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Review

Biosynthesis and action of neurosteroids

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Abstract

Over the past decade, it has become clear that the brain, like the gonad, adrenal and placenta, is a steroidogenic organ. However, unlike classic steroidogenic tissues, the synthesis of steroids in the nervous system requires the coordinate expression and regulation of the genes encoding the steroidogenic enzymes in several different cell types (neurons and glia) at different locations in the nervous system, and at distances from the cell bodies. The steroids synthesized by the brain and nervous system, given the name *neurosteroids,* have a wide variety of diverse functions. In general, they mediate their actions, not through classic steroid hormone nuclear receptors, but through other mechanisms such as through ion gated neurotransmitter receptors, or through direct or indirect modulation of other neurotransmitter receptors. We have briefly summarized the biochemistry of the enzymes involved in the biosynthesis of neurosteroids, their localization during development and in the adult, and the regulation of their expression, highlighting both similarities and differences between expression in the brain and in classic steroidogenic tissues.

Theme: Development and regeneration

Topic: Hormones and development

Keywords: P450scc; P450c17; Neurosteroid; Cholinergic neuron; Neurodegenerative disease; NP-C; Transcription; Gene expression

Contents

1. Introduction

The ever-growing field of neurosteroidogenesis began initially from the intersection of research in the neuropharmacology of ligand-gated ion channel receptors and research in steroid hormone synthesis. The demonstration that steroids could be synthesized de novo in the brain and the simultaneous experiments describing novel functions for certain steroidal compounds at nonclassical $GABA_A$ and NMDA receptors brought this new field to light. First, studies by Harrison and Simmonds and by Majewska demonstrated that certain steroids could modulate $GABA_A$ receptor function [31,39]. They did so by increasing the duration and frequency of $GABA_A$ channel opening, via a

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site that was distinct from the GABA site. This action of steroids was mediated by progesterone derivatives, and the action of those derivatives was stereospecific [1,2,49,51,67]. Other steroids could also modulate GABA-ergic function, but rather than act as a positive modulator, these steroids acted as negative modulators and inhibited GABA-ergic function.

A parallel series of experiments by endocrinologists placed those neuroactive steroids directly at their site of action in the brain. The fact that steroids could be synthesized in the brain came initially from observations made in the 1980s by Baulieu and colleagues who found that steroids such as pregnenolone, DHEA and their sulfate and lipoidal esters were present in higher concentrations in tissue from the nervous system (brain and peripheral nerve) than in the plasma. While these compounds could be due to peripheral synthesis and then sequestration in the brain, Baulieu and colleagues found that the steroids remained in the nervous system long after gonadectomy or adrenalectomy [15,16], suggesting that steroids could be synthesized de novo in the CNS and PNS. Such steroids were named 'neurosteroids' to refer to their unusual origin and to differentiate them from steroids derived from more classical steroidogenic organs such as gonads, adrenals and placentae.

Were these compounds synthesized in the brain or did they accumulate specifically in tissue from the nervous system? Several laboratories, including ours, determined directly if enzymes known to be involved in steroidogenesis, i.e. adrenals, gonads and placentae, could be responsible for neurosteroid synthesis (reviewed in [10,44]). These studies have established unequivocally that the enzymes found in classic steroidogenic tissues are indeed found in the nervous system. Depending upon the steroid synthesized, these steroids could affect gene expression through action at classic intracellular nuclear receptors, or could affect neurotransmission through action at membrane ion-gated and other neurotransmitter receptors.

While it is now well known that neurosteroids can modulate neurotransmitter receptors, what is the consequence of this action, and does this differ between what occurs during development and in the adult? In the adult, neurosteroid stimulation of neurotransmitter receptors results in behavioral effects associated with those receptors: stimulation of $GABA_A$ receptors results in decreased anxiety [67], sedation [21,56–58], and decreases in seizure activity [3,19,22–24,47,66]. Effects may also be mediated through other neurotransmitter receptors (reviewed in [10]). During development, neurosteroids have additional functions. Neurosteroids are involved in neuronal modeling, as DHEA and DHEAS stimulate embryonic axonal and dendritic growth, respectively [9], and allopregnanolone causes neurite regression [3]. Finally, there are effects of neurosteroids on neurotransmitter receptor expression, that ultimately affect the ability of the neurosteroids to mediate their effects [13,14,17,18,25,59].

