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(Article begins on next page)

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Neurosteroidogenesis today: Novel targets for neuroactive steroid synthesis and action and their relevance for translational research

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Abstract

Neuroactive steroids are endogenous neuromodulators synthesised in the brain that rapidly alter neuronal excitability by binding to membrane receptors, in addition to the regulation of gene expression via intracellular steroid receptors. Neuroactive steroids induce potent anxiolytic, antidepressant, anticonvulsant, sedative, analgesic and amnesic effects, mainly through interaction with the γ-amino-butyric type A ($GABA_A$) receptor. They also exert neuroprotective, neurotrophic and antiapoptotic effects in several animal models of neurodegenerative diseases.

Neuroactive steroids regulate many physiological functions such as stress response, puberty, ovarian cycle, pregnancy and reward. Their levels are altered in several neuropsychiatric and neurologic diseases and both preclinical and clinical studies emphasise a therapeutic potential of neuroactive steroids for these diseases, whereby symptomatology ameliorates upon restoration of neuroactive steroid concentrations. However, direct administration of neuroactive steroids has several challenges, including pharmacokinetics, low bioavailability, addiction potential, safety and

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tolerability that limit its therapeutic use. Therefore, modulation of neurosteroidogenesis to restore the altered endogenous neuroactive steroid tone may represent a better therapeutic approach.

This review summarizes recent approaches that target the neuroactive steroid biosynthetic pathway at different levels in order to promote neurosteroidogenesis. These include modulation of neurosteroidogenesis through ligands of the translocator protein 18 kDa (TSPO), and the pregnane xenobiotic receptor (PXR), as well as targeting of specific neurosteroidogenic enzymes like 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) or P450 side chain cleavage (P450scc). Enhanced neurosteroidogenesis through these targets may be beneficial for neurodegenerative diseases such as Alzheimer's disease and age-related dementia, but also for neuropsychiatric diseases, including alcohol use disorders.

Keywords

neuroactive steroids; 3α,5α-THP (allopregnanolone); translocator protein 18 kDa (TSPO); pregnane xenobiotic receptor (PXR); 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10); P450 side chain cleavage (P450scc); Alzheimer's disease; alcoholism

Introduction

Neuroactive steroids are endogenous neuromodulators that rapidly alter neuronal excitability by binding to membrane receptors (1). They can be synthesized in the brain *de novo* from cholesterol, in which case they have been termed neurosteroids (2), or can reach the brain from peripheral steroidogenic organs such as adrenals and gonads, and are locally metabolized (i.e. aromatization of testosterone into estradiol) (3). The synthesis of neuroactive steroids requires the translocation of cholesterol across the mitochondrial membrane, which occurs through a molecular complex formed by the translocator protein 18 kDa (TSPO), the steroidogenic acute regulatory protein (StAR), the voltage-dependent anion channel protein (VDAC), and the adenine nucleotide transporter protein (ANT). In the mitochondria, cholesterol is converted to pregnenolone by the P450 side-chain cleavage enzyme (P450scc); pregnenolone diffuses into the cytosol where it is further metabolized into different neuroactive steroids, as shown in Figure 1. Although TSPO, the rate-limiting step in neuroactive steroid synthesis, is highly expressed in microglia and astrocytes, but less abundant in neurons, neurosteroidogenesis occurs primarily in principal neurons of several brain areas that possess the necessary enzymatic machinery to convert cholesterol into neuroactive steroids (4).

The most potent neuroactive steroids are the progesterone metabolite (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and the deoxycorticosterone (DOC) metabolite (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC or allotetrahydrodeoxycorticosterone), which enhance γ-amino-butyric type A ($GABA_A$) receptor mediated neurotransmission and produce inhibitory neurobehavioral effects (5). The 3α,5α-reduced metabolites of testosterone and dehydroepiandrosterone (DHEA), 3α,5α-androstandiol and 3α,5α-androsterone, respectively, also potentiate $GABA_A$ receptors, albeit with less potency (6). Specific binding sites for neuroactive steroids have been identified on the α subunits of the $GABA_A$ receptor that allosterically modulate binding to

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GABA and benzodiazepine recognition sites (7). At nanomolar concentrations, 3α,5α-THP enhances affinity of GABA for its receptor, while at micromolar concentrations, it directly activates the receptor channel. 3α,5α-THP and 3α,5α-THDOC modulate both synaptic and extrasynaptic $GABA_A$ receptors albeit with higher potency at extrasynaptic receptors that contain δ subunits (5, 6). 3α,5α-THP also modulates serotonin type 3 receptors, neuronal nicotinic acetylcholine receptors, and voltage-activated calcium channels, although with micromolar potency (8). Another site of 3α,5α-THP action, the nuclear pregnane xenobiotic receptor (PXR) has been recently identified (9). In contrast, the sulfated derivative of pregnenolone inhibits GABA release, binds with high affinity to, and promotes trafficking of N-methyl-D-aspartate (NMDA) receptors, thus exerting excitatory actions (10). It is still unclear how neuroactive steroids act on their membrane receptor targets, whether it is through paracrine or autocrine mechanisms or by intracellular lateral diffusion through the cell membrane (11). Further, in addition to 3α,5α-THP's action at the aforementioned pharmacodynamics targets, it may also have homeostatic pharmacokinetic effects through its actions at nuclear PXR (9).

Pharmacological properties

Neuroactive steroids exert several psychopharmacological actions such as anxiolytic, antidepressant, anticonvulsant, sedative, anesthetic, analgesic and amnesic effects, likely due to their actions on $GABA_A$ receptors (12-18). Moreover, 3α,5α-THP promotes sexual behavior of female rodents (19). Neuroactive steroids also possess rewarding properties in rodents (20, 21), and can modulate ethanol or cocaine intake (22-24). Indeed, acute administration of several drugs of abuse, like alcohol, nicotine, morphine, γ-hydroxy-butyric acid (GHB) or Δ9-tetrahydrocannabinol, increases brain and plasma concentrations of 3α, 5α-THP and/or its precursors progesterone and pregnenolone in rats or mice (25-30) and this increase is thought to contribute to their rewarding effects.

In addition to these psychopharmacological effects, neuroactive steroids exert neuroprotective, neurotrophic and antiapoptotic effects in animal models of traumatic brain injury, spinal cord injury, peripheral neuropathy, cerebral ischemia, stroke, seizure disorder, and of neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, Nieman Pick type C disease (31-44). More recently, an anti-tumor effect of progesterone has been reported in experimental models of glioblastoma multiforme (45).

Physiological significance

Neuroactive steroid concentrations fluctuate in response to physiological conditions like stress, development, ovarian cycle, pregnancy and post-partum and their fluctuations have been associated with changes in $GABA_A$ receptor plasticity.

Neuroactive steroids are increased in rats and humans following acute stress and this effect may represent a homeostatic mechanism to restore the altered hypothalamic-pituitary-adrenal (HPA) axis function (46, 47). The neuroactive steroid response to stress is a complex phenomenon that involves adaptations in $GABA_A$ receptor plasticity and appears to differ across species (for recent reviews see (23, 48)).

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Neuroactive steroid concentrations also fluctuate across development. Brain levels of 3α,5α-THP are elevated in the embryonic rat, decrease around birth, show a transient increase on days 10-14, and remain low until puberty (49). The onset of puberty is associated with a rapid elevation in 3α,5α-THP levels and a marked increase in extrasynaptic α4βδ $GABA_A$ receptors, the main target of 3α,5α-THP, through which this steroid is thought to increase excitability and, thus exert the "paradoxical effect" of increasing anxiety in pubertal female mice (50).

Moreover, neuroactive steroids fluctuate across the ovarian cycle: progesterone and 3α,5α-THP levels are increased in mouse brain during diestrous (51) and in women's plasma during the luteal phase of the menstrual cycle (52). These changes are accompanied by increased expression of the δ subunit and decreased expression of the γ2 subunit of the $GABA_A$ receptor, with subsequent increase in tonic inhibition and decreased seizure susceptibility and anxiety (53).

Levels of progesterone and 3α,5α-THP increase markedly during pregnancy in both rats and women (54, 55) and decrease immediately prior to parturition in rats, before returning to baseline levels two days after delivery (54). These abrupt changes in steroid concentrations were associated with changes in $GABA_A$ receptor subunit expression that were thought to contribute to post-partum depressive symptoms (54, 56, 57).

