growth, spinogenesis and synaptogenesis, and motoneuron growth and recovery after injury. These effects of progesterone may be mediated through classic nuclear progesterone receptors, through membrane 25-Dx receptors, through other unidentified receptors. Some effects may additionally be mediated through conversion to other neuroactive steroids, such as allopregnanolone.

4. Allopregnanolone

4.1 General overview

Allopregnanolone is synthesized from progesterone via two enzymatic reactions: 5 alpha reduction of progesterone, yielding dihydroprogesterone, mediated by 5α reductase, followed by 3 alpha reduction of the C3 ketone, mediated by 3α HSD. Unlike progesterone, allopregnanolone is inactive at nuclear progesterone receptors. Allopregnanolone mediates its effects through modulation of $GABA_A$ receptors (Harrison and Simmonds, 1984; Majewska et al., 1986). Other steroids with a similar 3α hydroxy, 5α-ane structure, such as the derivative of deoxycorticosterone, have GABA-ergic effects that are similar to those described for allopregnanolone. Neurosteroids have been reported to modulate GABA-ergic function by increasing $GABA_A$ receptor opening frequency and duration receptors (Harrison and Simmonds, 1984; Majewska et al., 1986; Puia et al., 1990), at concentrations in the nanomolar range (Belelli et al., 1990; Gee et al., 1992; Lan et al., 1990; Morrow et al., 1990). More recent evidence suggests the mechanism by which neurosteroids modulate GABA-ergic function is through alteration of the kinetics of entry to and exit from desensitized states of the receptor (Zhu and Vicini, 1997; Zhu et al., 1996). A balance between unbinding, desensitization, and reopening of desensitized $GABA_A$ receptors underlies delay of inhibitory postsynaptic currents. Neurosteroids such as allopregnanolone slow the rate of recovery of $GABA_A$ receptors from desensitization and possibly increase the rate of entry into fast desensitized states. Desensitized states of $GABA_A$ receptors may be required for neurosteroids to modulate GABA responses, since neurosteroids most likely do not function by themselves, but rather function by potentiating inhibitory post-synaptic GABA-ergic transmission by prolonging slow deactivation. Recent data on a model of the structure of the neurosteroid:$GABA_A$ receptor complex has provided evidence for the sites of interaction between neurosteroids and the $GABA_A$ receptor subunits (Hosie et al., 2006). In this model, the authors propose two binding sites in the receptor's transmembrane domains that mediate the potentiating and direct activation effects of neurosteroids. This model posits that there are two neurosteroid binding sites: an activation site and a potentiation site, and that significant receptor activation relies on occupancy at both of these sites.

The behavioral and electrophysiologic effects of allopregnanolone and related compounds seem to be specific for $GABA_A$ receptors; these compounds have little or no action on glycine,

α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA), N-methyl-D-aspartate (NMDA) and 5-hydroxytryptamine type 3 (5-HT_3) receptors (Lambert et al., 2001).

In addition to mediating effects through $GABA_A$ receptors, recent evidence suggests that allopregnanolone is a ligand for the nuclear pregnane-X receptor (PXR). Unlike the nanomolar concentrations that are required to activate $GABA_A$ receptors, the PXR requires high micromolar concentrations of ligand to activate gene expression (Kliewer et al., 2002; Lamba et al., 2004; Langmade et al., 2006). It is unclear if such concentrations are attained *in vivo* in regions of the brain in which allopregnanolone is synthesized, or whether these effects would be purely pharmacologic. Our quantitative RT/PCR data suggest that PXR receptors are found in very low concentrations throughout the rodent brain, and that there are no significant changes in abundance from late gestation (E 18) to adulthood.