Since the enzymes that synthesize neurosteroids are the same as those that synthesize classic steroid hormones, we began studies aimed at determining if the transcriptional regulation of these genes was the same in classic steroidogenic tissues and in the nervous system. In addition, we have studied the regulation of neurosteroidogenic enzyme activity by selective serotonin reuptake inhibitors, and have also been using a mouse model of a neurodegenerative disease to study the roles of neurosteroids in development and maintenance of normal neuronal circuits.

2. Biosynthesis of neurosteroids

Steroid hormones are synthesized from cholesterol by a series of enzymes, both P450s and non-P450s, that act in concert to direct the synthesis of one or several distinct steroids in a particular cell. The determination of which steroid will be synthesized by a tissue depends, therefore, on the level of expression of a cohort of enzymes, and/or competition among enzymes for particular substrates. In the adrenals and gonads, the synthesis of androgens, estrogens, glucocorticoids and progestins follows the scheme presented in Fig. 1. In the adrenals, expression of P450c11β in the zona fasciculata/zona glomerulosa allows for the synthesis of glucocorticoids, while expression of P450c11AS in the zona glomerulosa allows for the synthesis of mineralocorticoids. Furthermore, in the human adrenal, P450c17 is a key branchpoint in steroidogenesis as its expression and enzymatic activity also dictates which steroid will be synthesized: lack of its expression in the zona glomerulosa allows for conversion of progesterone to 11-deoxycorticosterone, and together with expression of P450c11AS, ultimately leads to mineralocorticoid synthesis; expression of P450c17 with its 17α hydroxylase activity allows for 17α hydroxylation of pregnenolone, and together with P450c21 and P450c11β, leads to glucocorticoid (cortisol) synthesis in the zona fasciculata; expression of P450c17 with both its 17α hydroxylase and 17,20 lyase activities in the zona reticularis results in cleavage of the c17,20 bond, converting c21 to c19 steroids, and results in androgen synthesis.

While specific steroid synthesis in the adrenal may be regulated by the expression (and activity) of a particular set of steroidogenic enzymes, the ovary requires the participation of two distinct cell types, the granulosa and theca cells. Both of these cells are required for estrogen synthesis because granulosa cells lack P450c17 expression, while theca cells express P450c17. Hence, there must be coordinate expression of the steroidogenic enzymes and shuttling of steroid precursors and products into and out of both the granulosa and theca cells to result in estrogen synthesis. This is possible because theca cells surround granulosa cells, thereby making steroid shuttling relatively easy and efficient.

