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15. NEUROSTEROIDS AND BRAIN STEROLS

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Conventionally, steroids operate via transcription, but a subclass of brain-active steroids, dubbed neurosteroids, may govern cognitive processes via membrane-associated receptors. *De novo* synthesis of neurosteroids within the brain has been discussed; we suggest that these may derive primarily from the circulation. In contrast, the brain is largely self-sufficient in cholesterol. Synthesis and metabolism of cholesterol and its oxysterol derivatives appears to be crucial to brain development and function, emphasized by drugs (anti-convulsants, neuroleptics) and mutations (Smith-Lemli-Opitz, Niemann-Pick disease type C, cerebrotendinous xanthamatosis) that affect these pathways and have marked brain effects. Receptors for steroids and sterols are discussed, particularly those at cell-surface and intracellular membranes including sites of sterol metabolism and trafficking (including sigma-1, the emopamil binding protein [EBP], and the peripheral benzodiazepine receptor [PBR]). Potential overlaps between sterol and steroid signaling are discussed. In addition to regulating neuronal activity, we suggest that steroids and sterols may regulate proliferative and degenerative processes in the brain including apoptosis induction. Evidence is presented for cross-talk between activity of neurotransmitter receptors at the cell surface (e.g., $GABA_A$) and pathways operating within the cell; local sterol signaling could potentially extend between cells. We also address whether changes in neurosteroid signaling mediated by the adrenal steroid dehydroepiandrosterone (DHEA) could contribute to age-related cognitive impairments.

Definitions: Cerebrosterol, cholest-5-ene-3β, 24(S)-diol [24(S)-hydroxycholesterol]; DHEA, dehydroepiandrosterone; Oxysterol, hydroxylated derivatives of cholesterol (or dehydrocholesterol).

KEY WORDS: brain, hormone, metabolism, neurosteroid, steroid, synthesis.

INTRODUCTION

Signaling by steroids and sterols is widespread in vertebrates, insects, plants and fungi; the mammalian brain is no exception. In vertebrates, steroids produced from peripheral endocrine organs, including the adrenal, gonads and placenta, govern a diversity of physiological parameters including information processing in the brain. It has been suggested that steroid synthesis and metabolism take place in the CNS; brain-derived "neurosteroids" may play a role in cognitive processes. However, although brain function is modulated by steroids (and in turn the brain can govern peripheral (endocrine) steroid hormone production), what is unusual about the brain is its particular dependence on steroid/sterol synthesis and metabolism – deficits in cholesterol metabolism can be catastrophic for cognition while other brain and body systems continue to operate. Here we do not attempt to provide a comprehensive overview of brain steroid metabolism and action; the interested reader is referred to one of the excellent recent reviews

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that address specific topics (Baulieu, 1997, 1998; de Kloet *et al*., 1998; McEwen and Alves, 1999; Rupprecht and Holsboer, 1999; Mensah-Nyagan *et al*., 1999). Neither do we focus on well-trodden areas of neurotransmitter receptor targets, plasma cholesterol transport, and sulfation/desulfation of steroids. We have chosen to emphasise developmental and maintenance processes rather than acute modulation and, in particular, we have sought to emphasise new avenues of exploration rather than revisiting old territory. In the following we commence by examining the paradigm of estrogen-dependent masculinization of brain function. We then consider evidence for local synthesis and metabolism of cholesterol and steroids, and overview the targets, some unusual, that mediate their action. We continue by discussing the emerging contention that sterol mtabolism is pivotal to brain function and dwell on the role of neurosterols and neurosteroids in apoptotic and developmental processes in the mammalian brain. We conclude with a list of observations that emphasise fresh avenues of research.

STEROIDS GOVERN BRAIN DEVELOPMENT AND FUNCTION

Like other tissues, the brain responds to steroids, both locally produced and derived from the endocrine organs. This is exemplified by the masculinization of behavior produced by exposure to perinatal steroids (reviewed in Box 15.1). Perinatally, 17β-estradiol (estrogen) enters the brain only poorly. In males, gonadal testosterone (which enters the brain more freely) is converted to 17β-estradiol by aromatase (Box 15.2); estradiol then elicits male-type development and behavior. Conversely, estradiol in the adult (which does enter the brain) fails to cause male-type behavior. These observations highlight two important aspects of steroid regulation. First, steroid action is modified according to local delivery and metabolism in the target tissue. Second, steroids can have separate effects during development and in the adult brain. While the details of steroid imprinting of gender-specific behavior remain to be fully elucidated, an understanding of the effects of sex steroids on the brain is of great importance, particularly in view of the undoubted role that estrogens and related molecules play in modulating neuronal function in the adult brain and their potential for the therapy of neurodegenerative diseases including Alzheimer's (McEwen and Alves, 1999; Toran-Allerand *et al*., 1999).

Box 15.1 Sex steroid masculinization of brain function

The brains of males and females display significant structural differences, particularly in the sexually dimorphic nucleus of the medial preoptic area, but also in the base nucleus of the stria terminalis, the vomeronasal organ, hippocampus, amygdala and hypothalamus (Cooke *et al*., 1998). Sexual behavior in most mammalian species is also dimorphic, though the correlation between structural and behavioral differences is unknown.

Female rodents, in the presence of a male, display lordosis (i.e., adopt a characteristic posture that facilitates mating). The predisposition to the lordotic response is determined by two factors. First, by the absence of exposure to masculinizing androgens in the perinatal period; treatment of females with testosterone at this time impairs lordosis and leads to male-type behavior. Second, by the continued production of female sex steroids including estradiol and progesterone. Castration of male neonates produces animals that display lordosis when suitably primed with female hormones; normal males fail to show this behavior.

Perhaps counter-intuitively, the hormone responsible for masculinization of the brain and behavior is the female sex steroid estrogen (17β-estradiol), and defeminization can occur in the absence of a functional androgen receptor (Olsen, 1979). According to the paradigm for steroid masculinization of brain, maternal gonadal estradiol cannot enter the brain because it binds with high affinity to alpha-fetoprotein, a steroid-binding protein produced by the fetal liver (but not by the adult). However, brain estradiol can be produced, *in situ*, by enzymatic aromatization of testosterone derived from the bloodstream, mediated by brain aromatase enzyme (Box 15.2). Unlike estradiol, testosterone fails to bind to alpha-fetoprotein, and thus can cross into the brain. By this mechanism, gonadal testosterone reaches the brain in males and not in females, but only following conversion to estradiol produces masculinization of brain development and later life behavior (MacLusky and Naftolin, 1985).

In support, prenatal treatment of rats with an aromatase inhibitor can prevent masculinization (see, for instance, Clemens and Gladue, 1978) while in utero transfer of testosterone from male fetuses to female sibs via the bloodstream can elicit partial masculinization of the females (Houtsmuller and Slob, 1990). The unaromatizable derivative of testosterone, 5α-dihydrotestosterone (DHT) is rather ineffective at inducing brain masculinization despite playing a predominant role in the development of masculine body characteristics. The sexually domorphic nucleus (SDN) of the preoptic area is still dimorphic in rats with testicular feminization due to androgen receptor deficit (C.D. Jacobson, cited in Cooke *et al*., 1998), demonstrating that androgen receptor activation is not essential for sex-specific brain development. Mice lacking a functional estrogen receptor alpha (ERα) have clear impairments in male-type behavior (Wersinger *et al*., 1997; Ogawa *et al*., 1998), although female behavior is also disrupted. Testosterone, and its estradiol metabolite, appear to cause masculinization in part by inhibiting apoptotic mechanisms associated with sex-specific neuronal loss, for instance in the SDN. They can also have direct neurotropic effects (Davis *et al*., 1996).

However, the story is becoming more complicated-estradiol and its derivatives appear not to be the only natural steriods that affect masculinization. Although Honda *et al*. (1998) report selective deficits in male-type behavior in mice harboring a disruption of a brain promoter for the aromatase gene, in other studies young male mice lacking the aromatase enzyme were found to be capable of breeding and displayed normal mounting behavior (Fisher

Box 15.1 (*Continued*)

et al., 1998; Robertson *et al*., 1999) although these animals became physiologically infertile as adults due to testicular abnormalities. This finding argues that aromatase-mediated conversion of testosterone to estradiol is not essential for the development of male-type behavior, and suggests that brain testosterone may be partly responsible. More recent studies have shown that aromatase gene expression is regulated by androgens (see later). Furthermore, both estradiol and testosterone may be further metabolized in brain to active hormones. Estradiol may be hydroxylated in brain to generate hormonally-active catechol estrogens by hydroxylation at the 2 (or 4) position (Fishman and Norton, 1975), possibly by another enzyme or by additional aromatase-mediated metabolism (Osawa *et al*., 1993). Testosterone is potentially converted to dihydrotestosterone (DHT) in brain by 5α-reductase and thence to downstream metabolites including the product of 3α-hydroxysteroid dehydrogenase (3α-HSD) action on DHT, 5α-androstane-3α,17β->diol (see below and Figure 15.1); although not demonstrated, such steroids could cause masculinization independently of the androgen receptor. It is also likely that androgens and estrogens play multile roles in the developing brain, and interact with diverse nuclear and cell-surface receptors activating a variety of downstream signal-transduction pathways (Beyer, 1999; McEwen and Alves, 1999; Toran-Allerand, 1999).