Allopregnanolone synthesis changes during postnatal development (Griffin et al., 2004; Grobin et al., 2003). This is due to changes in expression of 5α reductase and 3α HSD (Agis-Balboa et al., 2006; Griffin et al., 2004). 3α HSD activity is high in the cortex, midbrain and hindbrain at birth and decreases to a steady level by 5 weeks of age. 5α reductase activity is greater in the hindbrain than in the cortex or midbrain, and its activity remains relatively stable from birth to adulthood. *In situ* hybridization and immunocytochemistry demonstrate co-localization of 5α reductase and 3α HSD mRNAs and proteins in pyramidal neurons in the cortex and hippocampus, glutamatergic granular cells of the dentate gyrus and mitral cells of the olfactory bulb, glutamatergic (dorsomedial) and GABA-ergic (reticular) cells of the thalamus, GABA-ergic cells of the striatum, glutamatergic neurons of the amygdala and GABA-ergic Purkinje neurons of the cerebellum (Agis-Balboa et al., 2006). Thus, in multiple regions throughout the rodent brain, 5α reductase and 3α HSD co-localize in the same neurons, and these neurons are both glutamatergic and GABA-ergic. These data confirm those found for expression of 5α reductase in neurons (Melcangi et al., 1994), but differ from earlier observations of its expression in glia (Tsuruo et al., 1996) or from data suggesting lack of neuronal expression of 3α HSD (Melcangi et al., 1994). Nevertheless, the data suggest that these two neurosteroidogenic enzymes are highly expressed in output neurons, glutamatergic pyramidal, GABA-ergic reticulothalamic, striatal and Purkinje neurons, are absent in GABA-interneurons in the telencephalon or hippocampus, and are present to a lesser extent in glutamatergic granular interneurons in the cerebellum and GABA-ergic granular cells in the olfactory bulb. The data also suggest that allopregnanolone synthesized in GABA-ergic neurons will be able to act at $GABA_A$ receptors postsynaptically or extrasynaptically (Belelli and Lambert, 2005). In glutamatergic neurons that express both 5α reductase and 3α HSD and that presumably synthesize allopregnanolone, the data suggest that allopregnanolone probably works in a paracrine fashion at $GABA_A$ receptors located on dendrites or cell bodies of those glutamatergic neurons, with or without secretion into the extracellular space, through plasma membrane lateral diffusion or through intracellular routes (Akk et al., 2005). Neurosteroid modulation of GABA-mediated currents involves G-protein and protein kinase activation (Brussaard et al., 2000; Fancsik et al., 2000; Harney et al., 2003; Hodge et al., 2002; Vicini et al., 2002; Wegner et al., 2006). Both protein kinase C (epsilon isoform) and protein kinase A have been suggested as being involved in neurosteroid action. It is unclear if neurosteroid binding increases protein kinase C or A activities, if kinase activities increase neurosteroid binding to $GABA_A$ receptors, or if neurosteroids prevent $GABA_A$ receptor modulation by protein kinase C, as different studies report different effects. In addition, it is unknown if the kinase directly phosphorylates the $GABA_A$ receptors or if it phosphorylates one of the $GABA_A$ receptor associated proteins.

4.2 $GABA_A$ Receptor Sensitivity to Allopregnanolone During Development

$GABA_A$ receptor subunit composition plays an important role in the physiologic and pharmacologic modulation of neuronal function, excitability and downstream sequelae (Gee and Lan, 1991; Lan et al., 1991; Zaman et al., 1992). Changes in sensitivity of $GABA_A$ receptors toward allopregnanolone, due to alterations in subunit composition, are induced by neurosteroid withdrawal (Biggio et al., 2006; Gulinello et al., 2002; Mascia et al., 2002; Smith et al., 1998), long term steroid administration (Follesa et al., 2002) and in animal models of epilepsy (Banerjee et al., 1998; Brussaard and Herbison, 2000; Cagetti et al., 2003; Devaud et al., 1996; Follesa et al., 2000; Gulinello et al., 2002; Mtchedlishvili et al., 2001; Reddy and Rogawski, 2000), as well as to changes in neurosteroid concentrations resulting from pregnancy (Concas et al., 1998; Fujii and Mellon, 2001; Herbison, 2001), stress (Barbaccia et al., 1996), social isolation (Pinna et al., 2004; Serra et al., 2000), as well as due to age of the animal (Shen et al., 2007)

4.3 $GABA_A$ receptor expression during embryonic development

GABAergic-signaling components appear and become widespread during embryonic vertebrate CNS development, suggesting that GABA may have developmental roles (Barker et al., 1998; Maric et al., 2001). GABA and its synthetic enzymes, the two isoforms of glutamic acid decarboxylase (GAD_{65} and GAD_{67}), appear throughout the embryonic rat neocortex together with $GABA_A$ receptor subunits (Cobas et al., 1991; Lauder et al., 1986; Laurie et al., 1992b; Poulter et al., 1992; Poulter et al., 1993; Van Eden et al., 1989). During the development of embryonic neocortex, GABAergic cells are distributed in the ventricular/subventricular zone that contains proliferating precursors and progenitors (Ma and Barker, 1995; Ma and Barker, 1998), and in the cortical plate/subplate region that contains differentiating neurons (Maric et al., 1997). $GABA_A$ receptor subunits show temporal- and region-specific expression (Laurie et al., 1992b). By contrast, cerebellar Purkinje neurons show no developmental switch in subunit expression from birth to adult. The changes of $GABA_A$ receptor subunit gene expression suggest a molecular mechanism for changing affinities in ligand binding. Hence, the composition, and presumably properties, of embryonic and early postnatal rat $GABA_A$ receptors differs markedly from those expressed in the adult brain.