In the brain, the situation becomes even more compli-

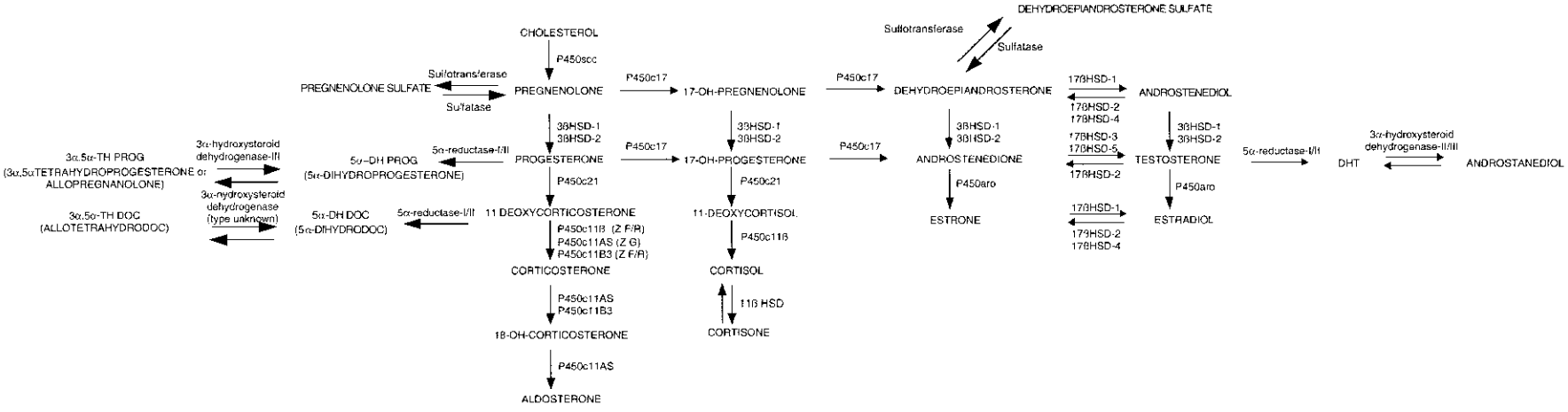

Fig. 1. Pathway for the biosynthesis of neurosteroids in the nervous system. The names for each enzyme are shown by each reaction. P450scc, mitochondrial cholesterol side-chain cleavage enzyme, mediates 20α hydroxylation, 22 hydroxylation, and scission of the c20–22 bond; 3β HSD, a non P450 enzyme(s) bound to the endoplasmic reticulum mediates both 3β hydroxysteroid dehydrogenase and Δ5–Δ4 isomerase activities; P450c11β, mitochondrial 11 hydroxylase, mediates 11 hydroxylation; P450c11AS, mitochondrial aldosterone synthase, mediates c11,18 hydroxylation and 18 oxidation; Z F/R and Z G refer to the adrenal zona fasciculata/reticularis, or zona glomerulosa, that express the particular P450c11 gene; 17βHSD (also called 17 ketosteroid reductase, or 17 KSR), mediates c17β reduction or c17 oxidation. Virtually all these enzymes have been identified in the brains of various species, with the notable exception of P450c21. 21 hydroxylating activity has been demonstrated, but may not be due to P450c21. Synthesis of steroid sulfates mediated by sulfotransferase and sulfatase are also shown.

cated. Not only are the steroidogenic enzymes expressed in distinct cell types (i.e. neurons vs. glia), but their distribution in the brain may not necessarily overlap, with respect to region as well as time in development. In addition, some enzymes may be active in the cell bodies while others may be active as well in the fibers extending from those cell bodies, and hence, steroid precursors/products may exist at sites far away from their cell body of origin.

The first, rate limiting, and hormonally regulated step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone (Fig. 1). This reaction is catalyzed by the mitochondrial enzyme cholesterol side chain cleavage, P450scc, in three successive chemical reactions: 20α-hydroxylation, 22-hydroxylation and scission of the c20–c22 carbon bond cholesterol. The products of this reaction are pregnenolone and isocaproic acid. A single P450scc species is found in all steroidogenic tissue, including the brain [42,43]. We initially used RNA analysis to determine directly if those enzymes known to synthesize steroids in adrenals, gonads, and placenta were also found in the brain [43]. By RT/PCR analysis, we indeed found that several of the genes encoding steroidogenic enzymes are found in a region-specific fashion in the brain. However, the abundance of the mRNAs was extremely low, and therefore we have focused on expression of the proteins, which are more easily detected by immunocytochemistry [7,8].

The molecular biology of steroid hormone biosynthesis, the enzymes involved in their synthesis, has been reviewed [46]. This will serve only as a brief summary of the pathways of steroidogenesis (Fig. 1), which have been detailed elsewhere [10]. Once pregnenolone is synthesized, it can be converted by the specific steroidogenic enzymes to form all the different types of steroid hormones. First, pregnenolone can be converted to progesterone, via 3βHSD, or it can be 17 hydroxylated by P450c17, a microsomal P450. There is substrate specificity for the 17 hydroxylase reaction, with some species preferring Δ5 steroids (i.e. pregnenolone) and others preferring Δ4 steroids (i.e. progesterone) (reviewed in [46]). 17-hydroxypregnenolone can be converted to DHEA by P450c17, which is then converted to androgens, mediated by tissue-specific 17βHSDs, to form androstenediol, and then to testosterone, via 3βHSD. P450aro, aromatase, converts testosterone to estradiol, while 5α reductase converts testosterone to DHT.

Progesterone is metabolized to glucocorticoids, mineralocorticoids, or to neuroactive steroids by a variety of enzymes. Synthesis of glucocorticoids (corticosterone in rodents, cortisol in human beings) and mineralocorticoids (aldosterone) requires 21 hydroxylation, mediated by microsomal P450c21, followed by the action of a zone-specific P450c11: P450c11β in the zona fasciculata/reticularis, to yield corticosterone, or by P450c11AS in the zona glomerulosa, to yield aldosterone. In human beings, glucocorticoid synthesis requires 17 hydroxylation of pregnenolone, but not 17,20 lyase activity, that would yield DHEA. Hence, regulation of 17 hydroxylase versus 17,20 lyase activity of P450c17 will dictate if the human adrenal will synthesize glucocorticoids (17 hydroxylase activity only) or DHEA (both 17 hydroxylase and c17.20 lyase activities).