Box 15.2. Aromatase

This enzyme is responsible for the conversion of testosterone to estradiol and ensuing brain masculinization subsequent to specific activation of estrogen receptors. The same enzyme catalyzes the conversion of androstenedione to estrone, a major pathway for estrogenic steroid production subsequent to the menopause. In rodents and in human a single gene appears to be responsible for CYP19 (P450arom) activity, though the existence of additional CYP19 genes in pig and goldfish (Choi *et al*., 1997; Tchoudakova and Callard, 1998) argues that additional rodent and human genes remain to be uncovered. CYP19 is somewhat unusual among the cytochrome P450s because CYP19 expression derives from several different upstream promoter regions. In all cases examined, however, the encoded protein is identical (Simpson *et al*., 1997). A major brain-specific promoter is utilized in the central nervous system (Honda *et al*., 1994) although another upstream promoter, specific to cortex, has also been reported (Kato *et al*., 1997) in addition to some expression from the promoter region employed in ovary. As expected for a key hormonal activation step, enzyme production is subject to a number of regulatory controls. Aromatase expression, principally in neurons, is high during pre- and peri-natal development, particularly in hypothalamic, limbic regions and developing sensory centres (Horvath and Wikler, 1999), but declines in adulthood (reviewed by Lephart, 1997). Although brain masculi-

nization reflects conversion of gonadal testosterone to estradiol, emerging evidence suggests that CYP19 expression is itself regulated by androgens as well as estrogens (Hutchinson *et al*., 1997), arguing that androgen and estrogen act in concert to produce brain masculinization. Aromatase enzyme activityis also modulated by natural breakdown products of testosterone and by environmental influences (reviewed by Hutchison, 1993). Brain aromatase is also reduced by prenatal stress (Jimbo *et al*., 1998); this could, plausibly, predispose to feminization in some species.

In addition to local metabolism (activation/inactivation) in the central nervous system, the brain has been suggested to be a site of *de novo* steroid synthesis, giving rise to local production of "neurosteroids". We next address the evidence that the ubiquitous steroid precursor, cholesterol (see Figure 15.1), is produced locally in brain.

CHOLESTEROL SYNTHESIS IN BRAIN

Cholesterol is a major component (~30–80%) of cell membranes, including those in the brain. In rats the major phase of brain membrane formation takes place during the perinatal period, during which many neurons become myelinated. Even during this period of peak demand, local synthesis seems to fulfil the cholesterol requirements of the brain. *In vivo*, cholesterol is rapidly produced in the brain from radiolabeled precursors (Sérougne and Chevallier, 1976) while, *in vitro*, primary cultures from newborn rat forebrain, consisting primarily of astrocytes and oligodendrocytes, convert [3H]-mevinolactone to cholesterol; here up to 10% of applied radioactivity could be recovered as cholesterol (Hu *et al*., 1989; Jung-Testas *et al*., 1989), while lesser amounts of pregnenolone and 20-hydroxypregnenolone (and their esters) were also produced. Local synthesis normally satisfies brain cholesterol demand in the brain and in the peripheral nervous system, both during development and during nerve regeneration (Jurevics and Morell, 1995; Morell and Jurevics, 1996; Jurevics *et al*., 1998).

Key cholesterol-synthesizing enzymes are expressed in brain. Cholesterol is produced from elementary precursors in an extensive series of reactions. The first and primary rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid by the enzyme hydroxy-methylglutaryl CoA reductase (HMGCR). In brain, HMGCR activity is abundant, particularly during development (Sudjic and Booth, 1976; Ness *et al*., 1979), peaking at postnatal day 4 in the rat (Ness, 1994) but continuing to be expressed during adulthood. In mice transgenic for an HMGCR fusion gene (Mehtali, 1988; Mehtali *et al*., 1990) reporter activity was highest in brain and was predominantly associated with oligodendrocytes in the adult (Duhamel-Clerin *et al*., 1994). Cholesterol production is subject to end-product repression, but while liver HMGCR transcription was repressed by cholesterol and induced by blockade of cholesterol synthesis, neither endogenous HMGCR gene expression nor that of the fusion transgene responded to manipulations of peripheral cholesterol demand (Mehtali, 1988;

Mehtali and Lathe, unpublished data). Thus cholesterol production in brain is regulated independently of other tissues including liver.

While the brain can satisfy the majority of its cholesterol demand, peripheral cholesterol can enter the brain (Spohn and Davison, 1972; Sérougne and Chevallier, 1976), and slow exchange between brain and plasma cholesterol is likely. In the adult, mature neurons downregulate cholesterol synthesis and an increasing proportion of brain cholesterol may be supplied by uptake of cholesterol via the apolipoprotein E (ApoE)/low density lipoprotein (LDL) receptor system (Poirier *et al.*, 1993; Poirier and Sevigny, 1998). Defective cholesterol synthesis due to Δ^7-reductase deficiency (Smith-Lemli-Opitz syndrome, mental retardation, developmental deficits and adrenal insufficiency) can respond to dietary cholesterol supplementation (Opitz and de la Cruz, 1994; Nowaczyk *et al.*, 1999) and, though the response is sometimes relatively weak, this may demonstrate that some cholesterol can enter the brain from the circulation, at least in adults. LDL receptor expression is found on cells of the blood-brain barrier, suggesting an involvement in cholesterol exchange (Meresse *et al.*, 1989; Dehouck *et al.*, 1998).

Moreover, the brain is often a net exporter of cholesterol (Bjorkhem *et al.*, 1997), and takes advantage of a unique 24(S) hydroxylation system operating in parallel with the high-density lipoprotein (HDL) export machinery (Bjorkhem *et al.*, 1999). The 24(S) hydroxylase enzyme, CYP46, is primarily expressed in brain, with lesser amounts in adrenal (Lund *et al.*, 1999; Bjorkhem *et al.*, 1999).

ARE STEROIDS SYNTHESIZED *DE NOVO* IN BRAIN?

What is the evidence that steroids are synthesized in the CNS? Though there is compelling evidence for local brain synthesis of cholesterol, and for steroid modification (exemplified by aromatization of testosterone to estradiol), data supporting *de novo* synthesis of pregnenolone and derivative steroids are more contentious. Steroids differ from sterols, such as cholesterol, in that the long hydrophobic side-chain is lost (see Figure 15.1), boosting both solubility and signaling potential. Side-chain cleavage (scc) can occur chemically, but *in vivo* is catalyzed by the enzyme P450scc (CYP11A1), converting cholesterol (and side-chain hydroxylated cholesterols) to pregnenolone, for onward conversion to generate DHEA, progesterone and derivative steroids. Evidence for brain steroidogenesis derives from four sources. First, that the brain naturally contains significant concentrations of steroids. Second, molecular data suggesting that key steroidogenic enzymes are expressed in brain. Third, from biochemical studies that address steroid conversions in brain. Fourth, that brain steroid levels may not be entirely subservient to peripheral plasma concentrations.

Steroids are present in brain

The first line of argument is that the brain naturally contains significant concentrations of steroids, particularly the adrenal androgens DHEA and pregnenolone.

These are present in blood and brain principally in the form of sulfates and fatty acid esters; free steroid is present at much lower concentrations (Table 15.1). Primates are unusual in that DHEA (and pregnenolone) sulfates circulate in blood at strikingly high levels compared with other species. Primate DHEA levels in the circulation usually exceed pregnenolone levels by a factor of 10 (though 17-hydroxypregnenolone is a major component in some species), and effective plasma levels of DHEAsulfate of up to 1–2 uM have been recorded. In human sciatic nerve, DHEAS remains the major form (in the order of 100 ng/g; Morfin *et al*., 1992) while the concentration of free pregnenolone can exceed the concentrations of its sulfate (~60 ng/g versus ~25 ng/g) with a significant contribution of fatty acid esters (~30 ng/g) (Morfin *et al*., 1992).

In rodent blood DHEA and pregnenolone levels are much lower, and pregnenolone is the major steroid, exceeding DHEA levels by 20-fold or more (Robel *et al*., 1987). Again the sulfates predominate (Table 15.1). In rat brain, both steroids are present (Corpéchot *et al*., 1981, 1983) but the concentration of pregnenolone (20–40 ng/g, equivalent to around 0.1 uM) was only marginally above that detected in kidney or spleen (9–15 ng/g) and substantially below the level in adrenal (5300 ng/g; Robel *et al*., 1987). In the study of Korneyev *et al*. (1993), steroid concentrations were highest in olfactory bulb (10–14 ng/g, equivalent to around 30 nM) but slightly lower in other brain regions (4–7 ng/g). Prasad *et al*. (1994) suggest that the apparent rat brain concentrations of pregnenolone and

Table 15.1 Consensus concentrations (ng/g) of neurosteroids in blood and brain[1].

		D[2,3]	*DS*	*DL*	*P*[3]	*PS*	*PL*
HUMAN	Blood	10	2000	na	2	100	na
	Sciatic nerve	na	150	na	60	25	30
	CSF	0.2	1	na	na	na	na
RAT	Blood	0.1	2	na	20	10	na
	Brain	3	2	30	20	10	70

Concentrations are compiled from published values for human blood (see Aso, 1976; Orentreich *et al*., 1984, 1992; Guazzo *et al*., 1996), human sciatic nerve (Morfin *et al*., 1992), human cerebrospinal fluid (Schwarz and Pohl, 1992; Guazzo *et al*., 1996) and rat plasma and brain (Corpechot *et al*., 1981, 1983, 1985; Jo *et al*., 1989). Multiple values have been averaged across different reports. D, free DHEA; DS, DHEA sulfate; DL, lipoidal/fatty acid esters of DHEA; P, free pregnenolone; PS, pregnenolone sulfate; PL, lipoidal/fatty acid esters of pregnenolone. na, values not available.