$GABA_A$ receptor subunit composition within specific regions of the brain, for example in neurons of the hippocampus, also changes during development and postnatally (Brooks-Kayal et al., 1998; Brooks-Kayal and Pritchett, 1993; Brooks-Kayal et al., 2001). Such changes not only occur in rodents, but similar changes also occur in human beings. These changes could result in different effects of allopregnanolone during development. During rodent embryogenesis, allopregnanolone had no effect on muscimol-stimulated binding to $GABA_A$ receptors, but at 5 days of age, an effect of allopregnanolone was seen. Unlike rodents, guinea pig $GABA_A$ receptors are affected by allopregnanolone during embryonic development (Bailey et al., 1999). Not only was muscimol binding to $GABA_A$ receptors seen during gestation, but effects of allopregnanolone increased to those seen in adult brains by late gestation. Changes in $GABA_A$ sensitivity toward neurosteroids during development may reflect changes in $GABA_A$ receptor subunit composition differences (Borodinsky et al., 1997). Other studies also suggested that there are post-natal, region-specific changes in neurosteroid sensitivity of $GABA_A$ receptors (Jussofie, 1995). In the medulla, $GABA_A$ receptor number did not change appreciably after birth, but sensitivity to neurosteroids increased after birth, while both $GABA_A$ receptor number and neurosteroid sensitivity changed dramatically in the frontal cortex and cerebellum. Maximal changes were observed approximately two weeks after birth. Electrophysiologic experiments suggested similar changes in $GABA_A$ receptor sensitivity to neurosteroid modulation (Mtchedlishvili et al., 2003). In those studies, GABA evoked larger currents from adult than from neonatal rat dentate granule cells, due to increased efficacy and current density, but not due to changes in potency.

However, allopregnanolone sensitivity of $GABA_A$ receptor currents increased during postnatal development due to an increased potency. Because of contemporaneous changes in $GABA_A$ α1 subunits, the authors suggested that this may result in changes in neurosteroid sensitivity.

More recent studies now suggest that there may be additional postnatal developmental switches that occur during puberty that may occur on particular subclasses of $GABA_A$ receptors (Shen et al., 2007). In studies in mice, the composition of CA1 hippocampal pyramidal neuronal α4βδ $GABA_A$ receptors increased at the onset of puberty. This may be due to decreased synthesis of allopregnanolone at this time (Fadalti et al., 1999), as allopregnanolone withdrawal has been shown to increase expression of α4 and δ subunits (Smith et al., 2006). These α4βδ $GABA_A$ receptors are typically expressed at low concentrations, are expressed extrasynaptically, have a high sensitivity to low concentrations of GABA, and have a relative lack of desensitization. In prepubertal mice, allopregnanolone decreased the outward current, was inhibitory and caused increased anxiety.

$GABA_A$ receptor stimulation also regulates expression of $GABA_A$ receptor subunits (Poulter et al., 1997). Expression of $GABA_A$ subunits in cultured cortical neurons paralleled their expression *in vivo*. Six subunits were studied; α3, α5, and β3 subunit expression declined, α2 expression increased, whereas β2 and *γ 2 subunit mRNA expression remained relatively constant during the culture period. $GABA_A$ receptor stimulation regulated these expression profiles; allopregnanolone augmented the rate at which the α3, α5, or β3 subunit mRNA expression declined and prevented the increase in α2 subunit mRNA expression and down-regulated β2 subunit mRNA expression. The $GABA_A$ receptor noncompetitive antagonist, tert-butylbicyclophosphorothionate (TBPS) increased expression of α3, α5, β2 and β3 subunit mRNA expression and decreased expression of α2 subunit mRNA. Neither allopregnanolone nor TBPS had any effect on γ*2 subunit mRNA expression. Hence, these cell culture experiments suggest that the developmental switchover of GABA receptor subunit mRNA expression is regulated by $GABA_A$ receptor activity, and further suggest that local synthesis of allopregnanolone may facilitate this developmental switch.