The synthesis of other neuroactive steroids involves two enzymes: 5α reductase and 3α hydroxysteroid dehydrogenase (3α HSD). Progesterone, deoxycorticosterone, or testosterone are all substrates for 5α reductase, and hence can be converted to their 5α reduced derivatives, 5α dihydroprogesterone (DHP), 5α dihydro deoxycorticosterone (DH DOC), or dihydrotestosterone, respectively. In turn, these steroids can be converted to neuroactive steroids by conversion to their 3α reduced derivatives, mediated by 3α HSD. Rodents have a single 3α HSD that can mediate all these reactions, while human beings have several 3α HSD genes that have substrate specificity [27]. Thus, the developmental and cell-specific expression of all these steroidogenic enzymes will ultimately dictate which steroid and neurosteroid will be synthesized in a particular tissue at a particular time in development.

Our studies focused on determining where these steroidogenic enzymes were found, the cell types in which they are expressed, and their pattern of expression during development [7–11]. Additional studies by others have also provided evidence of region-specific expression of

Table 1
Expression of steroidogenic enzymes in various regions of the brain

Enzyme	Cortex	Hippo-campus	Basal Ganglia	Hypo-thalamus	Thalamus	Cerebellum	Pons/ Medulla	PNS	Ref.
P450scc	++	+	++	+	+	+		++	[7,32,34,43]
P450c17	++	+	+(fibers)	ca	+	+(fibers)	++	++	[8]
3βHSD	+	+	+	+	+	++	++		[20,28,45]
5α reductase	+	+	+	+	+	+	+	+	[5,6,10,38,40,41]
3α HSD	+	+	+	+	+	+	+		[27,30,35–37,40]
STS	+	+	–	–	+	–	–	+	[11]

This table is a composite of data obtained from various species, and includes data from enzymatic activity, mRNAs, and proteins. The relative abundance of each enzyme in the different regions is indicated by one, two or three '+' marks.

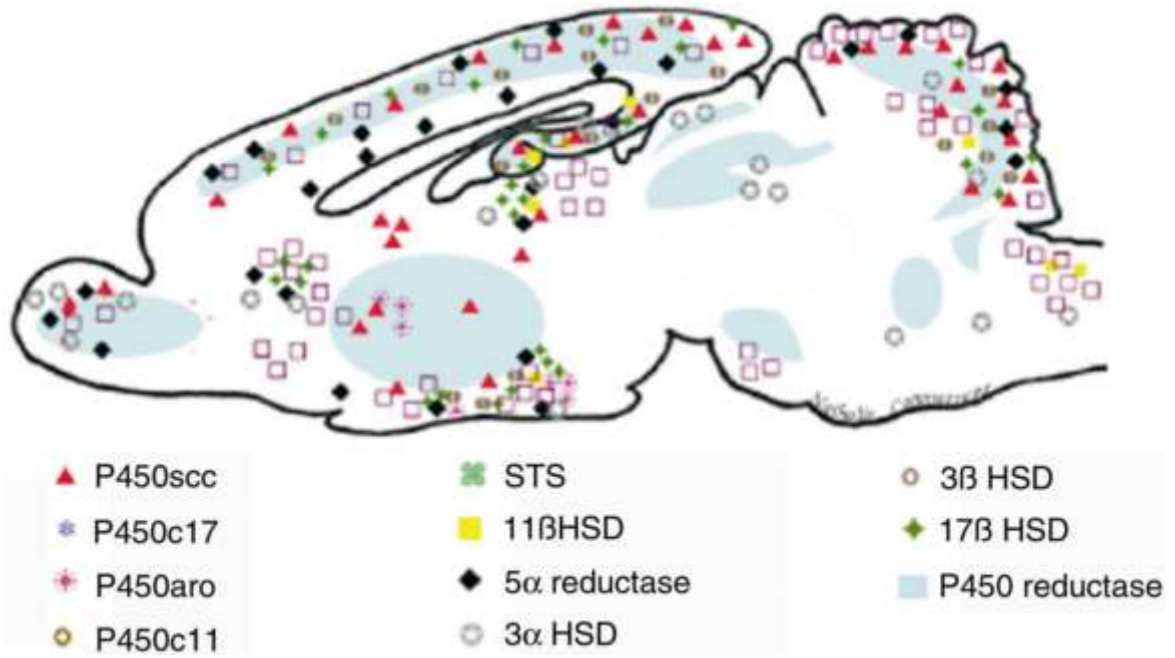

Fig. 2. Cartoon representation of an adult brain showing regional expression of enzymes involved in neurosteroidogenesis. The symbols for the different steroidogenic enzymes are shown on the bottom of the figure. The data from several species including rodent, primates and amphibians are represented. The locations in the various regions of the brain are only approximate, as most of these enzymes have not been co-localized with one another.