Notes:

1. Concentrations were generally obtained by radioimmunoassay; values obtained by mass-spectroscopy may be 100 to 1000-fold lower (Uzunova *et al*., 1998; Kim *et al*., 2000), for reasons that are not understood.
2. In human, concentrations of DHEA and its sulfate decline with age and differ between males and females (Orentriech *et al*., 1984, 1992). Concentrations can show marked diurnal variations (Robel *et al*., 1986) and active responses; hypothalamic but not amygdala or olfactory bulb DHEA levels were boosted 6-fold by exposure of male rats exposed to females (Robel *et al*., 1986).
3. Apparent brain concentrations of P and D can be increased up to 7-fold by pretreatment of extracts with organic base and $FeSO_4$ (Prasad *et al*., 1994).

DHEA may be boosted markedly (to ~450 ng/g; pregnenolone) by tissue treatment with organic bases or the reducing agent $FeSO_4$, suggesting that higher concentrations of these molecules (or intermediates in their synthesis) may be present, *in vivo*, as hydroperoxides. This result remains to be confirmed.

Although DHEA levels are markedly elevated in primates compared to rodents, it is of particular note that the overall concentrations of DHEA (free + sulfate) in rat brain (~5 ng/g, excluding esters) are not dissimilar from the concentrations recorded in human CSF (~1–2 ng/g, excluding esters), though substantially below those in human sciatic nerve (~150 ng/g, Table 15.1).

Molecular evidence for steroidogenic enzymes in brain

A second line of argument is that many steroidogenic enzymes, including the key cholesterol side-chain cleavage enzyme (P450scc, CYP11A1), are expressed in brain. However, these data can be difficult to interpret because some techiques are extremely sensitive (e.g., RT-PCR) and can detect trivial levels of mRNA expression. Le Goascogne *et al*. (1987) reported widespread detection of P450scc immunoreactivity in rat brain. Stromstedt and Waterman (1995) used RT-PCR to detect P450scc mRNA in all rat brain regions, but not in mouse brain. *In situ* hybridization detected P450scc mRNA in rat cortex, hippocampus, olfactory bulb and cerebellar granule, and also purkinje cells (Furukawa *et al*., 1998), with astrocytes, oligodendrocytes and neurons all expressing mRNA as assessed by RT-PCR (Zwain and Yen, 1999a). Generally, the level of expression seems to be far below that detected in classic steroid-synthesizing tissues such as the adrenals, gonads and placenta (Sanne and Krueger, 1995; Compagnone *et al*., 1995a,b; Kohchi *et al*., 1998).

The critical co-enzymes adrenodoxin and P450 reductase are detected throughout the brain (Oftenbro *et al*., 1979; Stromstedt and Waterman, 1995), while the sterol carrier protein SCP-2, that also participates in cholesterol synthesis and transport (e.g., Scallen *et al*., 1985) is well expressed in the central nervous system (Myers-Payne *et al*., 1996). The potential transport proteins, OPP1-6, related to the oxysterol binding proteins (OSBP) are widely expressed in the CNS (Laitinen *et al*., 1999). Furukuwa *et al*. (1998) also report *in situ* detection of mRNA encoding the intracellular cholesterol transport protein StAR, required for efficient cholesterol delivery to P450scc and steroid synthesis in adrenal, and suggest that StAR and P450scc (CYP11A1) are co-expressed in many brain regions. The brain and placenta homolog of StAR, MLN64, is also present (Watari *et al*., 1997). On the other hand, expression of steroidogenic factor 1 (SF1), a transcription factor required in other tissues for efficient expression of steroidogenic enzymes, is detectable by RT-PCR but does not appear to be abundant in rodent brain (see Stromstedt and Waterman, 1995). mRNA is rather more robustly and widely expressed in human brain, as assessed by Northern blotting and *in situ* hybridization, but again the levels are very much lower than those in conventional steroidogenic tissues (Ramayya *et al*., 1997). However, the dependency of brain steroidogenic enzyme production on SF-1 expression has not been demonstrated; SF-1 is not necessary for P450scc expression in developing mouse gut (Keeney *et al*., 1995).

The situation with CYP17 (17α-hydroxylase/c17,20-lyase), the enzyme that converts pregnenolone to DHEA, is not dissimilar – brain levels are low (Stromstedt and Waterman, 1995; Compagnone *et al*., 1995a; Kohchi *et al*., 1998) or undetectable (Le Goascogne *et al*., 1991). Here, however, it has been suggested that the brain may be able to convert pregnenolone to DHEA by a CYP17-independent pathway (Cascio *et al*., 1998); low levels of CYP17 might not preclude DHEA production *in vivo* from pregnenolone, or even, conceivably, by chemical oxidation of cholesterol (Prasad *et al*., 1994).

This summary above is not exhaustive; further overviews of key enzyme pathways are provided in Boxes 15.1–15.6 and in focused reviews elsewhere in this volume. In general, the brain expresses a surprising diversity of steroid-metabolizing enzymes, as revealed by biochemical conversions in brain extracts and enzymes detected by immunohistochemistry and molecular analysis of mRNA expression. Boxes 15.2–15.6 separately review the different conversions, focusing on aromatase (Box 15.2), 3β-HSD (Box 15.3), 3α-dehydrogenation and 5α-reduction (Box 15.4), 11-position modifications, particularly of the glucocorticoids (Box 15.5; see also chapters 7 and 12), and steroid 6 and 7 hydroxylation (Box 15.6).

Box 15.3 3β-hydroxysteroid dehydrogenase (3β-HSD)

The conversion of pregnenolone to progesterone by brain (Zhao *et al*., 1991) and by peripheral Schwann cells (Koenig *et al*., 1995) argues that this enzyme is active *in vivo*. The brain is far from unusual though – more than 10 different tissues can catalyse this conversion (Zhao *et al*., 1991). 3β-HSD both reduces the 3β-hydroxy group and isomerises the 5–6 unsaturated bond (to the 4–5 position; Figure 15.1); under some circumstances the reverse reaction may predominate. 3β-HSD converts pregnenolone to progesterone, but also metabolizes DHEA (produced by CYP17-conversion of pregnenolone; Figure 15.1) to androstenedione. These are important steroids in their own right, and are also precursors to other steroids including testosterone and estradiol (from androstenedione), the glucocorticoids and mineralocorticoids (from progesterone) and the 3α-hydroxy anesthetic steroids derived from them.

At least 6 different isoforms of 3β-HSD are known, in rodents (two in humans), but whereas types I, II and III catalyze progesterone and androstenedione synthesis, types IV and probably V catalyze a oxosteroid reductase reaction, reducing the 3-oxo group (Clarke *et al*., 1993), and convert, e.g., the 3-oxo steroid testesterone to the non-androgenic (but potentially hormonally-active) steroid 5α-androstane-3β,17βdiol (reviewed by Simard *et al*., 1995; Mason *et al*., 1997; Payne *et al*., 1997). In brain, oxidation seems to predominate (enzyme types I to III), and brain extracts metabolize pregnenolone and DHEA to progenesterone and androstenedione (Zhao *et al*., 1991; Guennoun *et al*., 1997). Gennoun *et al*. (1995), using a probe to a conserved region for *in situ* hybridization to rat brain, reported mRNA in cerebellum, cortex, striatum, thalamus, hypothalamus, olfactory bulb, and hippocampus. Sanne and Kreuger (1995) confirm 3β-HSD expression throughout rat brain,

Box (*Continued*)

and detected 3β-HSDs type I, II and III but not IV mRNA by PCR. In this study expression levels were generally reported to be low, although Ukena *et al*. (1999) suggest that 3β-HSD expression is age-dependent, being highest in the perinatal rat cerebellum.

Steroidogenesis in brain extracts

Third, steroid synthesis and metabolism in brain tissue extracts is well-documented. Newborn rat glia and purified mitochondria from young rats can produce pregnenolone from cholesterol or its precursors (Hu *et al*., 1987; Hu *et al*., 1989; Jung-Testas *et al*., 1989). Pregnenolone production by rat brain mitochondria is stimulated by ligands of the peripheral benzodiazepine receptor (Romeo *et al*., 1993; McCauley, 1995) that facilitates intracellular cholesterol transport; compounds activating cAMP production also increased pregnenolone formation in retina (Guarneri *et al*., 1994).

