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Sex, Sex steroids and Brain injury

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Abstract

Biologic sex and sex steroids are important factors in clinical and experimental stroke and traumatic brain injury (TBI). Laboratory data strongly show that progesterone treatment after TBI reduces edema, improves outcomes and restores blood brain barrier function. Clinical studies to date agree with these data, and there are ongoing human trials for progesterone treatment after TBI. Estrogen has accumulated an impressive reputation as a neuroprotectant when evaluated at physiologically relevant doses in laboratory studies of stroke, but translation to patients remains to be shown. The role of androgens in male stroke or TBI is understudied and important to pursue given the epidemiology of stroke and trauma in men. To date, male sex steroids remain largely evaluated at the bench rather than the bedside. This review evaluates key evidence and highlights the importance of the platform on which brain injury occurs, i.e. genetic sex and hormonal modulators.

Keywords

stroke; brain ischemia; estradiol; neurosteroids; progesterone; allopreganolone; androgens

1. Sex differences in brain injury

It is now clear that biological sex alters the incidence of, and outcome from, ischemic and traumatic brain injury. For example, male sex is an acknowledged risk factor for stroke, and in most epidemiological series, stroke occurs more frequently in men vs. women. This sexually dimorphic disease pattern remains apparent until ages well beyond the menopausal years [1, 2]. Nevertheless, stroke risk increases with age in both sexes, and there is broad evidence that outcome from an ischemic event is worse in aged women than in their male counterparts. Knowledge of mechanisms of ischemic cell death and neuroprotection is important for both sexes, but current evidence suggests that these mechanisms are not identical in males and females.

a. Sex differences in ischemic outcomes: animal models

Animal models of brain injury, typically rodents, have been used to evaluate side-by-side outcomes from ischemia or trauma. In most reports, females fare better than do their age-matched male counterparts. Early evidence in female vs. male spontaneously hypertensive, genetically stroke prone rats uncovered the male phenotype of “ischemia-sensitivity”[3]. This landmark study of 2000 animals showed that life expectancy is longer in the female, and the development of cerebral hemorrhage is delayed until an advanced age [3]. Subsequently, a

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number of studies have shown that outcome from experimental brain injury is clearly sex-linked. Female rats and mice of various inbred and outbred strains experience smaller tissue damage for an equivalent insult from focal or global cerebral ischemia [4-7] and improved functional outcome [8]. Similarly, male animals sustain greater injury than do age-matched females after traumatic brain injury [9]. We have explored complicated rodent models with genetic risk factors associated with human stroke, e.g. insulin-dependent genetic diabetes [10]. non-insulin dependent diabetes [11] and hypertension [12]. In each genetic strain and despite deleterious complications from diabetes or hypertension, females are less sensitive to cerebral ischemia than are males.

b. Sex-specific cultures: hormone independent cell death or survival

In vitro data more directly support the concept that cell death mechanisms can be sex-specific. This specificity is typically modeled in primary cell cultures grown without background steroids. Under such conditions, some molecular pathways of cell death or survival diverge based on the genetic sex of the tissue. For example, cultured female dopaminergic neurons tolerate exposure to toxic dopamine concentrations and survive twofold relative to male cells [13]. Similarly, female neurons from the cortical plate or ventricular zone have greater longevity in culture than do male cells, and differentially express higher levels of phosphorylated kinases such as Akt [14]. Sensitivity to glutamate, peroxynitrate (ONOO) and staurosporine in neuronal culture is sex specific, with male neurons being more susceptible to glutamate and ONOO than females. In contrast, response to oxidants such as H2O2 is gender neutral [15]. These observations are not limited to neurons. Cell death resulting from oxygen-glucose deprivation is less in female vs male cultured astrocytes or hippocampal slices [16, 17].

In summary, these findings suggest that the response to cerebral injury in vivo and in vitro is partially a function of the sex of the cell. However, this in no way discounts the importance of gonadal steroids or brain-derived neurosteroids as modulators of oxidant, toxic and ischemic challenges to the brain. The role of progesterone, estradiol and testosterone in shaping neuro-injury is reviewed in subsequent sections.

2. Neuroprotective effects of Progesterone

Progesterone is synthesized from cholesterol by the gonads, adrenal gland or placenta. In addition, progesterone can be generated within the brain as a neurosteroid, i.e. steroid hormones that accumulate in the central nervous system (CNS) either by *de novo* synthesis from cholesterol or *in situ* metabolism of precursors from the blood [18]. In contrast, the term neuro-active steroid refers to any steroid having a direct effect in the CNS, independent of source of production. Within brain, progesterone is metabolized to the highly neuro-active metabolites 5αDH-progesterone and 3α,5αTH-progesterone (allopregnanolone; ALLO). Progesterone's neuroprotective actions are mediated, in part, by these neuro-active metabolites.