steroidogenic enzymes either in the adult or developing brains from a variety of species (reviewed in [10,44])

Table 1 illustrates the expression of several steroidogenic enzymes (protein or mRNA) in different regions of the brain, irrespective of time of expression and species in which the studies were performed. The regional localization of steroidogenic enzymes in the adult brain (compiled from the data from various species) as well as in the developing brain are summarized in cartoon form in Figs. 2 and 3. The cartoon indicates that the expression of the

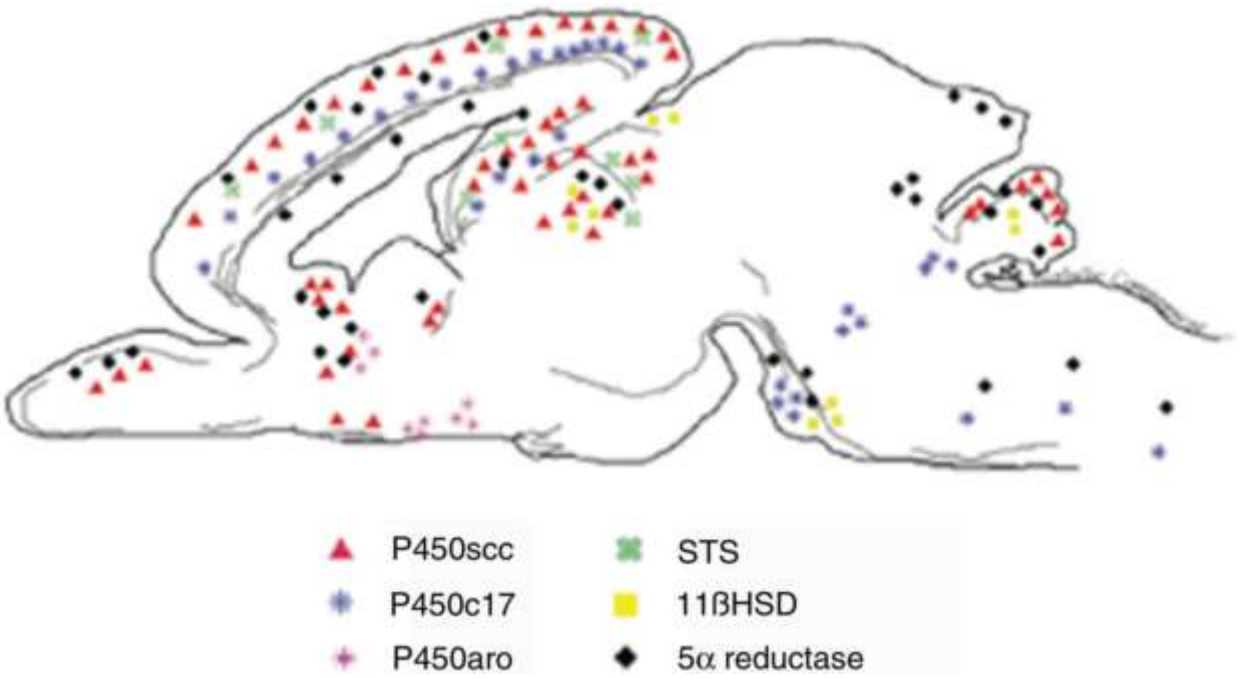

Fig. 3. Cartoon representation of a late embryonic brain (E18.5), showing the regional expression of the neurosteroidogenic enzymes. The symbols for each enzyme are indicated on the bottom.

steroidogenic enzymes is not random, but rather that their expression is localized to particular regions of the brain. As indicated in Table 1 and in Figs. 2 and 3, most of the steroidogenic enzymes have been found in the cortex, hippocampus, olfactory bulb, basal ganglia, hypothalamus, thalamus, cerebellum; the expression of some of the enzymes has also been identified in the tectum, pons, medulla, pituitary and spinal cord, as well as in various regions of the peripheral nervous system. The finding of steroidogenic enzymes in the specific regions of brains from a variety of species indicates that their role in synthesizing neurosteroids and the function of the neurosteroids may be similar throughout evolution. Co-localization of some of the steroidogenic enzymes has been established in whole brain sections or in cultured neuronal or glial cells (reviewed in [10,44], but these studies are far from complete. The demonstration that certain steroidogenic enzymes are expressed in glia, others are expressed in neurons, and still others are expressed in both neurons and in glia adds to the complexity of neurosteroidogenesis. Studies demonstrating the ability of specific brain regions to synthesize or metabolize particular arrays of steroids are also lacking, but will be important to understand more completely the function of specific neurosteroids in specific brain regions.