Pharmacological treatments with steroids, which affect brain 3α,5α-THP levels, modify $GABA_A$ receptor plasticity. Steroid withdrawal from long-term exposure of progesterone or 3α,5α-THP markedly increases hippocampal α4 and δ subunit expression, with subsequent changes in receptor function, sensitivity to benzodiazepines and increased anxiety and seizure susceptibility (58). Long-term administration of ethinyl-estradiol and levonorgestrel, two of the synthetic steroids most frequently used in hormonal contraceptives, decreased cerebral cortical and hippocampal pregnenolone, progesterone and 3α,5α-THP concentrations and these changes were associated with increased expression of the $GABA_A$ receptor γ2 subunit, and increased anxiety-like behavior in adult female rats (59-61). Neonatal administration of β-estradiol-3-benzoate to female rats, a treatment that affects sexual differentiation of the brain (62), induces a marked and persistent decrease in the cerebrocortical and hypothalamic concentrations of 3α,5α-THP in adulthood, which is associated with compensatory changes in $GABA_A$ receptor subunit expression (63, 64). Likewise, changes in 3α,5α-THP milieu during development, induced by neonatal finasteride administration, increase the expression of hippocampal α4/δ $GABA_A$ receptors and induce anxiety-like behavior in adulthood (65).

The molecular underpinnings for the steroid-induced plasticity of $GABA_A$ receptors are not quite understood. A recent study has reported increased phosphorylation and membrane trafficking of α4 subunit-containing $GABA_A$ receptors promoted by 3α,5α-THDOC (66), a novel mechanism by which neuroactive steroids may affect $GABA_A$ receptor expression and function.

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Pathological significance and therapeutic implications

Neurosteroidogenesis is impaired in several neuropsychiatric and neurodegenerative diseases (Table 1). For instance, a reduction in the concentrations of several neuroactive steroids (3α,5α-THP, 3α,5β-THP, 3α,5α-THDOC, 3α,5α- and 3α,5β-androsterone), or their precursors (pregnenolone, progesterone, DOC, DHEA) was reported in serum and/or cerebrospinal fluid of patients with major depression, premenstrual dysphoric disorder, post-traumatic stress disorder, schizophrenia, bipolar disorder, and in abstinent alcoholics (67-74). Likewise, brain concentrations of progesterone and 3α,5α-THP are altered in animal models of anxiety/depression (75-77) and post-traumatic stress disorder (78). Moreover, patients with male pattern hair loss treated with the 5α-reductase blocker finasteride report increased symptoms of depression and show altered levels of pregnenolone, progesterone, dihydroprogesterone, 3α,5α-THP, testosterone, dihydrotestosterone, 3α,5α- and 3α,5β-androstandiol, and 17β-estradiol even after drug discontinuation (79). It is not clear whether the impaired neurosteroidogenesis contributes to the disease outcome or whether it is the result of altered brain homeostasis. Interestingly, pharmacological treatment with antidepressants like fluoxetine, reboxetine, venlafaxine, imipramine or mirtazapine, but also with antipsychotics like clozapine and olanzapine, or mood stabilizers like carbamazepine or lithium, restores central and peripheral concentrations of 3α,5α-THP in rats and humans (68), suggesting that neuroactive steroids may contribute to the therapeutic efficacy of antidepressant medications.

Impaired neurosteroidogenesis has also been reported in patients with schizophrenia and it has been suggested that elevations of pregnenolone and 3α,5α-THP may contribute to the therapeutic efficacy of clozapine and olanzapine (80). Indeed, adjunct treatment with pregnenolone improves cognition and negative symptoms in patients with schizophrenia (81), suggesting that neuroactive steroids contribute to schizophrenia pathophysiology.

Given their anticonvulsant properties, neuroactive steroids have also been proposed as therapeutic agents for epilepsy. Clinical trials with ganaxolone are under way in subjects with epilepsy (82). Progesterone treatment is effective at reducing seizures among women with catamenial epilepsy and 3α,5α-THP mediates these effects (83). Neuroactive steroids are preferred over the classical benzodiazepine treatment because of their lack of anticonvulsant tolerance and because of their neuroprotective properties that contribute to reduce neuroexcitotoxicity and neuronal damage (84).

The discovery of the neuroprotective, neurotrophic and anti-inflammatory properties of neuroactive steroids has prompted numerous investigations into their therapeutic potential for neurodegenerative diseases. Impaired neurosteroidogenesis has been found in animal models as well as in humans with multiple sclerosis (39, 85, 86), Alzheimer's disease (42, 87-89), Parkinson's disease (90), traumatic brain injury (91), diabetes (92), suggesting that neuroactive steroids may contribute to the neuropathological processes of these diseases (93). Indeed, the reduction in 3α,5α-THP content correlated with severity of Alzheimer's disease in humans (89). Thus, restoring neuroactive steroid concentrations may represent a useful therapeutic approach for these neurodegenerative disorders. Several preclinical studies have been successful in this respect. For instance 3α,5α-THP promotes neurogenesis, improves learning and memory and ameliorated the pathology burden in the

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triple transgenic AD (3xTgAD) mouse model (42, 94, 95). 3α,5α-THP has also shown therapeutic efficacy in animal models of multiple sclerosis (39), Parkinson's disease (43), Niemann-Pick type C disease (44), diabetic neuropathy (96), traumatic brain injury (31), and stroke (97).

Taken together, this evidence suggests that neuroactive steroids may have therapeutic utility in several neuropsychiatric and neurologic disorders. However, direct administration of 3α, 5α-THP has several challenges that limit its therapeutic use. These include a short half-life (98), low bioavailability and poor solubility in aqueous formulations, which limits its oral administration (99), development of tolerance (100), side effects such as sedation (98), memory impairment (101), and addiction potential (20, 21, 102). Thus, different approaches to target the neuroactive steroid biosynthetic pathway have been explored. This review summarizes recent advances in neurosteroidogenesis and highlights the importance of modulating neuroactive steroid synthesis as a putative therapeutic approach for neuropsychiatric and neurodegenerative diseases that affect millions of people worldwide.

Translocator protein as a therapeutic target for Alzheimer's disease

The translocator protein (TSPO) is a five transmembrane structure located at the outer and inner mitochondrial membrane contact sites, expression of which is enriched in steroidogenic organs (103). Activation of TSPO by synthetic TSPO ligands elicits pleiotropic neuroprotective and cognitive benefits, mechanistically linked to regulation of mitochondrial function, including the facilitation of mitochondrial cholesterol import for steroidogenesis (104). Numerous endogenous ligands of TSPO have also been identified, including diazepam binding inhibitor (DBI), triakontatetraneuropeptide (TTN), phospholipase A2 (PLA2), and protoporphyrin IX, which also have the ability to stimulate mitochondrial cholesterol import and neurosteroidogenesis (104-106). Recent studies using TSPO-knockout mice indicate that TSPO function in steroidogenesis may be tissue specific, playing a crucial role in the adrenal but not the testes (107-111). Although the role of TSPO in steroidogenesis in the brain remains to be addressed using similar models, numerous studies have demonstrated the ability of TSPO ligands to increase neuroactive steroid hormone production in the brain by increasing cholesterol supply to P450scc at the inner mitochondrial membrane, which is the rate-limiting step in neurosteroidogenesis (112-118).

Alzheimer's disease is a neurodegenerative disorder that leads to memory loss and cognitive impairment; it is characterized by the accumulation of beta amyloid plaques and neurofibrillary tangles in the brain (119). Age-related depletion of neuroactive steroid levels is an established risk factor for Alzheimer's disease, with neuroactive steroids having numerous beneficial effects in animal models of Alzheimer's disease, including reducing accumulation of the toxic beta amyloid (Aβ) peptide, thought to be the toxic principle driving Alzheimer's disease degenerative cascades (119). TSPO ligands that stimulate the synthesis of protective neuroactive steroids directly in the brain offer a novel therapeutic approach aimed at harnessing the protective actions of neuroactive steroids for the prevention and/or treatment of Alzheimer's disease.

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In the brain, TSPO is predominantly expressed in astrocytes and microglia, with low levels also observed in neurons following injury or during repair (120). TSPO is a sensitive marker of gliosis, becoming markedly upregulated in glial cells during aging, following injury and in Alzheimer's disease (104). Although TSPO expression is closely linked to gliosis, the specific role of TSPO in glial function remains poorly understood. In Alzheimer's disease, elevated glial TSPO expression is observed early in the disease process and co-localizes with degeneration and neuropathology (121). Consequently TSPO ligands are also under widespread investigation as potential inflammatory biomarkers for *in vivo* PET imaging in Alzheimer's disease, leading to the development of many new-generation, safe TSPO ligands with the potential to be repurposed for Alzheimer's disease therapy.