In addition to synaptic $GABA_A$ receptors that are responsible for conventional phasic inhibition of action potentials, recent data demonstrate that there are extrasynaptic $GABA_A$ receptors that are highly sensitive to neurosteroids. These receptors are responsible for tonic inhibition and responses to GABA spillover (Brickley et al., 1996; Brickley et al., 2001; Hamann et al., 2002; Nusser and Mody, 2002; Rossi et al., 2003; Semyanov et al., 2003; Stell and Mody, 2002; Wall and Usowicz, 1997). $GABA_A$ receptors containing the delta subunit are restricted to these extrasynaptic locations (Nusser et al., 1998; Wei et al., 2003). Receptors containing the δ subunit are unique as they are benzodiazepine-insensitive (Shivers et al., 1989) and neurosteroid sensitive (Mihalek et al., 1999; Stell et al., 2003; Wohlfarth et al., 2002). $GABA_A$ receptors containing this subunit are highly abundant in the cerebellar granule cells, hippocampus and thalamus (Laurie et al., 1997; Laurie et al., 1992a) and cortex (Mihalek et al., 1999; Pirker et al., 2000). Ablation of the δ subunit *in vivo* in mice results in reduced sensitivity to neurosteroids (Mihalek et al., 1999), and results in concomitant reduced expression of α4 subunits in the hippocampus (Spigelman et al., 2003) and forebrain (Korpi et al., 2002; Peng et al., 2002) and increased expression in γ2 subunits in the forebrain (Korpi et al., 2002; Peng et al., 2002) and cerebellar granule cells (Tretter et al., 2001), but only in regions that express the δ subunit.

Ablation of the δ subunit in mice does not appear to result in gross neurodevelopmental abnormalities (Mihalek et al., 1999). Mice show increased decay time of synaptic $GABA_A$ currents, resistance to the neurosteroids alphaxalone and pregnanolone, reduced sleep time, reduced anxiolysis but normal behavioral response to volatile anesthetics, normal exploratory activities and normal pain sensitivity (Mihalek et al., 1999) and enhanced seizure susceptibility

to convulsants (Spigelman et al., 2002). Interestingly, ablation of the δ subunit results in reduced fertility. Unlike mice, ablation of the δ subunit in human beings may associated with abnormalities caused by terminal deletions of 1p36 (Windpassinger et al., 2002). These authors have mapped the human $GABA_A$ δ subunit to 1p36.33. Deletion of this region of chromosome 1 results in growth delay, facial anomalies, hearing and visual deficits, heart defects, body asymmetry, psychomotor retardation, epilepsy and self-abusive behavior. Since the δ subunit maps to this region, the authors suggest that deletion of human $GABA_A$ δ subunit likely contributes to the neurological and neuropsychiatric anomalies in patients with the 1p36 deletion. However, additional genes located in this region may also contribute to these anomalies.

4.4 Allopregnanolone effects in development

To study the role of allopregnanolone during development, we used a mouse model of a human neurodevelopmental disorder, Niemann Pick Type C (NP-C) (Griffin et al., 2004). NP-C is an autosomal recessive, lysosomal lipid storage disorder in which cholesterol trafficking is defective, resulting in accumulation of unesterified cholesterol (Patterson et al., 2001). All compounds that traffic through the same intracellular pathway, including gangliosides and other lipids, also accumulate in lysosomes or late endosomes (Cruz and Chang, 2000; Roff et al., 1992; Zervas et al., 2001). About 95% of human NP-C is caused by mutations in the *NPC1* gene (Carstea et al., 1997), and 5% is caused by mutation in the *NPC2* gene (Naureckiene et al., 2000). The naturally occurring mutant NP-C mouse (Morris et al., 1982) shows neuronal accumulation of cholesterol and gangliosides (Zervas et al., 2001), cerebellar degeneration (Morris et al., 1982), Purkinje cell degeneration (Higashi et al., 1993), irregular and diminished dendritic spines (Higashi et al., 1993), dysmyelination (Weintraub et al., 1987) and progressive loss of glia, neurons, and myelin (Ong et al., 2001). NP-C mice are hypoandrogenic and have undeveloped reproductive organs (Roff et al., 1993), suggesting a defect in the production of androgens from cholesterol. This suggested to us that there may likewise be a defect in the production of neurosteroids from cholesterol that may result in the neurodegeneration seen in this disease.