Developmental analysis of steroidogenic enzyme gene expression has also indicated at least one enzyme, P450c17, is developmentally regulated. It is expressed in various regions of the developing central nervous system, but it is not expressed in these same regions in the adult [8]. These findings indicate that this enzyme participates in the synthesis of specific steroids that may be important during the development of the nervous system, during neurogenesis or when connections among neurons are being established. Once neurons are differentiated or when neural pathways are established, these steroids may have no further function. What are these steroids and how do they function? These are testable hypotheses that we have only begun to study in detail (see below).

3. Action of neurosteroids on neuronal function

The sites of expression of the steroidogenic enzyme P450c17 indicated to us that products of this enzymatic activity, e.g. DHEA or DHEAS, may play a role in targeting thalamic axons to particular regions of the cortex during development. Hence, we sought to determine whether DHEA and DHEAS had an effect on embryonic cortical neurons [9]. We cultured neurons from the cortex of 16.5 day embryos, and determined directly if DHEA and DHEAS affected their morphology. We found that DHEA and DHEAS had distinct effects — DHEA caused axons to grow and DHEAS did not, and DHEAS caused dendrites to grow, and DHEA did not. These effects could be seen with concentrations as low as 10^{-12} M, and were dose-dependent.

The effects of DHEA appeared to be mediated through NMDA receptors. DHEA treatment of neocortical neurons caused a dose-related, sustained increase in intracellular calcium. These effects could be seen in only a small population of individual neurons from the neocortex, suggesting that the effect of DHEA was specific to a subset and not all neurons. DHEA could potentiate the effects of NMDA in increasing intracellular calcium in a synergistic fashion, indicating that DHEA and NMDA acted through different regions of the NMDA receptor. Studies using inhibitors of NMDA receptors, MK801 and D-AP5, both reduced the response of DHEA in a dose-dependent fashion. Interestingly, DHEAS did not follow the same course of action as DHEA. DHEAS did not increase intracellular calcium, as did DHEA, and hence it most likely does not affect the NMDA receptor or other calcium channels as well. The mechanism by which DHEAS stimulates dendritic growth is not via NMDA receptors, and is unknown.

4. Transcriptional regulation of steroidogenic enzymes in the CNS

We have been studying the transcriptional regulation of two genes, P450scc and P450c17, and have identified different regions of these genes that are transcriptionally active in classic steroidogenic tissues vs. the brain (Fig. 4). The 5′ flanking DNA of the rat P450c17 gene has several transcriptionally active domains in adrenal and Leydig cells, as well as in neurons. In adrenals and Leydig cells, the transcription factor SF-1 plays a major role in the regulation of P450c17 [26,69,70]. However, this factor is not found in regions of the nervous system in which P450c17 is expressed [33]. Those data therefore indicated to us that factors other than SF-1 are important for neural expression of P450c17. We have recently identified one factor, called SET, that is expressed in the nervous system in cells and regions that express P450c17 [8,12]. We have not found P450c17 expression in regions that do not express SET, suggesting that this factor is important for neural regulation of P450c17 during development.

In a manner similar to that described for P450c17, we have also identified factors that regulate P450scc in the nervous system during development [29,71]. We previously identified a transcriptionally active glial factor that bound to the -130-/94 region of the P450scc, and have now purified and identified it as the 70 and 80 kDa proteins that comprise the DNA binding proteins that are part of the multimeric DNA-dependent protein kinase Ku. We have also identified members of the Sp family of transcription factors as binding to P450scc and regulating its expression. There are at least 4 members of this family, and not all of these factors are co-expressed with P450scc. Like P450scc,