Onward metabolism is also reported, and in the above studies 20-hydroxypregnenolone (5-pregnen-3β,20α-diol), and 5-pregnen-3,20-dione were detected (Hu *et al*., 1987, 1989) while neurons from 17-day mouse embryos only formed 20-hydroxypregnenolone (Akwa *et al*., 1991). Here DHEA synthesis was not observed. However, although incubation of primary newborn rat forebrain cultures (primarily astrocytes and oligodendrocytes) with [3H]-pregnenolone led to the production of radiolabelled progesterone and 20-hydroxypregnenolone (Jung-Testas *et al*., 1989), the efficiency of conversion was low and progesterone represented only 3% of applied radioactivity after 24 hours incubation. Because the primary cultures were maintained *in vitro* for 3 weeks prior to assay, they may not accurately reflect the *in vivo* situation. However, 20-hydroxyprogesterone was also the predominant metabolite of progesterone in rabbit cornea (Navarro-Ruiz *et al*., 1987) and in whole brain extract (Carey *et al*., 1994) though a lesser amount of 5α-pregnane-3,20-dione was also produced. Kabbadj *et al*. (1993) report that 20-hydroxypregnenolone is a major product in isolated neuronal cultures, while astrocytes converted pregnenolone to 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone (THPROG). Steroidogenesis has also been reported in isolated rat retina where, in addition to pregnenolone, Guarneri *et al*. (1994) detected DHEA, progesterone, deoxycorticosterone, THPROG, and 3α,5α-tetraydrodeoxycorticosterone. Treatment of primary rat brain extracts with L-ascorbate boosted apparent levels of allotetrahydrodeoxycorticosterone and DHEA in addition to those of pregnenolone (Roscetti *et al*., 1998).

Box 15.4 3α-dehydrogenation and 5α-reduction; 17α and 20β dehydrogenase activities of 3αHSDs

A second but distinct pathway, mediated by 3α-hydroxysteroid dehydrogenase (3αHSD), often in conjunction with stereospecific reduction of the 4–5 unsatu-

rated bond (adding a 5α hydrogen), produces a separate class of steroids from the 3-oxo molecules produced by 3β-HSD. For the most part, such steroids are produced from progesterone, either directly to produce 5α-3α-hydroxy tetrahydroprogesterone (THPROG) or first via steroid 21β-hydroxlase (CYP21) to deoxycorticosterone, and then to 5α-, 3α-hydroxy tetrahydrodeoxycorticosterone (THDOC). Testosterone is also a substrate (Figure 15.1), producing the potent anesthetic steroid 5α-androstane-3α-,17β-diol.

Despite the functional linkage implied between the 5α reduction and 3αHSD steps, the enzymes are expressed separately. 5α reductase activity is found in many brain regions, particularly the hypothalamus and pons/medulla (Roselli and Snipes, 1984; Bertics *et al*., 1987; Li *et al*., 1997) and appears to be primarily neuronal (Melcangi *et al*., 1993, 1994). There exist at least 2 distinct 5α reductase genes. The lower affinity type I enzyme is widely expressed in adult brain, while the type II is expressed at significant levels during development, particularly in zones of neuronal proliferation and differentiation but, in the adult, only low mRNA levels were detected (Lauber and Lichtensteiger, 1996).

In contrast, 3αHSD activity is highest in olfactory bulb, with moderate levels in hypothalamus, cortex and cerebellum, and lower levels in limbic, thalamic, midbrain and brain stem structures (Cheng *et al*., 1994). The enzyme is predominantly expressed in astrocytes (Melcangi *et al*., 1993, 1994). However, four two types of 3αHSD enzyme that have been cloned in human (Matsuura *et al*., 1998). Type I is absent from brain, types II and III are expressed (Channa *et al*., 1995; Matsuura *et al*., 1998) while expression of the type IV enzyme in brain has not been studied. However, the type II enzyme harbors dual 3α- and 17β-HSD activities and only poorly converts dihydrotestosterone to 3α-hydroxy androstanediol (Lin *et al*., 1997), while the type III enzyme is more active on prostaglandins than it is on 3α- or 17β-hydroxylated molecules (Matsuura *et al*., 1998).

The difference in cellular expression and regional distribution could suggest that transport of intermediates is pivotal to the generation of "5α-3α" steroids in brain, or that these steroids make a significant contribution during development. For instance, maternal progesterone may suppress arousal of the fetal brain (Crossley *et al*., 1997). In the adult it is unclear whether the "5α-3α" anesthetic steroids play any functional role outside pregnancy. Stuerenburg *et al*. (1997) were unable to detect conversion of pregnenolone to progesterone in cortex *in vitro* while Young *et al*. (1996) failed to detect THPROG in normal mouse brain, but reported nanomolar concentrations in male mice treated with trilostane, a 3β-HSD inhibitor, and consequent suppression of aggressive behavior. Curiously, administration of progesterone brought about an even more dramatic increase in brain THPROG, but had no antiagressive effect. It may perhaps be suggested that THPROG activation of $GABA_A$ receptors is unlikely to play a major role in the adult brain; the same may not be true of 3α-hydroxy-5α molecules derived from adrenal steroids and further metabolized in the brain.

It is of note that many 3α-HSD enzymes are relatively promiscuous in the site-specificity of the reaction catalyzed. Type II 3α-HSDs also display 17β-HSD

Box 15.4 (*Continued*)

activity. Other enzymes classified as 3α-HSDs are members of the short-chain dehydrogenase/reductase family, rather than being aldo-keto/oxo reductases, and also possess 20β-hydroxylation activity (Pocklington and Jeffrey, 1968; Sweet *et al*., 1980; Ohno *et al*., 1991; see also Penning *et al*., 1997). 3α-/20β-HSD enzyme is reported in brain (Kobayashi *et al*., 1995) but no detailed studies have been reported. Finally, an epimerase catalyzing the isomerization of 3α-steroids to 3β-steroids has been reported in rat brain and other tissues (Huang and Luu-The, 2000). In view of the markedly different properties of 3α- versus 3β-steroids (for instance in $GABA_A$ modulation and anesthetic properties) this one enzyme could be a critical arousal determinant.

Are brain steroid levels independent of peripheral synthesis?

A final pointer for local steroidogenesis in the rodent nervous system is that brain steroid levels can vary independently of their concentrations in blood. The key thread in the argument is that adrenalectomy (adx) plus gonadectomy (gdx) can have little effect on steroid levels in brain. For instance, adx/gdx resulted in a 10-fold reduction in plasma steroid levels but only a slight depression (by 30–40%) in brain levels (Korneyev *et al*., 1993). In the same study peripheral benzodiazepine receptor ligands were able to increase brain pregnenolone levels (but not those of DHEA), but were without effect on plasma pregnenolone levels. This suggests that at least some brain synthesis of pregnenolone takes place *in vivo*.

However, the interpretation of such studies is not always easy. Firstly, it is difficult to prove that surgical gdx/adx is complete, with latent residua potentially of adrenal tissue becoming hypertrophic to replace the removed organ. The operation is also not neutral to the animal. In one study, adx/gdx increased rather than reduced brain steroid levels and, like stress, roughly doubled brain pregnenolone and DHEA (Robel *et al*., 1987). Secondly, studies on adx/gdx animals could be misleading if there are other non-brain sites of steroid synthesis, as noted by Le Goascogne *et al*. (1995). For instance, steroidogenesis has been detected in gut and skin during development (Keeney *et al*., 1995) and key enzymes P450scc, CYP17 and CYP21 are expressed in normal human skin (Slominski *et al*., 1996); some brain steroids in adx/gdx animals could potentially derive from such tissues. Finally, peripheral steroids tend to accumulate rapidly and selectively in fatty tissues such as brain (see Cheney *et al*., 1995) and brain levels of, e.g., pregnenolone can exceed plasma levels by a factor of 10–50 (Robel *et al*., 1987; Korneyev *et al*., 1993; Cheney *et al*., 1995).

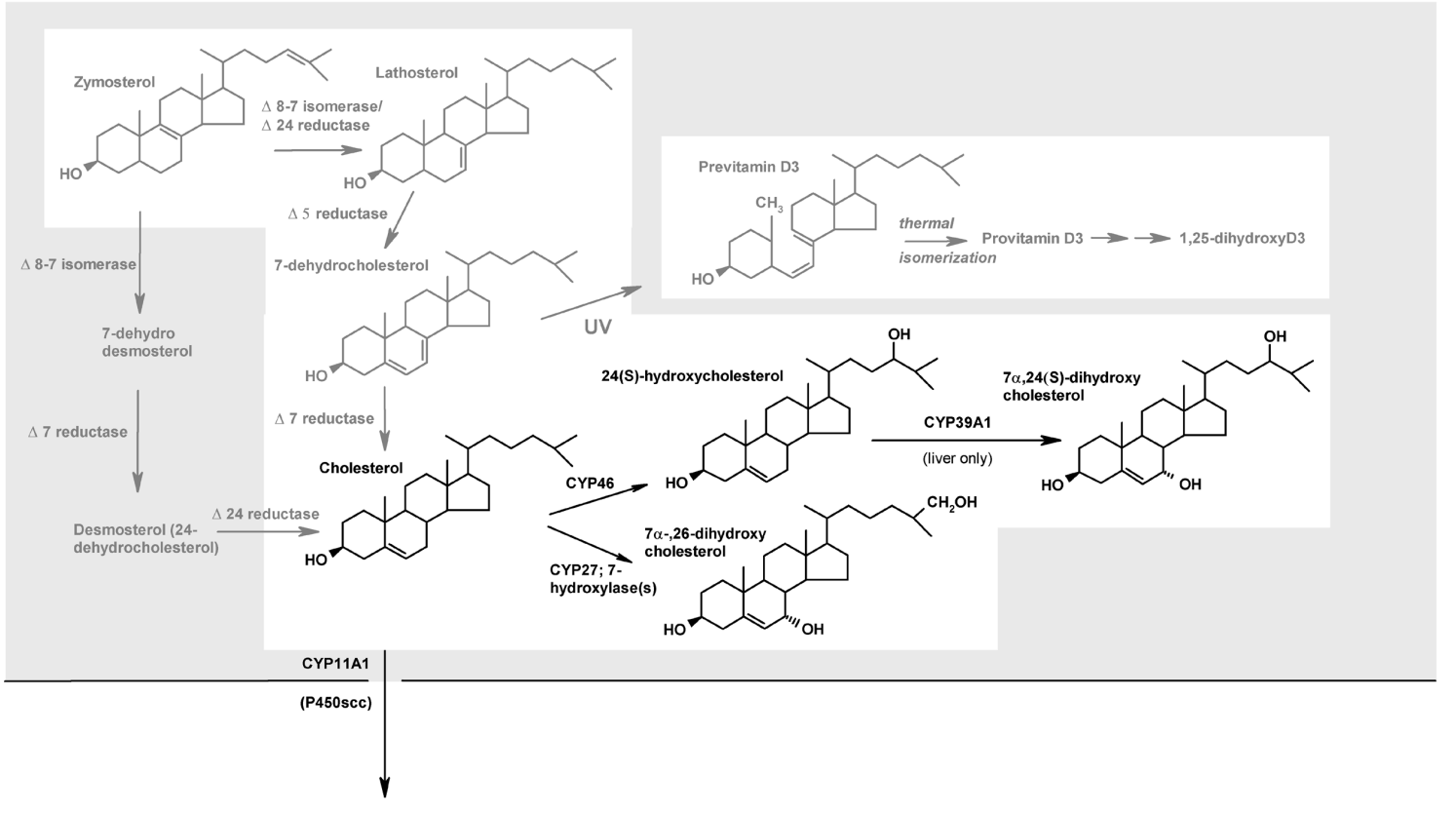
With these caveats in mind the data for local steroid production in brain are not unambiguous. Cheney *et al*. (1995) focussed on brain concentrations of progesterone (PROG) and its immediate derivatives 5α-dihydroprogesterone (DHPROG) and allopregnanolone (tetra-hydroprogesterone). Though concentrations were markedly higher in brain than in plasma, adx/gdx produced parallel decreases in brain and plasma PROG, suggesting that the majority of brain PROG