The translocator protein forms part of a complex with the VDAC and ANT, and has been implicated in mitochondrial cholesterol import (104). Mitochondrial cholesterol import is the rate limiting step in neuroactive steroid formation. Once localized at the inner mitochondrial membrane, cholesterol is converted to pregnenolone, the precursor to all other neuroactive steroids (see Figure 1). TSPO has a cholesterol recognition amino acid consensus sequence (CRAC) at the C-terminal identified as a cholesterol binding site (122), which is proposed to play a functional role in mediating the transportation of cholesterol across the hydrophilic intermembrane space. Structural studies indicate that ligand binding stabilizes the tertiary structure of TSPO facilitating cholesterol import (123). A common polymorphism at the C-terminal of human TSPO results in conformational changes interfering with the cholesterol binding site (124), which is associated with impaired cholesterol binding and metabolism (122) and reduced ability to produce pregnenolone (125). In addition to cholesterol import, TSPO has also been implicated in other important mitochondrial functions including mitochondrial respiration and ATP production (126), which may also contribute to the protective actions of TSPO ligands.

TSPO ligands have been shown to promote nerve regeneration, increase neuronal survival, reduce oxidative damage, inhibit apoptosis, attenuate gliosis and decrease Aβ accumulation in animal models of traumatic brain injury, excitotoxicity, axotomy and neuropathy, inflammatory disease and Alzheimer's disease (104, 116, 120). For example, in rats, the TSPO ligand, Ro5-4864, reduced reactive gliosis and neuronal loss following kainate acid-induced excitotoxicity (127). *In vitro* evidence indicates TSPO ligands act directly on glia to reduce inflammatory responses (128, 129), with the TSPO ligand, PK11195, decreasing pro-inflammatory cytokine production in cultured human microglia in response to LPS stimulation. Chronic inflammation and glial dysfunction contribute to the pathological degenerative cascade in Alzheimer's disease, and anti-inflammatory drugs have shown therapeutic promise for Alzheimer's disease (130). In the triple transgenic Alzheimer's disease mouse model (3xTgAD), the TSPO ligand, Ro5-4864, has been shown to reduce gliosis as well as lower accumulation of Aβ and improve functional behavioural outcomes (116). Interestingly, protection against Alzheimer's disease-related pathology was associated with increased brain levels of the protective neuroactive steroids in young-adult but not aged animals, suggesting that neuroactive steroid regulation may not be essential for the TSPO-mediated protective effects in this model.

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TSPO ligands also have diverse neuropsychiatric benefits, including anxiolytic, anti-depressive and cognitive-enhancing properties commonly attributed to their ability to increase levels of 3α,5α-THP, progesterone and testosterone (104, 116, 131). Consequently, TSPO ligands may offer symptomatic therapeutic opportunities for a range of neurological disorders, including Alzheimer's disease. For example, the new generation TSPO ligand, XBD-173, reduced anxiety-related behaviour in both rats and humans (117, 132). Mechanistically, the anxiolytic effect of XBD-173 was linked to enhanced GABA neurotransmission as a result of increased production of the neuroactive steroid 3α,5α-THP, which is an allosteric $GABA_A$ receptor modulator (117). Neuroactive steroids have well defined, potent, and broad cognitive, behavioural and psychological benefits including improved mood, and reduced anxiety and depression; this is coupled with a favourable side-effect profile, including the absence of sedation, tolerance, withdrawal symptoms or motor impairment (104). Therefore, neurosteroidogenic TSPO ligands may also be therapeutically useful for the treatment of not only the cognitive, but also the neuropsychiatric symptoms of Alzheimer's disease, including anxiety, agitation, depression, apathy and aggression. The treatment of neuropsychiatric symptoms in Alzheimer's disease is difficult and currently available therapies are not particularly effective in the management of these symptoms (133). These neuropsychiatric symptoms drive institutionalization; therefore improvement of currently offered pharmacotherapies has the potential to be valuable in terms of patient and caregiver quality of life.

TSPO offers a promising therapeutic target for Alzheimer's disease, with diverse neuroprotective and psychological benefits identified. Further, many new generation, safe TSPO ligands have been developed for *in vivo* imaging in humans, which may also prove therapeutically useful in the treatment of Alzheimer's disease. However, determination of the key mechanism(s) of TSPO action is critical for identifying the most efficacious TSPO ligands, optimally translating preclinical findings into clinical use, and rationally developing new TSPO ligands for therapeutic use (131). The recent development of numerous TSPO knockout mouse models will prove invaluable in addressing the role of TSPO in both normal and diseased states, as well as confirming the specificity of drug action.

Pregnane xenobiotic receptor (PXR): a novel target for neuroactive steroid synthesis and action

Neuroactive steroids can have rapid and robust actions in the central nervous system for behaviour. A model that has been utilized to elucidate the requisite role that production of pregnane steroids, such as 3α,5α-THP, have in the brain for behaviours has focused on reproductive endpoints. Several molecules, including TSPO, P450scc, StAR, 3α-hydroxysteroid dehydrogenase, and 5α-reductase, are downstream of cholesterol in the pregnane neurosteroidogenesis pathway (134-137). Pharmacologically blocking these at any point in this pathway reduces 3α,5α-THP formation in the brain and abolishes the lordosis response of female rodents (reviewed in (138)). More recently, the PXR has been identified as a potential factor upstream of cholesterol metabolism. PXR is a nuclear receptor that binds steroids, such as 3α,5α-THP, and influences transcription of cytochrome P450 enzymes that are involved in metabolism of many factors, including cholesterol and steroids (139-141). Although PXR is generally regarded for its role in the liver, it has been identified

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in brain regions, involved in motivation, affect and cognition, such as the ventral tegmental area, hippocampus, and cortex in mammals (9, 142-145). PXR is required for production of 3α,5α-THP and its actions for lordosis in the VTA, as well as for anxiety-related behaviours in the hippocampus (9, 143, 144, 146-149). Indeed, these effects of PXR may involve the well-known targets of 3α,5α-THP such as, NMDA and $GABA_A$ receptors (149). A question of interest is the role of PXR in other brain targets of steroids for behavioural plasticity, such as that underlying cognitive performance.

Pregnane steroids can enhance cognitive performance in young female rodents. Increased production of 3α,5α-THP with oestrous cycle fluctuations or replacement of progesterone or 3α,5α-THP to ovariectomised rodents is associated with better performance in hippocampal and cortical tasks, such as the object recognition task, as well as increased 3α,5α-THP in these regions (150). These effects persist in the absence of progestin receptors, which are not a typical target of 3α,5α-THP, but not with genetic knockout of 5α-reductase (151, 152). We have been examining the cognitive sequelae of rats with PXR knockout, on a Sprague-Dawley background, generated by Sage Labs. In the object recognition task, PXR-replete rats perform at chance (50 +/- 2% of object investigation time exploring the novel object); however, rats lacking functional PXRs perform below chance (40 +/- 2% of time exploring the novel object). Thus, capacity to form 3α,5α-THP may be critical for object memory among young female rodents.

With aging, there is a different pattern of effects of pregnane steroids in the hippocampus and cortex for cognitive performance, suggesting greater responses to progesterone decline and replacement in the cortex. For example, in addition to age-related decline in reproductive success, there is decline in 3α,5α-THP levels in frontal cortex of female rats (153). Variability in the cognitive response in this rat model of reproductive senescence or “menopause” is associated with 3α,5α-THP levels in the cortex (153). Thus, 3α,5α-THP can have pro-cognitive effects in healthy systems, but the target and patterns of these effects may be sensitive to aging; a question is whether pregnane steroids can improve hippocampal and cortical function in both healthy and compromised systems.

Another approach to investigate the role of pregnane neurosteroidogenesis as a critical target is to assess effects in age-related dementia. We, and others, have shown that people in the early stages of Alzheimer's disease or dementia have lower circulating levels of 3α,5α-THP than do non-demented peers matched for age, gender, education and socioeconomic factors (154). We have also used a double transgenic mouse model of early-onset familial Alzheimer's disease that co-overexpresses mutant forms of amyloid precursor protein and presenilin 1 Δ exon 9 mutation (APPswe+PSEN1Δe9; (155-157)). Comparisons were made following ovariectomy at 6 months of age and administration of chronic subcutaneous pellets of placebo (cholesterol) or progesterone for 6 months. In this mouse model, genetic mutations cause them to develop β-amyloid deposits after 5 months of age, and by 9 months old severe plaque deposits build up in the cortex and hippocampus (155). APPswe +PSEN1Δe9, compared to wild-type mice, had poorer performance on hippocampally-mediated tasks (worse object placement performance and more depressive behaviour in the forced swim task); progesterone improved responding in the wild-type, but not the APPswe +PSEN1e9, mice in these tasks (156, 157; Figure 2). Baseline differences were not observed

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in cortically-mediated tasks (object recognition or T maze) between the mouse strains, and progesterone improved performance of both wild-type and APPswe-PSEN1Δe9 mice in both of these tasks (156, 157; Figure 2). Additionally, progesterone, compared to vehicle, increased progesterone and 3α,5α-THP in the cortex of both wild-type and APPswe +PSEN1Δe9, but only increased 3α,5α-THP in the hippocampus of wild-type mice (156, 157). Thus, these data suggest that progesterone does not improve APPswe-PSEN1Δe9 behaviour in hippocampally-mediated tasks, like that of wild-type mice, and that they also show deficiencies in their capacity to form 3α,5α-THP in the hippocampus. Whereas, in the prefrontal cortex, they were able to form 3α,5α-THP, and wild-type and APPswe-PSEN1Δe9 mice performed similarly in these tasks, with progesterone administration improving their overall performance.