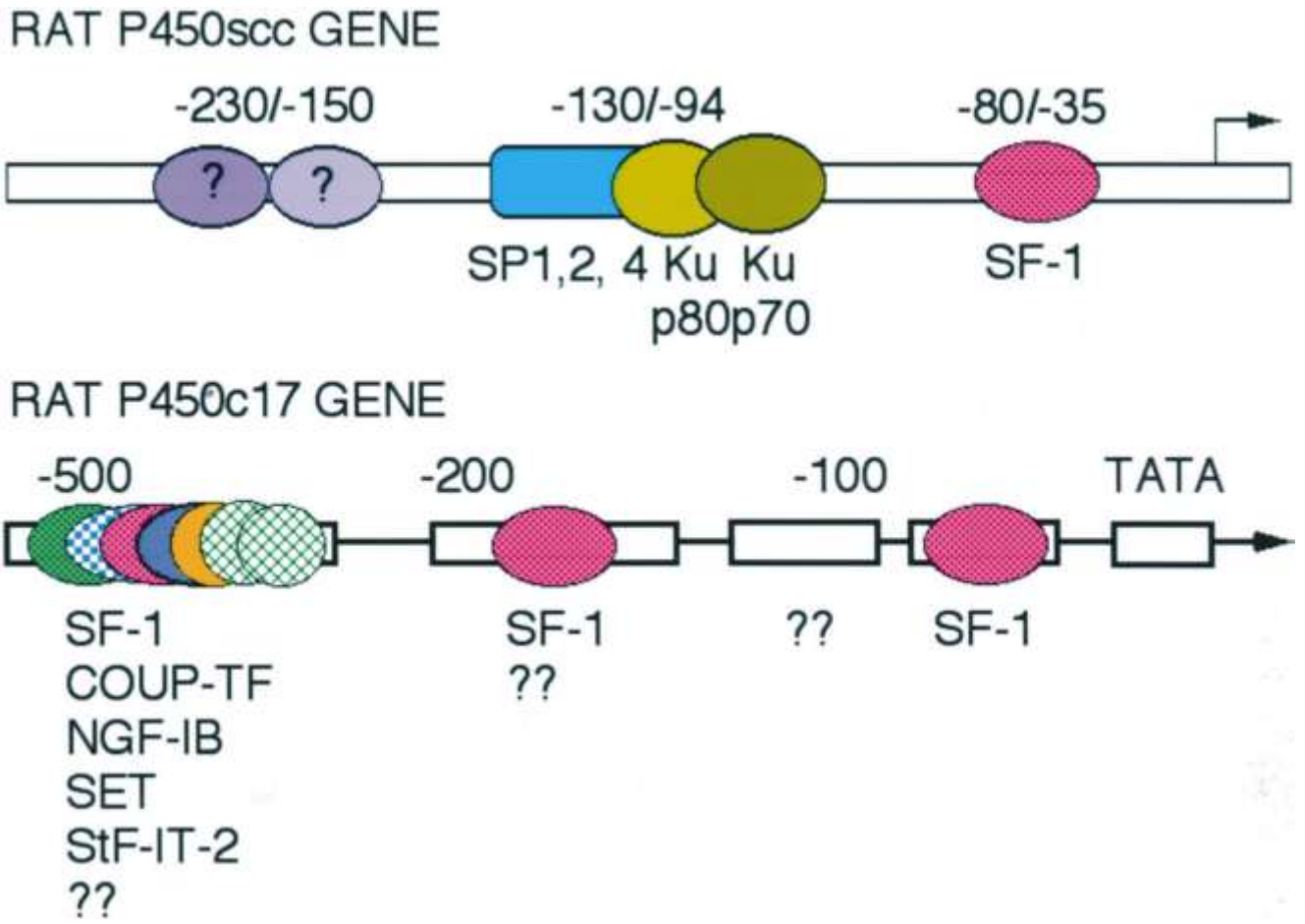

Fig. 4. Cartoon representation of the 5′ flanking regions of the rat P450scc and P450c17 genes, and the transcription factors that bind to these genes. These transcriptionally active regions were determined by transient transfection of gonadal, adrenal, and neural cells in culture, using specific regions of the 5′ flanking regions of these genes, ligated to a reporter gene (luciferase) [12,26,29,68–72].

the expression of Sp transcription factors is developmentally and regionally regulated.

5. Regulation of neurosteroidogenic enzyme activity

Studies by others indicated that neurosteroids may play a role in the etiology of some forms of depression. Initial studies in women with late luteal phase dysphoric disorder suggested that these women could be treated successfully for their depression with use of selective serotonin reuptake inhibitors (SSRIs) [60–62]. In other depressed patients, the levels of neurosteroids (specifically allopregnanolone) in the plasma [55] and cerebrospinal fluid [65] of patients, indicated that these levels were low in the depressed patients, compared with normal controls. Furthermore, the levels of neurosteroids could be increased in those patients by treatment with SSRIs, and that depression rating scales also improved with increased levels of neurosteroids. Thus, there appeared to be a correlation between SSRI treatment and neurosteroid levels. In animal studies, Guidotti's group demonstrated that SSRIs could increase allopregnanolone production in specific regions of rat brains or in brain sections placed in culture [64]. These increases in neurosteroid production occurred rapidly (within 10–15 min), indicating that SSRIs may act directly on the neurosteroidogenic enzymes to increase their activity, rather than through modulating gene transcription or translation.