derives from peripheral tissues. In the same study, adx/gdx was not accompanied by parallel declines in brain and plasma levels of allopregnanolone and DHPROG. Unexpectedly, adx/gdx produced decreases of 30–50% (allopregnanolone) and 70% (DHPROG) in brain, but failed to reduce plasma levels. This could mean that these steroids only enter the brain from the periphery if produced in excess. If so, this observation would argue against significant *de novo* brain synthesis of allopregnanolone and PROG from cholesterol. This interpretation is indicated by the fact that intravenous pregnenolone sulfate increased brain pregnenolone levels massively (50-fold); allopregnanolone levels increased 4–5 fold but, significantly, the increase factor was greater in adx/gdx animals (7–8 fold) (Cheney *et al*., 1995). These data suggest that, at most, only a modest proportion of brain allopregnanolone derives from local metabolism of progesterone.

Evidence that brain concentrations do not decline following adx + gdx (above) is crucial to the argument for brain steroid sythesis, but in one report not only did brain levels remain steady or increase, but blood levels of DHEA increased following operation. Blood DHEA sulfate levels in the intact animal were 0.26 ng/ml. This was unaffected by sham surgery (0.28 ng/ml) but rose to 0.36 ng/ml 15 days post adx + gdx (Corpechot *et al*., 1981). It would be unwise, therefore, to rely on such data to support the case for steroidogenesis in brain. On balance, if some *de novo* steroid synthesis does take place in rodent brain takes place, it is not very efficient. This is indicated by: (a) low levels of expression of key steroidogenic enzymes; (b) marginal levels of pregnenolone and DHEA synthesis in brain; and (c) failure of pregnenolone and DHEA levels to be independent of blood levels. If this is true, one must suppose that the majority of brain steroids (but not sterols) in the adult derive from the circulation. Nonetheless, a key issue is whether some local synthesis does take place, because peripherally- and locally-derived steroids could have quite different metabolic fates. Some derivative steroids are extremely potent regulators (for instance molecules with the 3a 5a configuration can activate GABAA receptors at concentrations orders of magnitude lower than, e.g., the high concentrations of DHEA and pregnenolone). Low levels of steroid synthesis could have dramatic effects – fast metabolism of steroids close to their sites of action could play a major role in modulating brain function (Balthazart and Ball, 2000). For *de novo* steroidogenesis in brain, while the interim verdict is "*not proven*", the jury is still out.

LOCAL STEROID METABOLISM AND THE MITOCHONDRION

The key step in steroid synthesis is the removal of the side-chain of cholesterol to produce the first steroid molecule, pregnenolone (Figure 15.1). What is unusual is that the enzyme responsible, P450scc or CYP11A1, is located in mitochondria, and substrate delivery to this enzyme is a key regulatory step (discussed later). While the vast majority of P450s are targeted to microsomes and, though some unusual enzymes have dual targeting specificity (e.g., CYP1A1: Addya *et al*., 1997; Bhagwat *et al*., 1999; CYP2E1: Neve and Ingelman-Sundberg, 1999), only three major P450 types are predominantly localized to mitochondria. These are the


Zymosterol
Lathosterol
Δ 8-7 isomerase/
Δ 24 reductase
HO
HO
Δ 5 reductase
Δ 8-7 isomerase
7-dehydrocholesterol
7-dehydro
desmosterol
UV
Previtamin D3
CH3
thermal
isomerization
Provitamin D3
1,25-dihydroxyD3
HO
Δ 7 reductase
Δ 7 reductase
24(S)-hydroxycholesterol
OH
7α,24(S)-dihydroxy
cholesterol
CYP39A1
(liver only)
Cholesterol
CYP46
Δ 24 reductase
Desmosterol (24-
dehydrocholesterol)
CH2OH
7α-,26-dihydroxy
cholesterol
CYP27; 7-
hydroxylase(s)
CYP11A1
(P450scc)

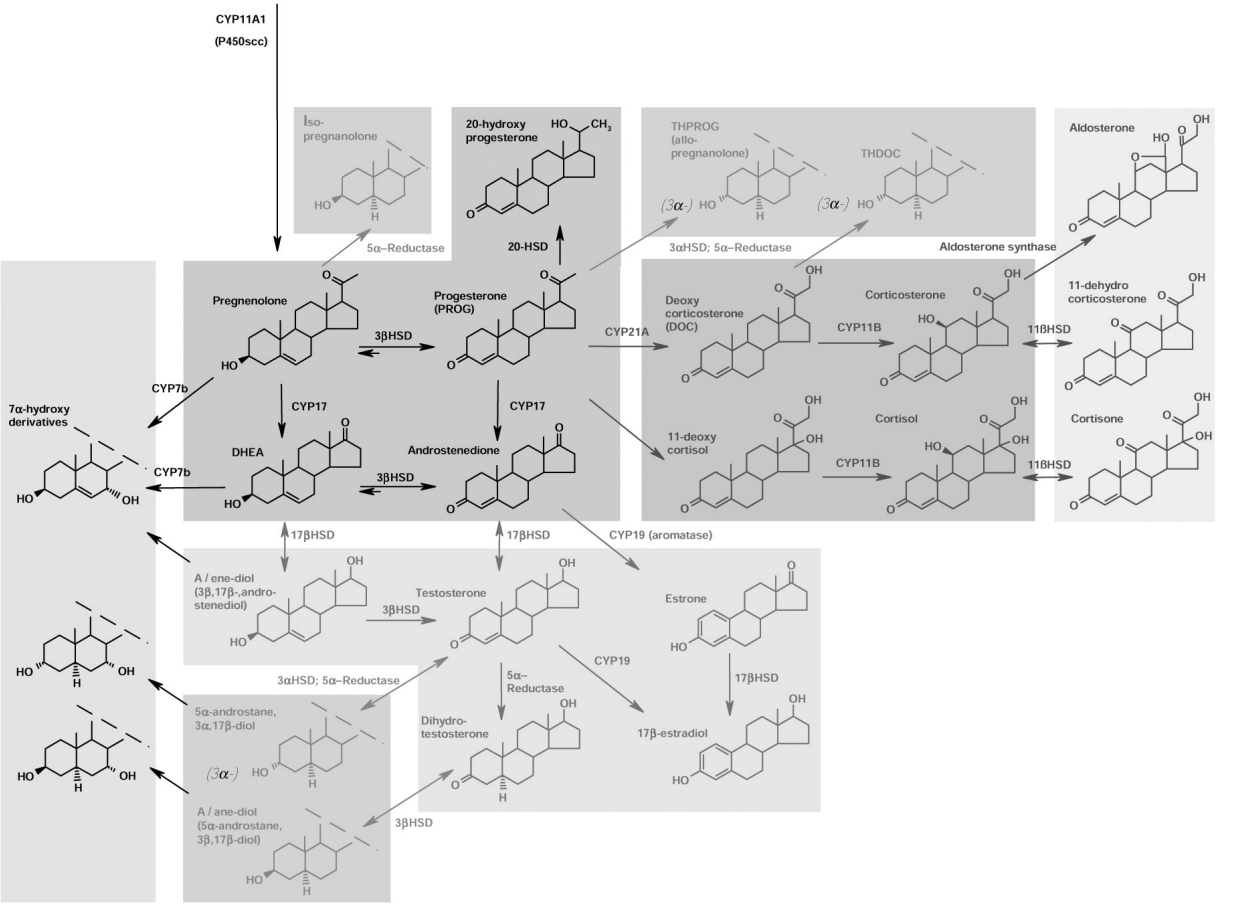


Figure 15.1 Pathways of steroid and sterol metabolism in brain (simplified).

cholesterol side-chain cleavage enzyme (CYP11A1), the 27-hydroxylase of cholesterol (CYP27; also 25-hydroxylase of Vitamin D), and the 11β-hydroxlases including the corticosterone-synthesizing enzyme CYP11B1 (with additional 18-hydroxylation activity) and the aldosterone synthase CYP11B2.