Studies in people that have been diagnosed with probable Alzheimer's disease and using other Alzheimer's disease mouse models suggests that there may be faulty metabolism effects in Alzheimer's disease relating to pregnane neuroactive steroids (as discussed herein and see (89, 99, 154)). A question of interest is the importance of PXR acting upstream of cholesterol for these effects. Levels of PXR as well as downstream enzymes required to 3α, 5α-THP formation, such as P450scc, StAR, 3β-hydroxysteroid dehydrogenase, and 5α-reductase, were measured by western blotting in the hippocampus and frontal cortex of behaviourally-assessed wild-type and APPswe+PSEN1Δe9 mice. The greatest increases in expression among the APPswe+PSEN1Δe9 mice, compared to their wild-type controls, was for PXR, P450scc, 3β-hydroxysteroid dehydrogenase, and 5α-reductase in the hippocampus (Figure 2). A different pattern was observed in the cortex with the greatest reductions in expression being for PXR and P450scc (Figure 2). This pattern of effects complements the behavioural effects observed as well as pregnane steroid levels in the hippocampus and cortex (Figure 2), and suggests the importance of additional consideration to neurosteroidogenic enzyme expression and activity in relation to behaviour and steroid levels in future studies. For example, an important follow-up study would be to assess levels of 3α-hydroxysteroid dehydrogenase. The findings that 5α-reductase was increased in the hippocampus of the APPswe+PSEN1Δe9 mice, with little difference in progesterone and dihydroprogesterone levels, but a large decrease in 3α,5α-THP levels, suggests that perhaps 3α-hydroxysteroid dehydrogenase activity is altered in the transgenic mice. Of further interest is the role of PXR in models of successful cognitive aging as well as neurodegeneration.

There may be beneficial effects of low body mass index and regular fasting to protect against Alzheimer's disease and promote longevity, albeit this is a controversial notion. Although a speculation, it may be that low body mass index and/or fasting may reduce burdens associated with switching from gluconeogenesis and lipolysis and demands for clearance, which would involve PXR and related factors, such as liver X receptor (42, 158, 159). A consideration about protein aggregation is faulty cholesterol metabolism may ultimately result in a neuropathological process. How PXR is related to pro-cognitive and protective effects of pregnane and other steroids and/or as pro-hormones for 3α,5α-THP is of interest. It may be that PXR is acting in the central nervous system, much like in the liver, to regulate metabolising enzymes, receptors, and efflux transporters, to promote homeostasis and brain health. Further investigations to directly address this are needed.

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Roles of 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) in neurosteroidogenesis

The circulating lipoprotein is a major source for neurosteroidogenesis even though cholesterol can be *de novo* synthesized from acetate in neurons and other brain cells. The molecular mechanism for lipoprotein transport into brain cells and for "free" cholesterol transport into mitochondrion has recently been reviewed (160). StAR on the outer mitochondrial membrane plays a key role in importing cholesterol into mitochondrion. StAR interacts with a complex machinery, of which the translocator protein TSPO serves as a downstream portal for cholesterol to move to the inner mitochondrial membrane where the side chain of cholesterol is cleaved by P450scc. As a result, cholesterol is converted to soluble pregnenolone. Since this is a rate-limiting step, overexpression of this side chain cleavage enzyme would generally elevate neuroactive steroid levels (161). Pregnenolone can be further oxidized to progesterone under the catalysis of 3β-hydroxysteroid dehydrogenase or exit the mitochondrion without active transport. It was reported (162) that in rat brain 3β-hydroxysteroid dehydrogenase is also present in the endoplasmic reticulum. However, the mitochondrion appears to possess a more favourable environment for an oxidative reaction catalyzed by 3β-hydroxysteroid dehydrogenase, since the ratio of NAD^+/NADH in mitochondrion is about two orders of magnitude greater than that in the endoplasmic reticulum. It was also reported (116) that TSPO ligands elicit pleiotropic neuroprotective and cognitive benefits. Whether these effects are linked to the regulation of neuroactive steroid synthesis remains to be determined.

Pregnenolone and progesterone become substrates for P450c17 in the endoplasmic reticulum, where they are converted to DHEA and androstenedione, respectively. As shown in Figure 1, DHEA can be oxidized to androstenedione under the catalysis of 3β-hydroxysteroid dehydrogenase, and then be further converted to testosterone or estrone, and subsequently to more potent androgen (5α-dihydrotestosterone) and estrogen (17β-estradiol), respectively. On the other hand, progesterone can be oxidized to DOC under the catalysis of 21-hydroxylase. Progesterone and DOC are reduced by NADPH in the endoplasmic reticulum under the catalysis of 5α-reductase and then by AKR1C2 sequentially to form 3α,5α-THP and 3α,5α-THDOC, respectively. 3α,5α-THP as well as 3α,5α-THDOC facilitate affective and motivated social behaviour through non-genomic targets, such as $GABA_A$ receptors, glutamate, and dopamine receptors (5, 6, 9). Human AKR1C2 (3α-hydroxysteroid dehydrogenase type III) plays almost no actual role in the oxidation of 3α,5α-THP and/or 3α,5α-THDOC, because its catalytic efficiency is 7-fold less than that for its backward reduction and only one seventh of its oxidative reaction catalyzed by 17β-HSD10 (163). A more unfavourable factor for its oxidation in the endoplasmic reticulum is the disparity of redox coenzyme concentrations, e.g., the concentration of NADPH is at least one order of magnitude higher than that of $NADP^+$. It seems unfeasible for 3α,5α-THP and/or 3α,5α-THDOC to be effectively inactivated by an oxidative reaction catalyzed by AKR1C2 (3α-hydroxysteroid dehydrogenase type III). Abnormal levels of 3α,5α-THP and/or 3α,5α-THDOC would certainly be harmful to brain functions. This puzzle was never really solved by implication of 3α-hydroxysteroid dehydrogenase catalyzing a reverse reaction between 5α-DHP and 3α,5α-THP as depicted in the figure 2 of Ref. (95). In fact, it was already found (163, 164) and emphasized (165-168) that mitochondrial 17β-HSD10 is essential for the maintenance of homeostasis of 3α,5α-THP and/or 3α,5α-

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THDOC (see Figure 1). 3α,5α-THP and/or 3α,5α-THDOC metabolism is mainly controlled by a dual enzyme molecular switch, composed of 17β-HSD10 and 3α-hydroxysteroid dehydrogenase type III (AKR1C2) localized in distinct subcellular compartments, mitochondria and endoplasmic reticulum, respectively (164, 168). With regard to the role of 17β-HSD10 in the metabolism of 3α,5α-THP, 17β-HSD10 had been twisted to ABAD (Aβ-binding alcohol dehydrogenase) for unknown reasons, as noticed in Figure 2 of Ref. (95).

The 17β-HSD10, a fascinating protein, is encoded by the *HSD17B10* gene, which was first cloned from human brain in 1997 (GenBank accession No. AF037438). The role of 17β-HSD10, after many debates and criticisms, has been clarified in more recent studies (163-174): it plays essential roles in neurosteroidogenesis as well as in the isoleucine degradation pathway. This explains why a mutation(s) of this gene, *HSD17B10*, may delay the brain development and/or result in the regression of brain functions even in the absence of Aβ peptide (167, 168, 174-176).

It was reported (165, 168-170, 177) that 17β-HSD10 can catalyze the oxidative reaction of 17β-estradiol to oestrone, an inactive oestrogen (see Figure 1). Binding of the oestrogen receptor-α to 17β-HSD10 would inhibit the enzymatic activities of 17β-HSD10 whereas 17β-estradiol itself facilitates the dissociation of a 17β-HSD10-estrogen receptor-α complex (178). As is well known, 17β-estradiol exhibits significant neuroprotective effects. Elevated levels of 17β-HSD10 found in Alzheimer's disease brains (164, 165, 168, 179) may take a part in the pathogenesis of Alzheimer's disease due to an imbalance of neuroactive steroid metabolism (164-170, 174, 177).