We performed a series of experiments to test this hypothesis directly [27]. We used recombinant proteins to determine whether SSRIs affected two enzymes: 5α reductase, which converts progesterone to 5α dihydroprogesterone (DHP), and 3α reductase, which converts DHP to allopregnanolone. Our experiments indicated that SSRIs had no effect on the enzymatic activity of 5α reductase, and did not increase the conversion of progesterone to DHP.

We therefore focused on 3α HSD. We cloned rat 3α HSD, and also cloned several human 3α HSD cDNAs to study the effects of SSRIs and other antidepressants on their activity [27]. Using these recombinant proteins in an in vitro assay, we found that the SSRIs fluoxetine and paroxetine decreased the Km of the rat 3α HSD and the

human type 3 3α HSD, such that there was much more conversion of DHP to allopregnanolone. The reverse oxidative reaction was unaffected by SSRI treatment. The antidepressant imipramine had no effect on the enzymatic activity of either the rat or human type 3 3α HSD.

Our data further showed that there were differences in the substrate specificity of the two 3α HSDs that we found in human fetal brain. Type 2b, a variant of type 2, did not use DHP as substrate, and hence was not involved in the synthesis of allopregnanolone. It did, however, use dihydrotestosterone as substrate. When DHT was substrate, the SSRIs had the same effect on this enzyme as they did on the human type 3 3α HSD enzyme: they decreased the Km of the enzyme, thereby synthesizing more androstanediol from DHT.

We also demonstrated by Northern blot analysis, that the type 2b and type 3 3αHSD mRNAs were regionally expressed in the human brain, suggesting that there are likely to be differences in the metabolism of DHP and DHT in different regions of the brain.

These data thus demonstrate that there is another level of regulatory control on the synthesis of neurosteroids — directly at the site of enzyme activity. These data further suggest that it may be possible to design drugs that will enhance the production of specific subsets of neurosteroids at their normal site of synthesis, rather than globally throughout the body. Such drugs may ultimately be useful for treatment of different types of depression, free from unwanted side effects.

6. Animal models for studying the function of neurosteroids

We have been using several animal models to study the endogenous function of neurosteroids in the development of the nervous system, including gene knock-outs and gene overexpression in transgenic mice. In addition, we have been using a mouse model of neurodegeneration, in which we believe that neurosteroids may play a role in the etiology of the disease. This mouse is a model for Niemann Pick Type C (NP-C) disease [48]. NP-C is an autosomal recessive neurodegenerative disease cause by mutations in the lysosomal NPC1 protein [4]. The lack of NPC1 protein in cells results in the accumulation of cholesterol esters in lysosomes (reviewed in [52,63]). Abnormal transport of cholesterol and other molecules in $NPC1^{+}$ vesicles may contribute to neurodegeneration [50]. Since there is accumulation of cholesterol esters in lysosomes, and since cholesterol esters are a source of steroid precursors in the adrenals and gonads, other investigators studied steroidogenesis in testes from NP-$C^{-/-}$ mice [54], and demonstrated that those mice have decreased androgen production, and subsequently, decreased expression of androgen-dependent proteins in the liver [53,54].

We have now studied the synthesis of neurosteroids in the brains of these mice from various ages, before and after the onset of the neuronal symptoms. Our data indicate that brain from adult NP-$C^{-/-}$ mice contains much lower concentrations of both pregnenolone and DHEA, when compared with normal littermates. We then studied the conversion of cholesterol to various neurosteroids, to determine which enzyme activity may be altered, when this alteration may occur, and whether there is regional specificity to these alterations. Our data indicate that by 9 weeks of age, the synthesis of allopregnanolone from progesterone is much less in NP-$C^{-/-}$ mice than in wild type mice. These differences can also be seen much earlier, before the onset of symptoms. Immunocytochemical analysis also indicates that expression of several neurosteroidogenic enzymes required for allopregnanolone synthesis are also decreased in specific brain regions of NP-$C^{-/-}$ mice. Thus we believe that decreased synthesis of neurosteroids may be a factor that ultimately leads to neurodegeneration in these animals. Further studies are underway to understand the roles of these steroids in the normal development of the nervous system.

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