We found that adult NP-C mice have diminished concentrations of pregnenolone and allopregnanolone in their brains, although concentrations of pregnenolone exceeded those of allopregnanolone. This suggested that steroidogenesis downstream of P450scc, the enzyme that converts cholesterol to pregnenolone, was also diminished in NP-C. We examined 5α reductase and 3α HSD activities in NP-C mice from gestation to adulthood, at ~ 70 days of age, when NP-C mice typically die. We found that 5α reductase and 3α HSD activities were similar in wild type and NP-mice during late gestation (E16.5), suggesting that reduction in allopregnanolone may not occur during development. However, we found that these enzyme activities were substantially reduced postnatally in all brain regions. 5α reductase activity was diminished by 3 weeks of age (at weaning), before onset of any neurological symptoms. This reduction in activity was seen in all regions of the brain, in male and female NP-C mice, but the greatest reduction (>50%) was seen in the cortex and midbrain. 5α reductase activity was reduced even further in all brain regions during the next 7 weeks of life.

While reduced 5α reductase activity was noted at 3 weeks of age, a more severe deficiency in 3α HSD was seen at birth (P0). This deficiency in 3α HSD was seen in all regions of the brain at birth, and was diminished further until 10 weeks of age, the end of life. This loss of enzyme activity at the end of life, but not at P0, was due to decreased protein.

The loss of both 5α reductase and 3α HSD activities at 7 weeks of age was due to loss of neurons expressing these enzymes. As indicated previously, these enzymes co-localize in many regions of the cortex, hippocampus, thalamus, striatum, and Purkinje neurons of the cerebellum (Agis-Balboa et al., 2006). At 50 days of age, neurons expressing these enzymes were lost in brains

from NP-C mice. In addition to loss of 5α reductase- and 3α HSD-expressing neurons, neurons that express P450scc and 3ßHSD were also lost in NP-C mice. While steroidogenic enzymes are expressed in cerebellar Purkinje neurons, studies co-localizing P450scc, 3ßHSD, 5α reductase and 3α HSD in other regions of the brain were not performed, so it is unknown if all these steroidogenic enzymes co-localize throughout the brain. Nevertheless, our data suggest that neurosteroidogenic neurons may be selectively lost in NP-C.

We hypothesized that loss of allopregnanolone was, in part, contributing to the loss of neurons in the NP-C mouse. We therefore determined whether treatment of NP-C mice would be beneficial in reducing the neurodegeneration. While treatment of NP-C mice at weaning was effective, we found that the most effective time to begin treatment was at postnatal day 7. This time corresponded to the time of increased allopregnanolone synthesis in wild type rodents (Grobin et al., 2003). We found that a single injection of allopregnanolone at postnatal day 7 increased survival from 67 to 124 days, delayed the onset of weight loss and onset neurological symptoms of ataxia and motor incoordination for about 4 weeks. In addition, analysis of cerebella from untreated and from allopregnanolone-treated mice showed that while adult untreated NP-C mice had > 80% loss of Purkinje neurons by 63 days of age, allopregnanolone-treated NP-C mice had substantially increased survival of those neurons. This was consistent with the neurological data demonstrating almost normal locomotor function at 9 weeks of age in allopregnanolone-treated mice.

When brains were analyzed for ganglioside accumulation, we found that allopregnanolone treatment resulted in significant reductions in ganglioside GM1, GM2 and GM3 at 60 days of age. Cholesterol accumulation was also diminished in brains from allopregnanolone-treated mice, and concentrations of cerebrosides were increased, suggesting that de-myelination was reduced as well. Thus, critically-timed treatment of NP-C mice with allopregnanolone in the early neonatal period results in long-lasting, beneficial biochemical and neurological changes in adulthood.

The mechanism by which allopregnanolone elicits its effects in the NP-C mice are not completely understood. Some of the beneficial effects are mediated through $GABA_A$ receptors (Griffin et al., 2004). When P0-P3 Purkinje neurons from wild type and NP-C mice are cultured *in vitro*, NP-C Purkinje neurons die within 72 hours of culture. However, treatment of those cultures with 50 nM allopregnanolone increased Purkinje cell survival to that found in wild type mice. Allopregnanolone, but not the $GABA_A$-inactive homologue 3ß5α tetrahydroprogesterone, could also increase survival of Purkinje neurons from wild type mice. Bicuculline blocked the effect of allopregnanolone. Thus, these *in vitro* data suggest that $GABA_A$ receptors play some role in mediating the beneficial effects of allopregnanolone.