Exogenous progesterone protects the CNS in a variety of experimental animal models of neurodegeneration, including spinal cord injury [19-24], penetrating or diffuse brain injury, traumatic brain injury (TBI) [25], transient global and focal cerebral ischemia {Stein, 2008 12702 /id;Schumacher, 2007 12749 /id}. Many of these experimental studies emphasize progesterone's attractive attributes from a clinical perspective, i.e. its relatively long therapeutic window that extends up to 2 hours after middle cerebral artery occlusion [27] and to 24 hrs after experimental TBI [28]. The ability of the active metabolite ALLO to mimic the beneficial effects of progesterone in many models points to metabolism in the CNS as a key factor in the steroid's neuroprotection. In contrast, recent clinical data showed no benefit or even harmful effects on incidence of cerebrovascular disease in postmenopausal women using estrogen/progestin formulations [29-31]. One explanation for this apparent discrepancy is that most synthetic progestins cannot be converted to neuro-active metabolites such as ALLO. In fact, the most

commonly used progestin in hormone replacement therapy, medroxyprogesterone acetate (MPA), appears to antagonize the beneficial effects of estrogen against glutamate toxicity in hippocampal neuronal cultures [32, 33]. In contrast, progesterone protects hippocampal neurons from glutamate toxicity and provides an additive benefit to estrogen-treated neurons [32, 34]. Similarly, MPA decreases estrogen protection against cerebral ischemia in subcortical brain regions in rat [35], while progesterone does not [36]. Therefore, our emerging knowledge of steroid synthesis and metabolism within the CNS may inform our clinical use of these complex substances.

a.Neurosteroids

Evidence began to emerge in the early 1980's that the brain is a steroidogenic organ, when Baulieu and co-workers found that the steroids dehydroepiandrosterone (DHEA) and pregnenolone, and their sulfated esters, were present in greater concentration in the brain than in circulation [37, 38]. Importantly, brain concentrations of all these steroids remained very high after adrenalectomy and gondadectomy. This gave rise to the now well accepted concept that steroids are produced in the brain, termed neurosteroids [18, 22]. The metabolic enzymes required to generate and metabolize steroids are present throughout the rodent and human central nervous system, although not expressed uniformly in all brain regions (For review see [39-41]). The non-uniform distribution of the metabolic enzymes points to the possibility of regional differences in generation and metabolism of neurosteroids, although this has not yet been thoroughly investigated.

Synthesis of neurosteroids begins with the conversion of cholesterol to pregnenolone within the mitochondria by the enzyme cytochrome P450scc (cholesterol side-chain cleavage enzyme) (Fig. 1). P450scc is the rate limiting enzyme in neurosteroid biosynthesis. Interestingly, P450scc, and its human counterpart Cyp11a1, is more highly expressed in female brain as compared to male [42, 43], indicating the possibility of sexually dimorphic synthesis of neurosteroids. Pregnenolone is the precursor of all neurosteroids, being converted directly into DHEA or progesterone (Fig. 1). Further metabolism of DHEA leads to the production of androgens (testosterone and DHT) as well as 17β-estradiol and derivatives. On the other hand, progesterone is predominantly converted to its highly active metabolites 5α-DHP and 3α,5α-THP (ALLO). While the focus of this review is on the beneficial effects of estradiol and progesterone, it is important to note that DHEA and its sulfated ester DHEAS are neuroprotective in a variety of experimental models. DHEA and DHEAS protect rat hippocampal neurons against N-methyl-D-aspartate (NMDA)-induced excitotoxicity [44, 45], β-amyloid peptide toxicity [46] and oxygen-glucose deprivation [47]. Importantly, DHEAS prevents ischemia-induced hippocampal neuron cell loss and impairment of hippocampal long-term potentiation following forebrain ischemia [48]. In addition, the PROG precursor PREG and PREGS also exhibit beneficial effects in experimental models of neurodegeneration [49, 50].