It was found that a weak androgen, 3α-androstanediol, could be effectively converted to the most potent androgen, 5α-dihydrotestosterone (5α-DHT), under the catalysis of 17β-HSD10 (172, 173). Mitochondrial 17β-HSD10 plays a key role in the ‘back door’ androgen synthesis pathway especially in castrated animals (165, 168, 172, 173).

17β-HSD10 is involved in the metabolism of several neuroactive steroids such as 17β-estradiol, oestrone, 3α-androstanediol and 3α,5α-THP (see Figure 1). It was demonstrated (180) that such neuroactive steroids are able to improve neuronal bioenergetics significantly. Missense mutation(s) would abolish the catalytic activity of 17β-HSD10 in the isoleucine degradation pathway such that an accumulation of tiglylglycine and 2-methyl-3-hydroxybutyric acid in blood and the excretion of such isoleucine metabolites from urine is a common symptom in patients with 17β-HSD10 deficiency, which was therefore designated as 2-methyl-3- hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency (181). It was reported (174) that mental retardation did not result from the accumulation of isoleucine metabolite, while an imbalance of neuroactive steroid metabolism could be a major cause of neurological handicap associated with brain-type 17β-HSD10 deficiency patients. Elucidation of the roles of 17β-HSD10 in neurosteroidogenesis provides further supports to this hypothesis.

Among the many factors essential for keeping mitochondria healthy, the homeostasis of neuroactive steroids is particularly significant because of its impact on the bioenergetics of brain cells (180). Abnormality of mitochondrial structure and function underlies

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pathophysiological basis of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Enzymes involved in neurosteroidogenesis are potential therapeutic targets in treatment of neurodegenerative disorders including brain-type HSD10 deficiency and Alzheimer's disease (168, 174-177, 179-181) as well as prostate cancer (172, 173).

Divergent neuroactive steroid responses across species: the case of ethanol-induced neurosteroidogenesis

Alcohol's effects on neuroactive steroid concentrations are the most well characterized among species. Pioneering studies in rats showed that systemic administration of ethanol (1-2.5 g/kg) increases plasma, cerebrocortical and hippocampal levels of the neuroactive steroids 3α,5α-THP and 3α,5α-THDOC in Sprague-Dawley rats (25, 182) and Sardinian alcohol-preferring rats (183). More recently, Cook et al. (184) showed that alcohol differentially affects cellular 3α,5α-THP immunostaining throughout the brain of Wistar rats. In fact, acute ethanol increased 3α,5α-THP immunoreactivity in the medial prefrontal cortex, the hippocampal CA1 pyramidal cell layer, the polymorph cell layer of the dentate gyrus, the bed nucleus of the stria terminalis, and the paraventricular nucleus of the hypothalamus. In contrast, ethanol decreased 3α,5α-THP immunoreactivity in the nucleus accumbens and the central nucleus of the amygdala. No changes were observed in the ventral tegmental area, dorsomedial striatum, granule cell layer of the dentate gyrus, or the lateral or basolateral amygdala (184).

The ethanol-induced increase in plasma neuroactive steroids is mediated by the HPA axis, since it is no longer observed in hypophysectomized and adrenalectomized rats (27, 185-187). However, ethanol has also been found to increase 3α,5α-THP in hippocampal minces from intact and adrenalectomized/gonadectomized rats (188, 189), suggesting that it is capable of inducing brain neurosteroidogenesis, independent from peripheral sources. Accordingly, the ethanol-induced increase in cellular 3α,5α-THP immunoreactivity is independent of adrenal activation in the CA1 pyramidal cell layer, dentate gyrus polymorphic layer, bed nucleus of the stria terminalis, and paraventricular nucleus of the hypothalamus of Wistar rats (190). Likewise, ethanol decreases 3α,5α-THP labelling in the nucleus accumbens “shore” (core-shell border), and central nucleus of the amygdala, independent of adrenal activation. However, in the medial prefrontal cortex ethanol increases 3α,5α-THP immunoreactivity in sham-operated animals, but not in adrenalectomized ones, suggesting that ethanol, directly and independently from peripheral sources, regulates local 3α,5α-THP levels in several subcortical regions, except for the medial prefrontal cortex (190).

Ethanol-induced elevations in neuroactive steroids reach physiologically relevant concentrations that enhance GABAergic transmission and thus, contribute to several behavioural effects of ethanol in rats. Neuroactive steroids modulate ethanol's anticonvulsant effects, sedation, impairment of spatial memory, anxiolytic-like and antidepressant-like actions (see (191) and (23) for review). Each of these behavioural responses is prevented by pre-treatment with the neuroactive steroid biosynthesis inhibitor finasteride and/or by prior adrenalectomy. The hypnotic effect of ethanol is partially blocked by adrenalectomy. Moreover, administration of 5α-dihydroprogesterone, the immediate

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precursor of 3α,5α-THP, to adrenalectomized rats restores effects of ethanol, showing that brain neuroactive steroid synthesis modulates effects of ethanol. Taken together, these studies suggest that elevations in neuroactive steroids influence many of the GABAergic effects of ethanol *in vivo* and contribute to sensitivity to behavioural effects of ethanol in rats (23, 191).

Studies on the neurosteroidogenic effects of ethanol in mice have yielded different results depending on the strains and the experimental conditions used. C57BL/6J mice have higher basal plasma neuroactive steroid levels compared to DBA/2J mice and that plasma 3α,5α-THP levels are decreased in C57BL/6J mice and not altered in DBA/2J mice following injection of 2 g/kg ethanol (182). Moreover, acute ethanol administration (1-4 g/kg) failed to alter cerebrocortical and hippocampal levels of 3α,5α-THP and progesterone in male C57BL/6J and DBA/2J mice, despite inducing a marked increase in brain and plasma corticosterone levels, a measure of HPA axis activation (192). Other studies reported that injection of 2 g/kg ethanol increased whole brain 3α,5α-THP levels in male DBA/2J (193) but not C57BL/6J mice (194), while orally consumed ethanol increased whole brain 3α,5α-THP levels in male C57BL/6J mice (194). Overall, these results highlight important species differences in ethanol's neurosteroidogenic effects between rats and mice.

The ethanol-induced changes in 3α,5α-THP content in mice appear to be related to the genetic background of the strain. In the genetic reference population of the C57BL/6J (B6) × DBA/2J (D2) (BXD) recombinant inbred strains, basal cerebral cortical 3α,5α-THP levels across selected strains ranged between 1.81 and 3.72 ng/g, while ethanol-induced changes in cerebral cortical 3α,5α-THP ranged between +4% and +63% (23). Both basal and ethanol-induced cerebral cortical 3α,5α-THP levels in the BXD strains were correlated with some phenotypes of ethanol intake, suggesting that neuroactive steroid responses to ethanol may be associated with excessive alcohol consumption (23).

The neurosteroidogenic effects of ethanol in humans have been examined in few studies, leading to inconsistent results. Elevated 3α,5α-THP plasma levels were reported in male and female adolescents seen in the emergency room for alcohol intoxication (195, 196), which likely resulted in high blood ethanol concentrations. In contrast, laboratory administration of low or moderate doses of ethanol was found not to alter plasma levels of 3α,5α-THP and other GABAergic neuroactive steroids (182, 197) or to decrease 3α,5α-THP levels (198) in healthy volunteers. Different ethanol doses, analytic methods to measure neuroactive steroids, age of the subjects or environmental factors that influence neuroactive steroid synthesis in humans may account for these inconsistent results. Indeed, the same dose of ethanol consumed in the human laboratory studies mentioned above (∼80 mg/dl) produced no effect in rats when administered systemically (182), suggesting that dose might be a key factor in the difference between rat and human studies. However, the possibility that ethanol may increase brain levels of 3α,5α-THP, without affecting its peripheral concentrations, remains open. In fact, some subjective effects of ethanol are diminished by prior administration of finasteride (199) or dutasteride (200), two inhibitors of 3α,5α-THP biosynthesis, suggesting that 3α,5α-THP may play a role in ethanol's actions in humans. Moreover, dutasteride reduced subsequent alcohol consumption in subjects classified as heavy drinkers (200). Likewise, men, who took finasteride for treatment of male pattern hair

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loss, reported a decrease in alcohol consumption, which was greater in those subjects who consumed the most alcohol (201). Taken together, these results further support the hypothesis that neuroactive steroids may mediate sensitivity to alcohol in humans.