The nuclear pregnane-X-receptor (PXR, NR1I2) may also mediate some of the beneficial effects of allopregnanolone (Lamba et al., 2004; Langmade et al., 2006). This nuclear steroid hormone receptor is the principal inducer of phase I and phase II drug-metabolizing/detoxifying enzymes and transporters. Consistent with this function, PXR is found in the greatest abundance in the liver. It is found in much lower concentrations in the brain, and its expression does not appear to change with age. We found that PXR-regulated genes could be activated in the brains of mice treated with allopregnanolone (Langmade et al., 2006). Unlike most steroid hormone receptors that have dissociation constants in the nanomolar range, and activate gene expression when steroids are present at 1-100 nM, the Kd for allopregnanolone binding to PXR is in the micromolar range, and requires 10-50 μM allopregnanolone for activation of gene expression in cell transfection assays (Lamba et al., 2004). PXR is activated by a wide range of structurally dissimilar compounds, consistent with its function in drug/xenobiotic sensing (Dixit et al., 2005; Ekins and Erickson, 2002; Kliewer et al., 2002; Poso and Honkakoski, 2006; Zhu et al., 2004), bile acid metabolism (Dussault et al., 2003; Goodwin et al., 2003),

owing to its large ligand binding pocket (Ekins and Schuetz, 2002; Orans et al., 2005; Schuster and Langer, 2005; Watkins et al., 2002; Watkins et al., 2001).

Studies using another $GABA_A$ inactive compound, the complete enantiomer of allopregnanolone, also showed that this compound was effective in treating NP-C mice (Langmade et al., 2006). Since the allopregnanolone enantiomer could not activate $GABA_A$ receptors but could activate PXR receptors, the data suggest that PXR may play a major role in mediating effects of allopregnanolone in the brain. However, the concentrations at which allopregnanolone activates $GABA_A$ (1-100 nM) versus PXR (50 μM) differ by more than 2 orders of magnitude. Endogenous concentrations of allopregnanolone has been reported as being in nanomolar and not micromolar concentrations in the brain. Hence, micromolar concentrations of allopregnanolone may not be achieved endogenously in the brain, and these effects of allopregnanolone treatment may be pharmacologic. Nevertheless, the data indicate that both $GABA_A$ and PXR may be involved in mediating effects of allopregnanolone. The data further indicate that both of these receptor systems play a role in ameliorating the effects of neurodegeneration in NP-C mice.

4.5 Neurogenesis and neuroprotection

Some of these neuroprotective and anti-neurodegenerative effects of allopregnanolone may be through increased neurogenesis, stabilization of synapses, and inhibition of toxin-induced cell death (Brinton, 1994; Keller et al., 2004; Wang et al., 2005). In cultures of hippocampal neurons, allopregnanolone causes regression of neurites from hippocampal neurons that have not yet made contact with other neurons or glia, but has no effect on neurites that have made these connections (Brinton, 1994). In this system, allopregnanolone also protects neurons from picrotoxin-induced cell death. Allopregnanolone also induces significant increases in proliferation of neuroprogenitor cells from rat hippocampus and human neural stem cells derived from the cerebral cortex (Wang et al., 2005). These effects are dose-dependent, isomer-specific and steroid-specific; 3ß5α tetrahydroprogesterone and other related steroids are without effect. Concentrations of allopregnanolone eliciting these effects are in the 100 nM range, consistent with a $GABA_A$ receptor-mediated mechanism. Allopregnanolone also increases expression of genes that promote mitosis and inhibits expression of genes that repressed cell proliferation. The effects of allopregnanolone may also involve voltage-gated L-type calcium channels (VGLCC), as some effects are blocked by the VGLCC blocker nifedipine. These effects are consistent with allopregnanolone inducing rapid increases in intracellular calcium via stimulation of $GABA_A$ receptor-activated VGLCC. Others have reported similar findings of allopregnanolone stimulating granule cell neurogenesis. In those studies, the effects of allopregnanolone were shown to be mediated through $GABA_A$ receptor-activated VGLCC (Keller et al., 2004).

Thus, changes in allopregnanolone biosynthesis, $GABA_A$ receptor subunit expression, and perhaps PXR or other receptor expression during development and in the early neonatal period, may affect neurogenesis, neuronal proliferation, survival and migration. Appropriately timed pharmacologic treatment with allopregnanolone may also promote similar effects *in vivo*.