b. Progesterone and Stroke

Acute administration of progesterone either immediately before or after transient focal cerebral ischemia decreases infarct volume and improves behavioral outcome in male mice [51, 52] and rats [53-57] and reduces hippocampal neuron loss following global cerebral ischemia (four vessel occlusion; 4-VO) in male rats [58]. The therapeutic window for acute progesterone in male rats has been shown to be 2 hours after focal cerebral ischemia [27] and 24 hr after global ischemia[58], making it a promising molecule for pharmacological intervention following stroke. Notably, progesterone is an effective neuroprotectant in the more complex cat brain [59]. While most of the studies to date have focused on male animals, progesterone is effective in reproductively senescent aged and young ovariectomized female rats [53, 54]. However, others have observed either no benefit [36] or worse outcome [60] following chronic progesterone treatment, suggesting that dose and duration of steroid administration is important to outcome.

c. Progesterone and Traumatic Brain Injury

Progesterone has been well-studied in models of TBI, for example controlled contusion of the medial frontal cortex (For review see [25]). Combined pre-and post-injury treatment decreases cerebral edema and neuronal loss at 72 hours in both young and aged male and female animals[61, 62]. Similar results are observed with doses administered up to 48 hours post-injury, although the steroid was most effective when administered within 2 hr of trauma[28]. Similarly, physiological levels of progesterone provide protection in ovariectomized female rodents[63]. The main effect observed in these studies is that of restricting post-injury edema. As with ischemic brain injury, progesterone's benefits are dose dependent [64] and may be lost at very high doses [65]. Importantly, progesterone treatment improves functional recovery following experimental TBI [62, 66-68].

These experimental observations have been extended to clinical trials involving patients with TBI [69]. Patients of both sexes (n=100) with moderate to severe brain damage from blunt head trauma were enrolled in a prospective, randomized, placebo controlled study. Progesterone was continuously administered by intravenous infusion for 3 days post-injury, and mortality was reduced in patients receiving progesterone as compared to placebo-treatment. Functional recovery was also significantly better in moderately injured patients who received progesterone. Similar positive results have been recently reported with severe traumatic brain injury [70]. While these results must be confirmed in a larger, fully powered trial, they are the first evidence that progesterone may be effective for the treatment of clinical TBI.

3. Mechanisms of Progesterone Neuroprotection

Studies of experimental TBI again provide the best clues as to progesterone's mechanisms of protection. For example, PROG decreases vasogenic and cytotoxic cerebral edema following TBI [28] and focal stroke [51, 52, 71]. Recent evidence suggests that the steroid regulates expression of aquaporin-4, a water permeable channel that likely plays a significant role in edema formation following brain injury [72]. Additional evidence implicates progesterone's transcriptional actions, by increasing expression of anti-apoptotic genes such as Bcl-2, and decreasing pro-apoptotic genes such as bax, bad and caspase-3 activity [73, 74]. Inhibition of sigma1 receptors has also been implicated [75], as has anti-oxidant actions as revealed by a reduction in lipid peroxidation after TBI [76]. Progesterone is thought to have anti-inflammatory actions by reducing microglia activation, toll-like receptor expression [68] and pro-inflammatory cytokines [77 78 67, 79, 80].

In addition to neuroprotection, progesterone may play a role in post-injury remyelination and repair [26]. The steroid increases the rate of myelin formation in cultured Schwann cells [81] and improves regeneration of lesioned mouse sciatic nerve [82]. Within the central nervous system, progesterone stimulated oligodendrocyte proliferation and subsequent myelination [20, 83-85], possibly by progesterone receptor dependent mechanisms [83]. Interestingly, the latter is the only effect to date directly attributed to classical nuclear progesterone receptor signaling.

4. Allopreganolone

The ability of the nervous system to metabolize progesterone complicates our understanding of its mechanism of protection. To date few studies have demonstrated that progesterone, rather than a metabolite, is the active molecule responsible for protection in brain ischemia and injury. In fact, Sayeed et al. recently demonstrated that ALLO is more effective than progesterone in transient ischemic injury, suggesting that it is the metabolite that is of greatest potency [55]. Further, we have observed that progesterone protection of cerebellar Purkinje cells treated with oxygen and glucose deprivation requires metabolism to ALLO [86].