All these enzymes are expressed in brain, though at modest levels (see text and Box 15.5). Although co-expression in the same cells has not been formally demonstrated, it is possible that the improved solubility of hydroxycholesterols may facilitate delivery to P450scc (e.g., Jones and Hsueh, 1982; Brand *et al*., 1998) and 27-hydroxylation could therefore cooperate with side-chain cleavage in steroid synthesis. Note that the cholesterol 24(S) hydroxylase, CYP46, is microsomal (Lund *et al*., 1999).

Co-expression of P450scc (CYP11A1) and CYP11B (Box 15.5) would suggest preferential synthesis of 11β-hydroxypregnenolone, a conversion that has some antecedents (Maschler *et al*., 1975; Murphy, 1981; see also Ainsworth and Nitchuk, 1975) but which is not generally regarded as a major route of steroid metabolism. However, 3β-hydroxysteroid dehydrogenase is distributed richly in both the mitochondrial and microsomal compartments (see Thomas *et al*., 1999), arguing that a major local product could be 11β-hydroxyprogesterone (Figure 15.1), a natural glucocorticoid receptor ligand and potential substrate for further local metabolism.

Box 15.5 11β-hydroxylation/aldosterone synthase, and 11β-HSD

Adrenal corticosteroids: glucocorticoids (cortisol in humans and most mammals, corticosterone in rats and mice) and mineralocorticoids (aldosterone), have potent actions on the central nervous system, affecting a wide range of electrophysiological, structural and metabolic parameters and thereby altering functions such as mood, cognition, neuronal development and survival (de Kloet, 1991).

11β-hydroxylation and corticosteroid synthesis. Until recently glucocorticoid synthesis via 11β-hydroxylation of deoxycorticosterone and deoxycortisol was thought to be the exclusive property of the adrenal. However, the 11β-hydroxylase (CYP11B1) is expressed in brain, as assessed by immunohistochemistry (Ozakie *et al*., 1991) and RT-PCR (Gomez-Sanchez *et al*., 1996; MacKenzie *et al*., 2000), and confirmed by *in situ* hybridization, albeit at low levels (Erdmann *et al*., 1995, 1996). Ozakie *et al*. (1991) reported enzyme activity in brain (cortical white matter) at about 15% of the level seen in adrenal, while Gomez-Sanchez *et al*. (1996) describe conversion of deoxycorticosterone to corticosterone in brain extracts. However, Stromstedt and Waterman (1995) suggest that CYP11B1 is expressed in rat brain but not in mouse brain.

Aldosterone is generally produced by concerted 11β-hydroxylation and 18 hydroxylation/dehydrogenation that converts inert deoxycorticosterone directly to aldosterone. The enzyme responsible, aldosterone synthase (P450aldo; CYP11B2), appears also to be expressed in some brain regions, including hippocampus (Gomez-Sanchez *et al*., 1997); these authors detected aldosterone synthesis in brain minces. Together these suggest that at least some glucocor-

ticoid and mineralocorticoid synthesis might take place in brain, though the prevalent view is that glucocorticoids principally derive from the circulation.

Glucocorticoid inctivation/reactivation by 11β-hydroxysteroid dehydrogenase. Plasma glucocorticoid levels are tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis and its suprahypothalamic inhibitory regulators. Notable is the hippocampus which highly expresses both corticosteroid receptors, the lower-affinity glucocorticoid receptor (GR) and the higher-affinity mineralocorticoid receptor (MR), and appears to act in glucocorticoid inhibitory control upon HPA activity (Jacobson and Sapolsky, 1991).

Until recently the main determinants of corticosteroid action within the brain were thought to be the prevailing levels of the steroids, mostly derived from the circulation, and the relevant densities of the two intracellular receptors, GR and MR. Recently it has become apparent that a hitherto arcane enzyme complex, 11β-hydroxysteroid dehydrogenase (11β-HSD), plays an important role in determining intracellular glucocorticoid action (Seckl, 1997). 11β-HSD catalyzes the interconversion of active physiological 11-hydroxy glucocorticoids (cortisol, corticosterone) and their inert 11-oxo forms (cortisone, 11-dehydrocorticosterone). The latter are not known to bind to receptors. The physiological importance of the enzyme was only recognised a decade ago when renal 11β-HSD was found to inactivate glucocorticoids and prevent them from binding to the innately non-selective MR, allowing aldosterone alone to bind (Edwards *et al*., 1988; Funder *et al*., 1988). In the absence of the enzyme or the presence of liquorice-based inhibitors, cortisol gains access to and activates MR causing sodium retention, potassium loss and severe hypertension. Subsequently, it was discovered that there are two distinct 11β-HSD isozymes, the product of distantly related genes, which play very different roles.

11β-HSD Type 2 is a high-affinity, NAD-dependent 11β-dehydrogenase, which rapidly inactivates glucocorticoids to their inert 11-oxo forms. It is this activity that engenders MR specificity in the kidney (Albiston *et al*., 1994). Mutations in the 11β-HSD2 gene result in the rare hypertensive syndome of apparent mineralocorticoid excess (Mune *et al*., 1995) and transgenic mice homozygous for targeted disruption of the 11β-HSD2 gene show an analogous hypertensive phenotype due to illicit occupation of MR by corticosterone (Kotelevtsev *et al*., 1999). 11β-HSD2 is also highly and widely expressed in the developing midgestational brain at a time preceding that of MR expression, but coinciding with GR expression (Brown *et al*., 1996; Diaz *et al*., 1998). It has thus been hypothesized that this may be important in attenuating glucocorticoid effects on neuronal and glial development. Postnatally, 11β-HSD2 is only expressed in a few selective regions of the rat brain, such as the nucleus tractus solitarius, the subcommissural organ and scattered neurons in the central nucleus of the amygdala (Robson *et al*., 1998). In these regions aldosterone appears to exert selective effects on salt appetite and blood pressure which are not mimicked by corticosterone (Gomez-Sanchez, 1986; McEwen *et al*., 1986), suggesting that 11β-HSD2 protected MR exist

Box 15.5 (*Continued*)

in these sites. This notion, supported by the hypertensive effects of central infusion of enzyme inhibitors (Gomez-Sanchez and Gomez-Sanchez, 1992), nevertheless remains to be formally established.

In contrast, 11β-HSD Type 1 is widely expressed in the adult CNS including the hippocampus, and predominantly in neurons, as well as in the pituitary and hypothalamus (Lakshmi *et al*., 1991; Moisan *et al*., 1990, 1992). Whereas the enzyme is bidirectional in cell homogenates, in intact cells it is often a predominant 11β-reductase (Jamieson *et al*., 1995) (although other studies have argued that the hippocampal enzyme catalyzes bidirectional interconversion; Jellink *et al*., 1999). This reaction direction, far from inactivating glucocorticoids, regenerates active steroids from inert circulating 11-oxo forms. Recent data suggest that cortisone and 11-dehydrocorticosterone levels are around 10–50 nmol/l in human and rat plasma. The 11-oxo steroids circulate largely unbound to plasma proteins, unlike the active 11-hydroxy glucocorticoids, which are around 95% bound to CBG and albumin (Dunn *et al*., 1981). Thus, although total cortisol or corticosterone levels are between 20 and perhaps 500 nmol/l, with marked diurnal variation, "free" levels are lower than those of 11-oxo steroids for at least part of the day. The potential importance of 11β-HSD1 in the CNS remained moot until studies in primary hippocampal neurons showed predominant 11β-reductase-mediated glucocorticoid reactivation (Rajan *et al*., 1996), so contributing to glucocorticoid neurotoxicity (Ajilore and Sapolsky, 1999). The recently described 11β-HSD1 knock-out mouse (Kotelevtsev *et al*., 1997) should allow determination of any biological importance of this *in vivo*. Indeed, the mutant mice show elevated basal corticosterone levels, suggesting that glucocorticoid negative feedback control of the HPA axis is attenuated, raising the possibility that 11B-HSD1 could contribute to age-related changes in HPA axis activity (Meaney *et al*., 1993). Indeed, very recent data directly support the notion of blunted glucocorticoid feedback control of the HPA axis in 11B-HSD1 null mice (Harris *et al*., 2000). In this light it is intriguing that hippocampal 11β-HSD1 activity is downregulated by chronic stress (Jamieson *et al*., 1997), perhaps acting to help to protect vulnerable neurons from the deleterious effects of chronic glucocortoicoid excess. 11β-reductase represents a potential target for manipulating glucocorticoid action therapeutically.