5. Dehydroepiandrosterone and dehydroepiandrosterone sulfate

5.1 General overview

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) were among the first neurosteroids identified in rat brains (Corpechot et al., 1981; Corpechot et al., 1983). P450c17, the enzyme that is required for synthesis of DHEA from pregnenolone, is found in specific neurons of embryonic rodent brains (Compagnone et al., 1995b). P450c17 expression is mainly neuronal; its expression can be found as early as embryonic day 9.5, and persists in the central

nervous system during development. However, P450c17 was not found in the central nervous system in adult rodents, suggesting that this enzyme, and its neurosteroid product, functions mainly during development. Others have shown that P450c17 was found in adult male rat hippocampi (Hojo et al., 2004). In the hippocampus, P450c17 was localized to pyramidal neurons in the CA1-CA3 region, and also in granule cells of the dentate gyrus. In these cells, P450c17 was localized in pre- and post-synaptic locations and in the endoplasmic reticulum. Interestingly, although P450c17 steroidogenic enzyme activity was low in the hippocampus, steroid synthesis could be enhanced by exposing neurons to NMDA, suggesting that neurosteroidogenesis may be activity-dependent. Similar findings for NMDA stimulation of neurosteroidogenesis were also reported for enhancement of pregnenolone synthesis in the hippocampus (Kimoto et al., 2001).

5.2 Neurite growth

DHEA and DHEAS have been shown to have dramatic effects on growth of embryonic rodent cortical neurites (Compagnone and Mellon, 1998). DHEA at low nanomolar concentrations increased the length of Tau-immunopositive neurites. These neurites were identified as axons. DHEA had much less effect on MAP-2 immunopositive neurites (dendrites). By contrast, DHEAS at low nanomolar concentrations had no effect on axonal growth, but stimulated dendritic growth. DHEA stimulation of embryonic cortical neurons caused a dose-dependent increase in calcium entry into cells. This effect was blocked by MK801 and D-AP5, suggesting that DHEA's effects involved NMDA receptors. These data, together with the data suggesting activity-dependent neurosteroid synthesis, suggest that DHEA may be synthesized and act locally to cause axonal growth in cortical embryonic neurons. Similar studies could not be done in adult neuronal cultures, so it remains unknown if DHEA's effects on axonal growth are limited to embryos. However, DHEA did have effects on synapse formation in hippocampal neurons (Hajszan et al., 2004). Treatment of ovariectomized rats with 1 mg DHEA/day for 2 days increased CA1 spine synapse density more than 50%. However, this effect of DHEA was likely mediated through local aromatization to estradiol as the aromatase inhibitor letrozole inhibited the effect of DHEA.

P450c17 is also expressed in the adult rat spinal cord (Kibaly et al., 2005). Immunohistochemical studies localized P450c17 in both neurons and glial cells in the spinal cord. Slices of spinal cord tissue containing P450c17 protein could convert [^{3}H] pregnenolone into [^{3}H] DHEA, and this conversion was reduced by ketoconazole, an inhibitor of P450c17 activity. Thus, the spinal cord is one region of the adult CNS that expresses P450c17 and can synthesize DHEA endogenously from a pregnenolone precursor. These data suggest that DHEA synthesized locally may control spinal cord neurite growth and other activities.

5.3 Neuroprotection

DHEA and DHEAS also have neuroprotective effects. Both DHEA and DHEAS protect the hippocampus against glutamate, AMPA or kainate toxicity (Kimonides et al., 1998). This protection was greater when given before NMDA insult, and effects were seen both *in vitro* and *in vivo*. Others showed that neuroprotection by DHEA, but not DHEAS, was mediated through inhibition of nitric oxide (NO) production and inhibition of calcium-sensitive NO synthase (NOS) activity, caused by NMDA stimulation (Kurata et al., 2004). This neuroprotective effect of DHEA may be mediated via the sigma-1 receptor, as sigma-1 receptor antagonists rimcazole or BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) partially, but significantly, reversed the neuroprotective effect. However, high concentrations of DHEA (micromolar concentrations) may be neurotoxic, mediating these effects through inhibition of complex I of the mitochondrial respiratory chain (Safiulina et al., 2006). Thus, DHEA concentrations may be crucial in promoting neuroprotection versus neurotoxicity.