Studies on the role of GABAergic neuroactive steroids in alcohol dependence have shown that chronic ethanol consumption in rats induces tolerance to the neurosteroidogenic effects of ethanol and ethanol-dependent rats have a blunted elevation in plasma and brain neuroactive steroid content, likely the result of a blunted HPA axis function (191). In ethanol-dependent C57BL/6J mice, cellular 3α,5α-THP immunoreactivity is increased in the CA3 hippocampus, but decreased in medial prefrontal cortex, ventral tegmental area, nucleus accumbens core, dorsolateral striatum and lateral amygdala (202). Likewise, 3α,5α-THP and 3α,5α-THDOC serum levels are decreased in human alcoholics during alcohol withdrawal and return to normal levels upon recovery (73). Overall, these findings suggest that chronic ethanol consumption leads to a dysregulation in neuroactive steroid biosynthesis, with a blunted neuroactive steroid tone that might attenuate ethanol sensitivity and thus contribute to alcohol dependence. Indeed, risk of alcohol dependence is associated with polymorphic variation in the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase implicated in the conversion of progesterone and deoxycorticosterone to their neuroactive metabolites 3α,5α-THP and 3α,5α-THDOC, further providing indirect evidence that neuroactive steroids may contribute to alcohol sensitivity in humans (203).

Ethanol-induced elevations of GABAergic neuroactive steroids may protect against the risk for alcohol dependence (191). Diminished elevations of GABAergic neuroactive steroids following ethanol exposure would result in reduced sensitivity to the anxiolytic, sedative, anticonvulsant, cognitive-impairing and discriminative stimulus properties of ethanol. Reduced sensitivity to ethanol is associated with greater risk for the development of alcoholism; thus, restoration of ethanol sensitivity by re-establishing the neuroactive steroid tone in alcohol-dependent subjects may have therapeutic utility for prevention of alcohol consumption and/or relapse. In agreement, preclinical studies have found that administration of endogenous (epiallopregnanolone) or synthetic (3α,5β-20-oxo-pregnane-3-carboxylic acid) neuroactive steroids, as well as of the precursor pregnenolone, reduced ethanol self-administration in alcohol-preferring rats (204, 205). Likewise, recombinant adeno-associated serotype 2 vector mediated over-expression of P450 side chain cleavage (P450scc; the rate limiting enzyme in steroid synthesis) in the ventral tegmental area of alcohol preferring rats reduced ethanol reinforcement and consumption (161). This effect was long-lasting and was associated with an increase in 3α,5α-THP immunoreactivity in this brain area, suggesting that GABAergic neuroactive steroids may contribute to ethanol reinforcement. Indeed, P450scc over-expression in the nucleus accumbens, a brain region that plays a key role in ethanol reinforcement, but is insensitive to ethanol-induced neurosteroidogenesis (184), did not alter operant ethanol self-administration or 3α,5α-THP immunoreactivity (190), suggesting that the neuroactive steroid response to ethanol plays a role in the mechanisms that regulate its voluntary consumption. Increased 3α,5α-THP levels modulate the activity of neurons in the ventral tegmental area or influence ethanol's action upon these cells, thereby reducing ethanol reinforcement and consumption.

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Thus, targeted modulation of neuroactive steroid synthesis through administration of neuroactive steroid precursors or through increased expression of specific neurosteroidogenic enzymes may represent a useful therapeutic approach for alcoholism, as well as for other neurological or psychiatric diseases associated with altered neurosteroidogenesis. Species differences in the neurosteroidogenic effects of ethanol may be the result of genetic diversity, which is also often observed across individuals of the same species. Thus, it will be important to consider genetic diversity in neuroactive steroid biosynthesis for their therapeutic actions.

Conclusions

The concept that neuroactive steroids may represent potential protective agents for different pathologies of the central and peripheral nervous system has been explored in detail in several experimental models. However, since steroid receptors are widely expressed in many tissues, a therapeutic strategy that uses exogenous neuroactive steroids could also evoke endocrine side effects. Therefore, alternative strategies, based on pharmacological agents or gene therapy tools, able to increase the synthesis of endogenous neuroactive steroids directly in the nervous system have been recently explored. Thus, the present review discussed the potential therapeutic activity elicited by targeting of steroidogenic enzymes, like 17β-HSD10, or P450scc, as well as that elicited by inducers of steroidogenesis, like for instance the TSPO and PXR. Indeed, even if the role of TSPO in steroid hormone biosynthesis has been recently challenged (108, 111, 206), the protective effects exerted by ligands of this mitochondrial receptor remain a promising field of research for neurodegeneration occurring in central and peripheral nervous system (207, 208). On this line of thinking, together with PXR, it is important to recall that also the activation of liver X receptor has been demonstrated to be an interesting pharmacological tool. Indeed, liver X receptor in adrenal glands modulates StAR (209), and its activation induces genes involved in cholesterol efflux, promoting cholesterol utilization (209). Moreover, as recently demonstrated in an experimental model of diabetes, activation of liver X receptor increases neuroactive steroid levels directly in the central (210) as well as the peripheral nervous system (211). In agreement, activation of liver X receptors exerts protective effects in diabetic peripheral neuropathy (211), global or focal cerebral ischemia (212), as well as in neurodegenerative diseases, such as multiple sclerosis, Alzheimer's and Parkinson's diseases (93, 213).

Altogether, the observations here reported indicate that the assessment of neurosteroidogenesis and of its physiological and pharmacological control may represent a promising topic of research that deserves further exploration in preclinical and clinical studies.

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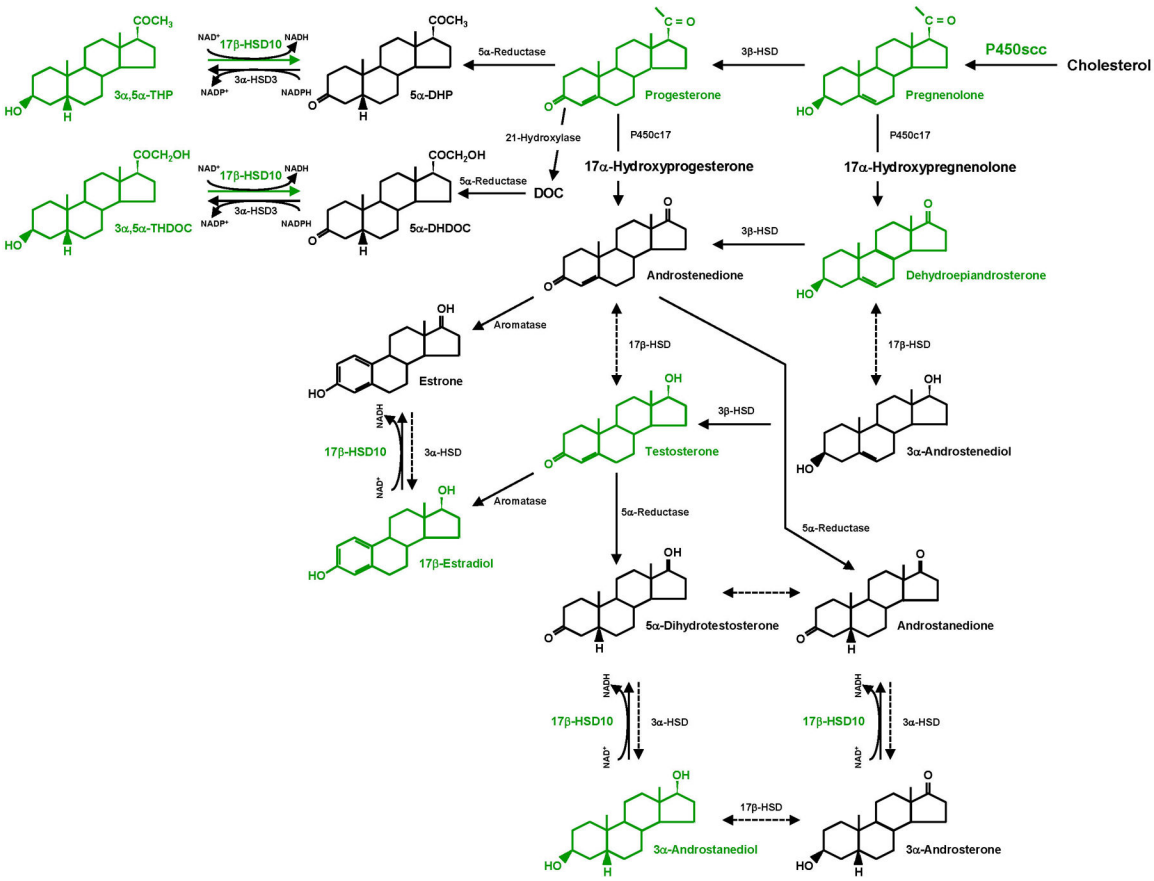

Figure 1.