STEROID HORMONE NUCLEAR RECEPTORS ARE ABUNDANT IN BRAIN

Peripherally-produced molecules that can access the brain include the sex steroids (testosterone, DHT, estradiol, progesterone), adrenal steroids including the corticosteroids (glucocorticoids and aldosterone), pregnenolone and dehydroepiandrosterone (DHEA), and non-steroidal molecules including the hydroxycholesterols, vitamin D, thyroid hormones (triiodothyronine (T3) and thyroxine

(T4)). These are thought to bind to classic steroid hormone receptors present in brain cells, whereby they modulate transcription. The family of steroid hormone receptors includes the androgen receptor (AR), estrogen receptors (ERα and ERβ), the receptors for glucocorticoids and mineralocorticoids (GR and MR), progesterone (PR), vitamin D (VDR), thyroid hormone (TR subtypes) and other diverse members including similar but poorly-characterized receptors such as the estrogen-receptor related molecules ERR1 and ERR2, and also receptors responding to oxysterols (e.g., LXRα, FXR, CAR-β), fatty acids and prostenoids (PPARs) (reviewed by Kumar and Thompson, 1999; Shibata *et al*., 1997; see in particular the evolutionary trees assembled by Laudet, 1997; Laudet *et al*., 1992; Baker, 1997). Many if not all of these receptors are expressed in the brain, with expression of AR, ERα, ERβ, GR and MR being recorded in hypothalamus, cortical and limbic regions, including the hippocampus, and other brain structures.

According to the traditional view of steroid hormone action, ligand binding to the cognate cytoplasmic receptor is followed by transport of the complex to the nucleus wherein transcription of specific hormone-responsive genes is induced or repressed. This is exemplified by the glucocorticoid hormones.

Two receptors exist, GR and MR, with different affinities for the major circulating glucocorticoid, corticosterone in rodents and cortisol (hydrocortisone) in man. MR binds corticosteroids avidly, while the lower affinity receptor GR only becomes fully occupied under conditions of stress. The principal effect of MR or GR ligand binding is stimulation or repression of specific gene expression (reviewed by Vreugdenhil *et al*., 1996; de Kloet *et al*., 1998); note that the precise classes of genes induced by MR and GR are overlapping but are to some extent distinct. In addition to genomic actions on transcription, many steroids have fast actions on neuronal excitability that cannot be explained by relatively slow changes in transcription. For other steroids (e.g., pregnenolone and DHEA) no nuclear receptors are known; instead these appear to influence neuronal activity by modulating non-nuclear receptors. These aspects are considered separately.

MEMBRANE-ASSOCIATED STEROID RECEPTORS

While the molecular evidence argues that steroids target specific cytosolic receptors that subsequently translocate to the nucleus, the pharmacologic evidence is sometimes very different. Extremely rapid effects on neuronal excitability (seconds to minutes) can be detected, a timescale inconsistent with *de novo* transcription of hormone-responsive genes. To give one example, estradiol induces rapid changes in the electrical activity of cells without evident functional nuclear estrogen receptors (Gu *et al*., 1999). Other steroidal molecules have been shown to influence neuronal function despite coupling to large molecules such as serum albumin, arguing that, in general, some functional receptors for steroid hormones reside at the cell surface (reviewed by Schmidt *et al*., 2000). This underlines an earlier report of a specific high affinity cell-surface receptor for aldosterone (Wehling *et al*., 1993) and a cell-surface target for corticosterone in amphibian brain (Moore and Orchinik, 1994). Wagner *et al*. (1999), working with novel inhibitors of the progesterone and

glucocorticoid receptors RTI3021-012 and 022, found that, while these two new molecules displayed avid binding to GR, they failed to block corticosteroid action, possibly suggesting that some glucocorticoids may also act through non-GR pathways so far uncharacterised. Gametchu *et al*. (1999) discuss a cell-surface variant of the glucocorticoid receptor GR that they term mGR; this species appears to be an isoform of the classic nuclear receptor. It is of note that glucocorticoid entry to cells appears to rely on a specific and saturable uptake system at the cell surface (Allera and Wildt, 1992a,b; Lackner *et al*., 1998), but photoaffinity labeling rvealed a 53 kD membrane protein in rat liver plasma membrane (Ibarrola *et al*., 1992) that was competed by cortisol, deoxycorticosterone and progesterone. This is probably distinct from mGR on molecular weight grounds.

Evidence has also been provided for a cell surface estradiol receptor. Monje and Boland (1999) report that estradiol binding to the high-affinity surface receptor of rabbit uterus was reduced by anti-ER antibody; this would suggest that the cell-surface receptor comprises a structurally-modified form of the classic nuclear receptor ERα. However, this study does not rule out the possibility of a discrete class of cell surface estrogen receptors unrelated to the nuclear receptors ERα and ERβ. An intriguing report by Razandi *et al*. (1999) describes how 2% of ERα or ERβ appears at the cell surface, and moreover is functionally coupled to inositol polyphosphate production and adenyl cyclase activity. Clarke *et al*. (2000) report that ERα immunoreactivity is principally associated with neurites (and not the nucleus) of rat hippocampal neurons, arguing for non-genomic actions at the cell membrane. Molecular characterization of the inferred cell surface glucocorticoid and estrogen receptors remains an important research objective and if, as so far seems likely, they derive from the classic nuclear receptors, the elucidation of their cell-surface signaling pathways may provide some surprises.

NEUROSTEROID ACTION AT NEUROTRANSMITTER RECEPTORS

Some steroids, such as DHEA and pregnenolone, seem to lack a nuclear receptor, though the plethora of "orphan" nuclear receptors affords many candidates. There have been reports of high-affinity DHEA binding sites in peripheral tissues such as T-cells (Meikle *et al*., 1992; Okabe *et al*., 1995), cervical fibroblasts (Imai *et al*., 1992) and liver cytosol (Yamada *et al*., 1994). In the absence of demonstrated functionality these might possibly represent transport proteins rather than receptors per se (although it may be incorrect to make a firm distinction between the two). However, it has been well established that DHEA and structurally-related steroids can modulate neuronal function via an interaction with cell membrane receptors and ion channels.

Diverse neurotransmitter receptors have been shown to respond to steroids (including the AMPA, glycine, kainate, nicotinic acetylcholine, NMDA and oxytocin receptors; reviewed by Rupprecht and Holsboer, 1999). However the best studied interaction is with the ligand-gated ion channel responding to the principal inhibitory neurotransmitter, gamma-amino butyric acid (GABA).

Two types of steroid-GABA receptor interaction have been described, agonistic and antagonistic, dependent on the structure of the steroid ligand. Potentiation of

the $GABA_A$ receptor by the synthetic steroid anaesthetic alfaxolone was reported by Harrison and Simmonds (1984), while natural steroid metabolites of corticosteroids and progesterone that also bear a 3α-hydroxy group are potent agonists of the $GABA_A$ receptor (Majewska *et al*., 1986). These latter steroids, produced by 3α-reduction of deoxycorticosterone and progesterone, increase the inhibitory action of the GABA receptor, so suppressing neuronal activity and producing anesthetic, anticonvulsant, sedative and anxiolytic properties. Conversely, 3β-hydroxysteroids such as DHEA and pregnenolone are natural antagonists of the $GABA_A$ receptor (Majewska *et al*., 1990), thus promoting neuronal activity (reviewed by Majewska *et al*., 1992; Lambert *et al*., 1995, 1996; Belelli *et al*., 1999). Metabolic switching between 3β-hydroxylated steroids that antagonise the $GABA_A$ receptor (such as pregnenolone and DHEA) and 3α-hydroxylated steroids (particularly those that are also 5α reduced) may represent an additional level of control of neuronal activation in the brain *in vivo*.

As discussed elsewhere, it is noteworthy that the binding site on the $GABA_A$ receptor complex that interacts with steroids appears to be distinct from the modulatory binding sites on this same receptor for either benzodiazepines or barbiturates. Recent data also suggest that the binding sites for steroid agonists and antagonists may differ according to the subunit composition of the $GABA_A$ receptor (Maitra and Reynolds, 1998).

Steroid modulation of the N-methyl D-aspartate (NMDA)-type channel for the excitatory amino acid glutamate has also been recorded. In particular, pregnenolone appears to enhance the action of the NMDA receptor (Wu *et al*., 1991), while an anesthetic 3α-hydroxysteroid is reported to antagonize the same receptor (Park-Chung *et al*., 1997). Thus, natural 3β-hydroxysteroids such as pregnenolone enhance neuronal excitability in two ways, by dampening inhibitory $GABA_A$ channel activity and by boosting excitatory NMDA currents. Anesthetic 3α-hydroxysteroids have the reverse action, enhancing $GABA_A$ channels and inhibiting NMDA activity, suggesting that the local ratio of 3α- and 3β-hydroxysteroids is critical to neuronal function. As noted earlier, steroid modulation of neurotransmitter receptors is found on many ligand-gated ion channels and is not restricted to the GABA and NMDA receptors (reviewed by Rupprecht and Holsboer, 1999).

A further level of complexity is added by the finding that neurosteroids may also act via intracellular targets. Two such targets have come to the fore: the peripheral benzodiazepine receptor and the sigma site. Both seem to be involved, perhaps counter-intuitively, in aspects of sterol transport and metabolism, and have the potential to be modulated by both sterols and steroids. These two targets are important because ligand binding may, by circuits that are largely unknown, result in alterations to ion channel activity.

THE PERIPHERAL BENZODIAZEPINE RECEPTOR; CHOLESTEROL TRANSPORT

Benzodiazepines, exemplified by diazepam, are potent anticonvulsant and anxiolytic agents and are widely used in the clinic. These selectively target and generally

enhance the activity of $GABA_A$ receptors at the cell surface, though regional differences in subunit affinity and expression may explain somewhat diverse pharmacologic actions (reviewed by Teuber *et al.*, 1999; Whiting *et al.*, 1999). However, biochemical studies revealed selective benzodiazepine binding in tissues not known to express functional GABA receptors, giving rise to the concept of the "peripheral benzodiazepine receptor", PBR (also referred to as the "omega" receptor).