5.4 Anti-apoptosis

DHEA, DHEAS, and allopregnanolone also protect chromaffin cells and the sympathoadrenal PC12 cells against apoptosis induced by serum deprivation through mechanisms independent of NMDA and NOS inhibition (Charalampopoulos et al., 2005; Charalampopoulos et al., 2004). The effects of DHEA, DHEAS and allopregnanolone are time- and dose-dependent with EC_{50} in the low nanomolar range. The steroid specificity of this response requires a 3ß/Δ5 configuration for the androstenes and a 3α hydroxy configuration for the pregnanes. The prosurvival effect of DHEA(S) appears to be mediated by G-protein-coupled-specific membrane binding sites, and does not involve NMDA, $GABA_A$ or sigma-1 receptors. It involves the antiapoptotic Bcl-2 proteins, and the activation of transcription factors CREB and NF-κB, upstream effectors of the antiapoptotic Bcl-2 protein expression, as well as PKCalpha/beta, a posttranslational activator of Bcl-2. Furthermore, DHEAS and allopregnanolone directly stimulate biosynthesis and release of neuroprotective catecholamines norepinephrine and dopamine from PC12 cells. This effect is due to a direct transcriptional effect on tyrosine hydroxylase. In addition, DHEA, DHEAS and allopregnanolone regulate actin depolymerization and submembrane actin filament disassembly, a fast-response cellular system regulating trafficking of catecholamine vesicles. Thus, DHEA, DHEAS and allopregnanolone may promote neuroprotection through multiple mechanisms.

5.5 Neurogenesis and neuronal survival

DHEA also promotes neurogenesis and neuronal survival (Karishma and Herbert, 2002; Suzuki et al., 2004). Treatment of rats with DHEA pellets increases neurogenesis in the dentate gyrus. This effect appears to be specific for DHEA, since neither pregnenolone nor androstenediol has these effects. In addition to stimulating hippocampal neurogenesis, DHEA also blocks the reduction in neuronal survival due to corticosterone treatment. Some of the effects on neuronal survival may be due to decreases in apoptosis (Zhang et al., 2002). Using cultured neural precursors from rat embryonic forebrains, DHEA activated the serine-threonine protein kinase Akt, which is widely implicated in cell survival signaling. Interestingly, DHEAS had the opposite effect and decreased Akt and increased apoptosis. Thus once again, DHEA and DHEAS have different effects on neural survival, morphology and function, suggesting that the balance between these two neurosteroids may play a critical role in nervous system development and maintenance.

The effect of DHEA on increasing neurogenesis and neuronal survival was also found in cultures of human neural stem cells (Suzuki et al., 2004). In the human cell system, both epidermal growth factor and leukemia inhibitory factor were required to elicit these effects of DHEA, suggesting that human and rodent systems may require slightly different survival or growth factors. As seen in the rodent culture system, pregnenolone had no effect on proliferation of human neural stem cells, and the effect of DHEA could be blocked by blocking NMDA and sigma 1 receptors. Thus, DHEA, whose concentrations are high during human gestation, greatly diminish after birth, increase pre-pubertally until the 30's, and then decline rapidly thereafter, may play a critical role in enhancing neuronal survival and hippocampal neurogenesis. Similarly, reduction in DHEA concentrations, for example in some people with major depressive disorders, may result in reduced neurogenesis and reduced ability to inhibit effects of neurotoxicity.

6. Conclusions

This review highlighted only a few of the neurosteroids that have been identified in the embryonic and adult brain. Novel functions for these and other neurosteroids in the development and maintenance of the nervous system are continually being discovered. These effects are identified and tested not only in rodent, but in human systems and cell culture

models, highlighting the similarities of these effects among various species. Thus, the effects observed in rodents are not unique to rodents. Use of rodents to ablate genes involved in the production of specific neurosteroids, in a temporal- region- and cell-specific fashion may provide further insight into endogenous functions of these compounds during critical developmental periods. The initial characterization of the mechanisms through which these neurosteroids mediated their effects suggested that a single mechanism may be involved. However, as more and more functions are attributed to these varied compounds, there are likely to be multiple mechanisms by which these neurosteroids elicit their effects, rather than through a single receptor-mediated event. Future studies will continue to dissect the mechanisms of action of pregnenolone, progesterone, allopregnanolone and DHEA, and will continue to identify additional physiologic and pharmacologic functions of these intriguing compounds.

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