Outline of neurosteroidogenesis. Neuroactive steroids and neurosteroidogenic enzymes that are potential key therapeutic targets are shown in green. The side chain of cholesterol is cleaved by P450scc as cholesterol is transported to the inner mitochondrial membrane and thus converted to pregnenolone. Soluble pregnenolone can enter into the endoplasmic reticulum unaided. 17β-HSD10 catalyzes the oxidation of neuroactive steroids in mitochondria with NAD^+ as the coenzyme. This enzyme most effectively catalyzes the oxidation of 3α,5α-THP and 3α,5α-THDOC such that it is essential for the homeostasis of these neuroactive steroids, which was controlled by a dual enzyme molecular switch, composed of 17β-HSD10 and 3α-hydroxysteroid dehydrogenase type III (AKR1C2) localized in distinct subcellular compartments, mitochondria and ER, respectively (164, 168). The catalytic efficiencies (k_{cat}/K_m) of 17β-HSD10 are as high as 427 and 1,381 min^{-1} •mM^{-1} for the oxidation of 3α,5α-THP and 3α,5α-THDOC, respectively (163, 164). Abbreviations: 5α-DHP, 5α-dihydroprogesterone; DOC, deoxycorticosterone; 5α-DHDOC, 5α-dihydrodeoxycorticosterone; 3α,5α-THP, (3α,5α)-3-hydroxypregnan-20-one or allopregnanolone; 3α,5α-THDOC, (3α,5α)-3,21-dihydroxypregnan-20-one or allotetrahydrodeoxycorticosterone; HSD, hydroxysteroid dehydrogenase.

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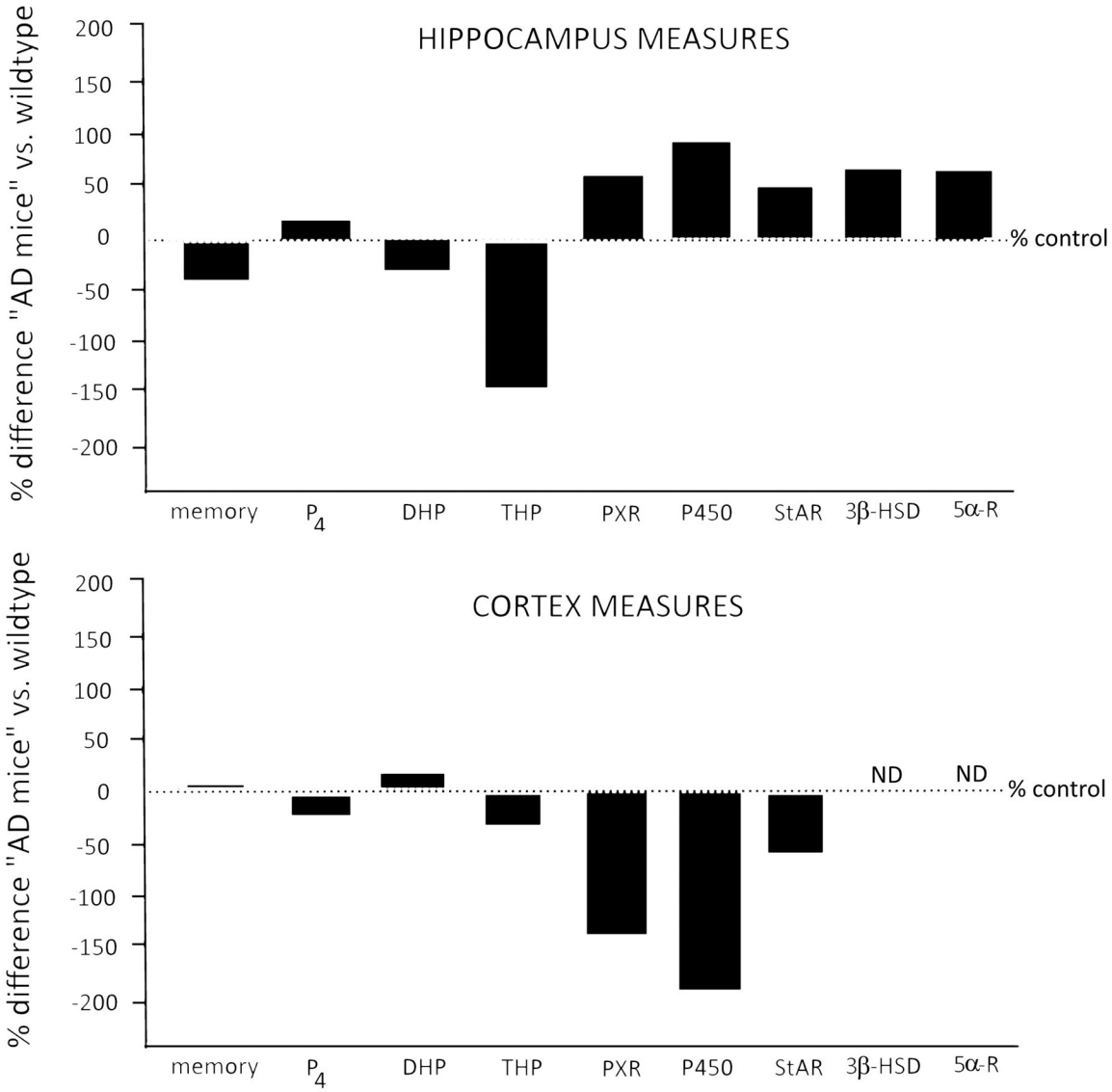

Figure 2.

Figure depicts behaviour, pregnane steroid concentrations, and expression patterns in the hippocampus (top panel) and cortex (bottom panel) for 12 month old transgenic mice that co-overexpress mutant forms of amyloid precursor protein and presenilin 1 Δ exon 9 mutation (APPswe+PSEN1Δe9; a murine model of early-onset familial Alzheimer's disease-AD), compared to their age-matched wild-type controls (n = 4-6). For all measures, mice had data collected at 12 months of age, following 6 months of continuous progesterone (P_4) administration via subcutaneously implanted pellets (25 mg, 90-day release at 6 months of age and then 9 months of age; purchased from Innovative Research of America). *Behaviour:* Performance in a memory task assessing the hippocampus (object placement) and cortex (object recognition) is depicted and based upon comparing the Alzheimer's disease mice to wild-type controls (methods and raw data published in (146)). *Pregnane steroid concentrations:* Levels of P_4, dihydroprogesterone (DHP), and 3α,5α-THP (THP) were measured using radioimmunoassay of dissected out hippocampus and cortex (methods and