ALLO is synthesized in the brain by sequential reductions by 5α-R and 3α-HSD. 5α-R reduces progesterone to 5αDH-PROG in a uni-directional reaction that is rate-limiting in the production of ALLO. 3α-HSD converts 5αDH-PROG into 3α,5αTH-PROG (ALLO), and is also capable of oxidizing ALLO into 5αDH-PROG. ALLO is neuroprotective in several animal models of neurodegeneration and injury, including Alzheimers [87], Niemann-Pick C disease [88], kainate excitotoxicity [89], focal cerebral ischemia [55], and TBI [90]. ALLO improves post-injury neurogenesis [91], decrease apoptosis via activation of PKB/Akt kinase [92], and like progesterone, increases anti-apoptotic gene transcription [93, 94] and decreases inflammation [79]. Because ALLO is among the most abundant and potent endogenous positive modulators of $GABA_A$ receptors, most physiological effects of ALLO are attributed to this mechanism (For review see [95]). Interaction with $GABA_A$ receptors may also hold the key to ALLO's neuroprotection, as we have recently demonstrated in cultured neurons treated with *in vitro* "ischemic" insults [86].

5. Summary

The brain is a steroidogenic organ, possessing the metabolic enzymes necessary to produce most steroid hormones. Progesterone, among the most abundant neurosteroids in the brain, is neuroprotective in a variety of experimental animal settings. This protection may be mediated by altering specific gene expression via binding to the classical progesterone receptor (PR). Alternatively, neuro-active progesterone metabolites interact directly with neuronal membrane receptors and intracellular signaling cascades. In fact, the active metabolite ALLO mimics PROG neuroprotection in most experimental settings. Few studies have attempted to determine the relation between the parent hormone progesterone and it conversion to metabolites in animal studies of brain injury, but our recent data suggests that progesterone neuroprotection is completely mediated by metabolism to active metabolites, likely ALLO.

6. Estradiol: The best studied sex steroid in neuroprotection

The estrogens, particularly E2, have been widely studied in experimental ischemic and hemorrhagic brain injury. Almost without exception, these studies report that E2 reduces tissue damage, improves functional recovery and may stimulate repair processes. Interest in the estrogen steroid family arose, in part, from clinical use of contraceptives and of perimenopausal hormone replacement therapy in women with potential cerebrovascular disease. In addition, two key observations fueled ongoing interest in estrogens as neuroprotectants. First, outcome from experimental stroke in female animals is influenced by the stage of the estrous cycle. Proestrus is associated with smaller tissue damage (quantified as infarct size) after focal cerebral ischemia in rat, and this association is generally attributed to the high levels of E2 produced during this stage. In contrast, metestrus (low E2 production) is associated with greater brain damage [96]. Second, female animals sustain less tissue damage than do males after similar ischemic or traumatic insults [5, 6, 53, 97]. The benefit of female gender is lost in reproductively senescent animals or after ovariectomy but can be restored by estrogen supplementation [5, 53]. These data emphasize that estrogens are highly important to outcome from ischemic brain injury in the female.

a. Basis for estrogen as a neuroprotective therapy

Subsequently, a large number of laboratories focused on the estrogens as a form of endogenous neuroprotection or as a potential therapy for stroke [98]. Estrogen therapy of a variety of types and formulations has been shown to be beneficial in the male ischemic brain [99-101]. While most of these animal studies emphasized tissue damage quantified as infarct size or cell loss early after the insult, chronic E2 supplementation also improves functional outcome [8, 102].

Despite the mass of evidence that E2 is neuroprotective, it should be noted that some investigators have found no effect or even detrimental effects of E2 in experimental stroke. Such findings may be related to the dose of E2 used since neuroprotection may be lost or detrimental effects may occur at higher doses [12 103]. E2 may also be less beneficial with increased severity of injury, e.g., prolonged or permanent vessel occlusion as opposed to transient occlusion [104, 105]. Age may also be a confounding factor, as steroid receptor expression is altered with reproductive senescence [106]. A recent study showed that E2 replacement reduced infarct size, systemic and brain inflammation only if it was initiated immediately after ovariectomy, but not after a prolonged period of hypoestrogenicity [107]. This observation, if translated to the human, may have clinical implications for postmenopausal women. Overall, experimental data supports a beneficial effect of E2, however dissenting findings suggest that E2 mediated neuroprotection may depend on the specifics of the experimental and clinical situation.

One final consideration is that acute steroid injection has been protective in some studies, while in others only chronic therapy produces beneficial results [108, 109]. The latter issue influenced subsequent investigations into E2's protective mechanisms, i.e. if such mechanisms are mediated by the steroid's cognate estrogen receptors (ER) of either subtype and if transcriptional processes are required for benefit. A profound understanding of these mechanisms is required if we are to develop drugs that mirror estrogen's neuroprotection without the undesirable hormonal effects.