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raw data published in (146)). *Protein expression:* Expression patterns in hippocampus and cortex were determined by western blotting, of specific proteins (pregnane xenobiotic receptor (PXR), cytochrome P450-dependent side chain cleavage- P450scc, steroidogenic acute regulatory protein- StAR, 3β-hydroxysteroid dehydrogenase- 3β-HSD, and 5α-reductase- 5α-R). The mean of relative intensity (relative density of protein of interest to actin control) in hippocampus and cortex of Alzheimer's disease mice were compared to that determined in wild-type controls. A standard western blotting protocol (146) was employed to assess these factors in hippocampus and prefrontal cortex tissues (a description of tissue collection from animal subjects, brain storage, dissection and preparation is described in (146)). Protein concentration for each sample was determined with a Nanodrop spectrometer. Samples of equal protein concentrations were then prepared for loading on to NuPAGE Bis-Tris Mini Gels (4-12% SDS Polyacrylamide) by combining them with 2.5 μl of NuPAGE LDS (4×) sample buffer, 1 μl of NuPAGE Reducing Agent (10×), 6.5 μl of deionized water (Invitrogen). Electrophoresis was then conducted with running gels with 1× MOPS running buffer with one lane reserved for the protein ladder and one for the positive control (liver homogenate). Protein was then transferred to nitrocellulose using 1× NuPAGE Transfer buffer. The blots were blocked in 5% milk PBS-10% tween solution. All blots were probed with primary antibodies (Ab) at 4°C overnight. Primary (1°) Ab for PXR, P450, StAR, and 5α-reductase were purchased from Santa Cruz Biotechnology; 3β-HSD was received from Dr. Penning, and actin was purchased from Sigma. The secondary (2°) Ab was a Goat Anti-Mouse IG (H+L) Horseradish Peroxidase Conjugate (Bio-rad, Hercules, CA, USA). Concentrations of Ab were: PXR (1° Ab concentration 1:1000; 2° Ab concentration 1:2500); P450 (1° Ab concentration 1:2500; 2° Ab concentration 1:2500); StAR (1° Ab concentration 1:1000; 2° Ab concentration 1:2500); 3β-HSD (1° Ab concentration 1:2500; 2° Ab concentration 1:2500); 5α-reductase (1° Ab concentration 1:1500; 2° Ab concentration 1:2500), actin (1° Ab concentration 1:500; 2° Ab concentration 1:2500). Blots were probed with 2° Ab for 1 hour on a shaker at room temperature (Bio-rad). Results were visualized using DuoLuX Chemiluminescent/Fluorescent Substrate Kit for Peroxidase (Vector Laboratories), imaged on a ChemiDoc XRS (Bio-rad), and analyzed using ImageJ software. Expression was not detected (ND) for 3β-HSD in either group or for 5α-reductase in the wild-type controls, albeit Alzheimer's disease mice showed 2.0 relative intensity. Relative intensity values in the hippocampus (mean ± sem) for PXR (wildtype 0.4 ± 0.2; AD 1.0 ± 0.2), P450 (wildtype 0.3 ± 0.1; AD 1.4 ± 0.3), StAR (wildtype 0.6 ± 0.1; AD 1.0 ± 0.3), 3α-HSD (wildtype 0.4 ± 0.2; AD 0.8 ± 0.5) and 5α-reductase (wildtype 0.5 ± 0.2; AD 1.0 ± 0.7). Relative intensity values in the cortex (mean ± sem) for PXR (wildtype 2.2 ± 1.2; AD 1.0 ± 0.3), P450 (wildtype 1.7 ± 0.8; AD 0.6 ± 0.1), StAR (wildtype 1.4 ± 0.8; AD 0.9 ± 0.2), 3α-HSD (wildtype ND; AD ND) and 5α-reductase (wildtype ND; AD 2.0 ± 1.2).

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Table 1

Preclinical and clinical evidences for dysregulation in neurosteroidogenesis in neuropsychiatric and neurologic disorders.

Disease	Experimental studies	
	Mouse / Rat	**Human**
Neuropsychiatric disorders		
Anxiety disorders	Anxiolytic-like effects of 3α, 5α-THP in several animal models (12, 68). Anxiolytic-like effects of TSPO ligands in animal models (117).	Increased serum levels of 3α, 5α-THP and 3α, 5β-THP, and decreased 3β, 5α-THP levels in patients with panic disorder (68). No change in 3α, 5α-THP levels in patients with generalized anxiety disorders (68). Decreased TSPO expression in platelets and lymphocytes (104). Anti-panic effects of TSPO ligands in experimental induced anxiety (117).
Depression	Antidepressant-like effects of 3α, 5α-THP (13). Decreased brain 3α, 5α-THP levels in animal models of depression, and normalization by antidepressant treatment (75-77).	Decreased cerebrospinal fluid and serum 3α, 5α-THP levels, and normalization by antidepressant drugs (214, 215). Decreased serum levels of pregnenolone, progesterone, 3α, 5α-THP, 3α, 5β-THP, DHEA, 3α, 5α-androsterone and 3α, 5β-androsterone in women with a history of depression (69).
Premenstrual dysphoric disorder (PMDD)	Progesterone withdrawal model of PMDD associated with a upregulation of extrasynaptic α4/δ $GABA_A$ receptors and decreased sensitivity to benzodiazepines (58).	Symptoms start at ovulation, paralleling the rise in progesterone and 3α, 5α-THP levels, and severity reaches its maximum at the neuroactive steroid peak (70). Administration of progesterone or 3α, 5α-THP exacerbates negative mood symptoms in PMDD patients (70).
Post-traumatic stress disorder (PTSD)	Decreased brain 3α, 5α-THP in the socially isolated mouse model of PTSD (78). Ganaxolone improves behavioral deficits in the socially isolated mouse model of PTSD (216).	Decreased cerebrospinal fluid 3α, 5α-THP concentrations (217). Decreased TSPO expression in platelets (104).
Bipolar disorder	--	Increased pregnenolone and DHEA levels in posterior cingulate and parietal cortex (72). Increased plasma progesterone and 3α, 5α-THP levels (218). Decreased TSPO expression in platelets (104).
Schizophrenia	Increased cerebral cortical 3α, 5α-THP following olanzapine and clozapine administration in rats (80).	Increased pregnenolone and DHEA levels in posterior cingulate and parietal cortex (72). Decreased 3α, 5α-THP levels in parietal cortex (72). Decreased TSPO expression in platelets (104). Pregnenolone treatment improves cognition and negative symptoms in patients with schizophrenia (81).
Addiction	3α, 5α-THP has rewarding properties (20, 21, 219). Neuroactive steroids have ethanol-like discriminative stimulus properties (22, 102, 191). Acute administration of psychoactive drugs with abuse liability increases neuroactive steroid levels (25-30). Neuroactive steroids modulate ethanol and cocaine intake (22, 204, 205, 220-222). Overexpression of P450scc in the ventral tegmental area increases 3α, 5α-THP and reduces ethanol reinforcement and consumption (161).	Increased plasma 3α, 5α-THP levels in adolescents following alcohol intoxication (195, 196). Decreases serum 3α, 5α-THP and 3α, 5α-THDOC during alcohol withdrawal (73). Neuroactive steroids mediate subjective effects of ethanol (198, 200).
Neurological disorders		
Epilepsy	Anticonvulsant effects of 3α, 5α-THP in several animal models (14, 84).	Clinical trials with ganaxolone (82) and progesterone (83). 3α, 5α-THP treatment for pediatric super-refractory status epilepticus (223).
Alzheimer's disease	Decreased neuroactive steroid precursors in brain of 3×TgAD mice (88).	Decreased prefrontal cortex 3α, 5α-THP levels, which are inversely correlated with neuropathological disease stage (89).

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Disease	Experimental studies	
	Mouse / Rat	**Human**
	Neuroprotective and neurotrophic effects of 3α, 5α-THP in 3×TgAD mice (42, 94). Up-regulation of glial TSPO (104). TSPO ligands reverse AD-related neuropathology in 3×TgAD mice (116). Progesterone administration increases progesterone and 3α, 5α-THP levels in the cortex of APPswe+PSEN1Δe9 mice; it also improves cortically-mediated but not hippocampal-mediated cognitive tasks (156, 157). Increased 17β-HSD10 expression in brains of Alzheimer's disease mouse models (179).	Decreased plasma 3α, 5α-THP levels in people in the early stages of AD (154). Elevated glial TSPO expression appears early in disease and co-localizes with neuropathology (121). Increased expression of 17β-HSD10 in activated astrocytes (164).
Parkinson's disease	3α, 5α-THP restores tyrosine hydroxylase neurons and improves motor performance in MTPT-treated mice (43).	Decreased dihydroprogesterone and 3α, 5α-THP levels in plasma and liquor (90).
Multiple sclerosis	Decreased expression of 3α-HSD and 3α, 5α-THP levels in brain of mice with experimental autoimmune encephalomyelitis (39). 3α, 5α-THP treatment attenuates experimental autoimmune encephalomyelitis neuropathology in mice (39). Neuroactive steroid levels in rats are altered in a brain region and sex dependent manner (85).	Decreased 5α-reductase expression and 3α, 5α-THP levels in the white matter (39). Increased TSPO expression in white matter lesions correlates with brain damage (104). Increased levels of neuroactive steroid precursors and decreased levels of dihydroprogesterone, 3α, 5α-THP, and dihydrotestosterone in plasma and cerebrospinal fluid of male patients (86).
Niemann-Pick type C disease	Decreased steroidogenic enzymes expression, as well as pregnenolone and 3α, 5α-THP brain levels in NP-C mice (44). Neuroprotective effect of 3α, 5α-THP in NP-C mice (44).	--
Diabetic neuropathy	Decreased brain and peripheral neuroactive steroid levels in rats with streptozotocin-induced diabetes (92). Neuroprotective effects of progesterone, dihydroprogesterone and 3α, 5α-THP in streptozotocin-induced diabetic neuropathy in rats (96).	--
Traumatic brain injury	Neuroprotective effects of progesterone and 3α, 5α-THP in rats (31). Progesterone and DHEA levels correlate with neurological recovery from TBI in mice (91).	Negative outcome of clinical trials with progesterone (224).
Stroke	Neuroprotective effects of progesterone and 3α, 5α-THP in mice (97).	Increased TSPO expression in primary lesion and remote areas (104).