7. Mechanisms of Estrogenic Neuroprotection

It is now well-recognized that E2 elicits both genomic and non-genomic protective actions following an ischemic insult **(Fig.2)**. These actions include stabilizing the blood-brain barrier and subsequently reducing brain edema [110 111], increasing blood flow during and after the ischemic insult [112, 113], engaging anti-inflammatory molecules [114, 115], and increasing expression of cell-survival mediators such as bcl2 and the novel cocaine and amphetamine regulated transcript (CART) [116, 117]. In addition, E2 can act as a concentration-dependent antioxidant and anti-lipid peroxidation agent [118-120]. The steroid also interacts with N-methyl-D-aspartate (NMDA) receptors, producing receptor activation at low doses (Connell BJ et al., 2007), but inhibiting receptors and subsequently blunting excitotoxicity at higher E2 doses [121]. Lastly, E2 not only acts as an acute protectant after ischemia but can also improve regeneration and plasticity of new neurons [107]. This may contribute to improved post-ischemic memory after ischemia that is seen in estrogen-supplemented animals [102].

A plethora of literature documents E2 mechanisms that are transcriptional, involving genes that do or do not carry estrogen response elements (EREs), as well as non-transcriptional rapid signaling action through interaction with membrane-bound G-proteins, modification of protein phosphorylation and of intracellular second messenger levels such as cAMP or calcium. The relative importance of these mechanisms in explaining E2's ability to improve ischemic and traumatic neuronal injury is unclear. For example, whether classical nuclear ERs are essential for neuroprotection has remained controversial for some time, and there is disagreement amongst studies that use pharmacological ER agonists vs. genetically ER deficient mice. It is known that both ER-α and ER-β are widely expressed in all cell types throughout the brain, i.e., neurons, glia, and endothelial cells, and are present in ischemia-sensitive areas such as neocortex and hippocampus [122-124]. Not surprisingly, concentrations of ER-α and ER-β are higher in adult females as compared to males [122]. We showed in a very early study that generalized pharmacological blockade of ERs with ICI182,780 exacerbated ischemic brain injury in female mice, suggesting that nuclear receptors play a role [125]. However, ERα gene deletion in female mice was paradoxically associated with decreased infarct size, which may be related to the enhanced brain tissue perfusion observed in ERα knockout mice [126]. In a

model of permanent cerebral vessel occlusion, E2 failed to protect cortical tissue in ERα, but not ERβ, knockout mice, suggesting that E2 may signal through ERα to reduce stroke damage [127]. However, a selective ERβ, but not ERα, agonist reduced hippocampal ischemic damage in ovariectomized mice [12], suggesting that different receptor subtypes may mediate E2 signaling in a model-specific and possible brain-region specific manner. Clearly, further work is required to translate our extensive understanding of E2 signaling under physiological conditions to those of brain injury.

8. Androgens and Brain Injury in the Male

Despite the relative paucity of data that address "male sensitivity" to brain injury, emerging evidence suggests that androgens strongly impact outcome and mechanisms of cell death. In men, low testosterone levels have been associated with poor outcome after acute ischemic events [128]. Androgen levels are inversely associated with stroke severity, infarct size, and 6-month mortality; and total and free testosterone levels tend to normalize within 6 months following stroke. These data do not necessarily suggest a direct causal relationship because brain injury provokes an acute stress reaction that causes a reduction in plasma testosterone. Animal studies are starting to address this issue. In male rats, androgen replacement in castrates increases histological damage from stroke [100, 129, 130], while stressors that reduce pre-ischemic testosterone level also reduce stroke damage [131]. In contrast, testosterone replacement in male castrates *after* stroke accelerates functional recovery [67]. Beneficial effects of androgens following peripheral nerve damage or brain trauma have also been reported in animals [132-134]. These apparently conflicting results may be reconciled by the hypothesis that androgens are deleterious during acute injury but beneficial during the recovery phase. Potential mechanisms by which androgens could enhance post-stroke recovery include normalization of reperfusion, promotion of axonal regeneration, synaptogenesis, and neurogenesis [135].

9. Conclusion

Biologic sex and sex steroids are important factors in clinical and experimental brain injury. Our understanding of these factors is relatively recent, in part, because many pre-clinical and mechanistic studies have been carried out only in male animals and mixed sex cell cultures. Estrogen has accumulated an impressive reputation as a neuroprotectant in physiologically relevant doses in laboratory studies, but translation to patients remains to be shown. Laboratory data strongly show that progesterone treatment after TBI reduces edema, improves outcomes and restores blood brain barrier function. Clinical studies to date agree with these data, and there are ongoing human trials for progesterone treatment after TBI. The role of androgens in male stroke or TBI is understudied and important to pursue given the epidemiology of stroke and trauma in men. To date, male sex steroids remain largely evaluated at the bench rather than the bedside. Further studies of genetic sex and of the hormonal platform on which brain injury occurs are needed and should provide new insights for science and health care.

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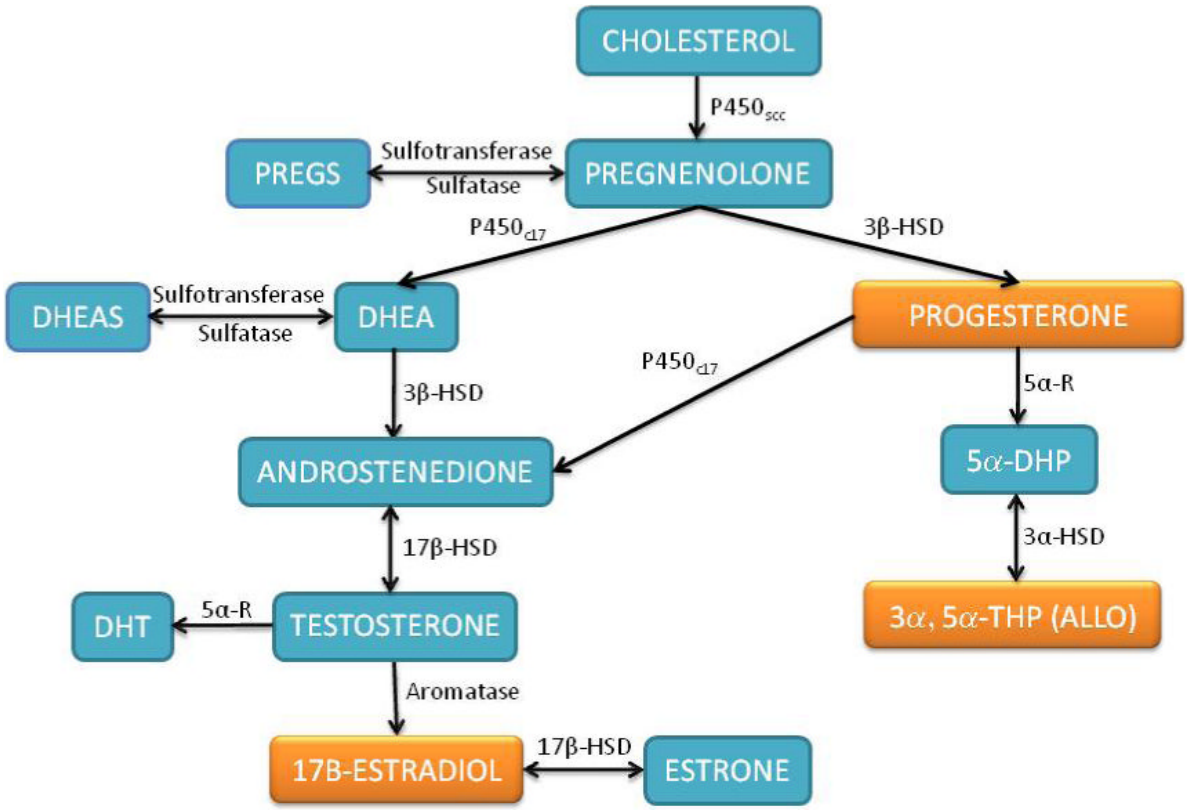

Figure 1. Overview of Neurosteroid Synthesis

Cholesterol is converted into pregnenolone within the mitochondria and then further converted into DHEA via $P450_{c17}$ or progesterone by 3β-HSD. DHEA is converted into testosterone via the intermediate androstenedione. Progesterone can also be converted to testosterone by $P450_{c17}$. Testosterone is converted to its more potent analog DHT by 5α-R or converted into 17β-estradiol by the enzyme Aromatase. Progesterone is converted to its highly active metabolites 5α-DHP and 3α,5α-THP (ALLO) by consecutive reductions by 5α-R and then 3α-HSD. Both pregnenolone and DHEA can be interconverted to sulfated esthers by sulfotransferase and sulfatase enzymes. P450scc, mitochondrial cholesterol side-chain cleavage enzyme; P450c17, microsomal 17 hyrdoxylase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5α-reductase; 5α-HSD, 5α-hyrdoxysteroid dehydrogenase; 17β-HSD, 17β-hyrdoxysteroid dehydrogenase; Aromatase, P450-aromatase; DHT, dihydrotestosterone; 3α,5α-THP, Allopregnanolone.