The cloned PBR (Sprengel *et al.*, 1989) polypeptide is an 18 kD protein with 5 trans-membrane domains stationed on the outer mitochondrial membrane and widely expressed in steroidogenic tissues, liver and brain. The receptor turns out to be a cholesterol transport protein that can cooperate with StAR-type sterol transporters (Sugawara *et al.*, 1997), probably including brain MLN64, to translocate cholesterol from the outer to the inner mitochondrial membrane (Besman *et al.*, 1989; Krueger and Papadopoulos, 1990). Thus, PBR delivers cholesterol to the key initial enzyme in steroid synthesis, mitochondrial P450scc (CYP11A1), and also the mitochondrial cholesterol 27-hydroxylase, CYP27. Cholesterol, a highly hydrophobic molecule, might be expected to diffuse freely in membranes, but this is not the case. In fact, membranes can act as an impermeable cholesterol barrier; this is best illustrated by the near-total block of steroid synthesis in individuals harboring mutations in the intracellular cholesterol transport protein StAR (see Chapter 5). Indeed, all aspects of cholesterol movement seem to require dedicated transport systems (reviewed by Liscum and Munn, 1999). Thus PBR ligands can dramatically increase the rate of steroid synthesis in diverse tissues (Papadopoulos and Brown, 1995) and also in brain (McCauley *et al.*, 1995).

Although PBR is thought to be regulated by porphyrins (hemin and protoporphyrin IX; Verma *et al.*, 1987), an insight into the role of PBR was provided by the characterization of an endogenous polypeptide ligand for the receptor. This is a conserved 87/107 amino acid polypeptide that competes with diazepam for binding, hence its name: "diazepam binding inhibitor" (DBI) or endozapine (Gray *et al.*, 1986; Webb *et al.*, 1987; Costa and Guidotti, 1991). On binding PBR, the DBI polypeptide (like benzodiazepines themselves) stimulates cholesterol transport and promotes steroidogenesis (e.g., Besman *et al.*, 1989; reviewed by Papadopoulos *et al.*, 1997); disruption of the PBI gene in cultured cells prevents cholesterol transport and steroid synthesis (Culty *et al.*, 1999). Although DBI appears to lack a secretion signal sequence, there are alternative forms, some of which are membrane-associated (Webb *et al.*, 1987; Todaro *et al.*, 1991). DBI is widely expressed during development (Burgi *et al.*, 1999), in the brain and peripheral tissues (Alho *et al.*, 1988), and binds to both $GABA_A$ and PBR targets (reviewed by Barbaccia *et al.*, 1990; Costa and Guidotti, 1991; Papadopoulos *et al.*, 1991). DBI is predominantly non-neuronal in origin (Tong *et al.*, 1991); potentially, DBI released from glia could act at $GABA_A$ receptors to modulate neuronal metabolic activity. Such a trans action has not been demonstrated.

Drug targeting at PBR could be dismissed as purely circumstantial, except that binding is reported to elicit similar anticonvulsant and anxiolytic effects to binding at $GABA_A$ (Sanger *et al.*, 1994), and can similarly impair learning in rodents (McNamara and Skelton, 1991, 1992). In decided contrast, Gunther *et al.* (1995) report that mice lacking the gamma2 subunit gene of the $GABA_A$ receptor

are refractory to diazepam. This would seem to argue that $GABA_A$-gamma2, rather than PBR, provides the principal *in vivo* target for benzodiazepines. This conclusion should not go unchallenged, because while diazepam targets both the peripheral (omega/PBR) and central ($GABA_A$) receptors, other agents with undoubted pharmacologic activity can selectively target the peripheral receptor (e.g., Ro5-4864 and PK11195) or $GABA_A$ (e.g., clonazepam). Selective PBR ligands can exert potent amnestic, anxiolytic and proconvulsant effects *in vivo* (McNamara and Skelton, 1992; Romeo *et al*., 1994; Ferrarese *et al*., 1995; Okuyama *et al*., 1999).

To complicate the picture, pharmacologic evidence argues for a multiplicity of omega/PBR-type binding sites in brain (e.g., Woods and Williams, 1996; Rao and Butterworth, 1997). The molecular basis of this diversity is not known. Interactions between PBR and accessory proteins such as PRAX-1 that are selectively expressed in some sub-regions of the brain (Galiegue *et al*., 1999) could be responsible, as could different phosphorylation states of PBR (Whalin *et al*., 1994).

Overall, this work raises a pressing question. By what route do alterations in sterol transport modify brain function? Sterol transport is likely to regulate local neurosteroid synthesis; benzodiazepine binding to PBR improves cholesterol delivery to P450scc, and these could act by boosting levels of GABA-active neurosteroids derived from pregnenolone, though the contribution of *de novo* steroid synthesis remains to be confirmed. Sterol transport may be intimately coupled with aspects of mitochondrial function. PBR copurifies with a family of related voltage-dependent anion channels (porins) of the outer mitochondrial membrane (McEnery *et al*., 1992, 1993). It is not known whether PBR regulates these ion channels but we should note that DBI/endozepine seems to have two targets. Firstly, it modulates a cell-surface ion channel that harbors a steroid binding site. Secondly, it activates a mitochondrial transport system involved in steroid synthesis; plausibly it could also regulate mitochondrial membrane polarisation. Cross-talk between the two systems is likely (see below). $GABA_A$ agonists, like PBR ligands, were reported to increase pregnenolone synthesis in isolated rat retina (Guarneri *et al*., 1995).

SIGMA RECEPTORS, EMOPAMIL BINDING PROTEIN AND STEROL METABOLISM

The involvement of PBR in sterol transport, and its targeting by neuro-active drugs, argues that sterols and sterol/steroid metabolism may regulate brain function. This conclusion is reinforced and emphasized by the identification of further intracellular sites sensitive to steroidal molecules, the "sigma" receptor and the emopamil-binding protein (EBP).

Pharmacologic sigma ligands encompass a group of chemically unrelated neuroleptic and neuroprotective drugs, such as haloperidol, pentazocine and emopamil, used for example in treating schizophrenia (reviewed by Walker *et al*., 1990). Although some such agents also target the dopamine receptor (e.g., haloperidol, trifluoperazine) or the NMDA receptor (e.g., ifenprodil), others seem to be fairly selective for the sigma site (e.g., [+] pentazocine, NE100, SR31747A, PD144418)

though interaction with other receptors is not excluded. Interactions with (+)-opiates and phencyclidine have also been suggested.

Sigma sites have been pharmacologically classified into sigma-1 and sigma-2 sites (Hellewell and Bowen, 1990; Quirion *et al*., 1992), with different distributions in brain (McCann *et al*., 1994; Bouchard and Quirion, 1997). A sigma-3 receptor has also been suggested (Myers *et al*., 1994) but the molecular basis for this diversity is not yet clear. In fact, recent cloning experiments have led to the discovery of two coding sequences which share at least some pharmacologic properties with the sigma-1 site. Although both have properties that strongly suggest an involvement in sterol metabolism, they are, perhaps surprisingly, unrelated to each other.

The sigma-1 receptor

Clones encoding a *de facto* sigma-1 receptor were identified on the basis of affinity for sigma ligands including haloperidol and SR31747A (Hanner *et al*., 1996; Kekuda *et al*., 1996; Jbilo *et al*., 1997). This 25 kD protein has a perinuclear localization, harbors a single transmembrane domain, and shares very significant homology (~30% sequence identity) with the yeast Erg2 (ergosterol synthesis; C8-C7 sterol isomerase) enzyme. Puzzlingly, it has not yet been possible to demonstrate any catalytic activity associated with the cloned polypeptide (Jbilo *et al*., 1997). No inherited deficits in sigma-1 have been reported.

Table 15.2 Brain targets for steroids.

Cell surface receptors	*Sterol synthesis* enzymes and regulation*	*Transporters*	*Nuclear receptors*	*Signaling molecules*
$GABA_A$	HMGCR	Peripheral benzodiazapine	GR, MR	*Sonic*
NMDA	HMGCS	receptor (PBR)	PR, AR	*Hedgehog* (Shh)
AMPA	Sterol 8-7 isomerase	NPC-1, -2	ERα, β	*Patched* (Ptc)
Kainate	(sigma)	StAR, MLN64VDR	TR subtypes	
Glycine	EBP	Caveolin	ERR1,2, PPAR, PXR	
Acetyl choline		FKBP56	CAR, PXR, LXR, FXR	
sigma-2, sigma-3				
Oxytocin	SCAP/SREBP	Cyclophorins	SF-1, SXR, CPF, FTZ-F1	
mGR?		LDL-receptor family	NUR1, NUR77, COUP-TF	
		Apolipoproteins B,E, other	LHR-1	
		Tout-velu		
		OPP1-6		

* Other potential targets include enzymes involved in sterol and steroid metabolism, including cholesterol and steroid hydroxylases (CYPs 7, 11), 5α-reductases and hydroxysteroid dehydrogenases (3β-HSD, 11β-HSD).

Emopamil-binding protein

This polypeptide was cloned on the basis of affinity for emopamil, a neuroprotective agent effective in cerebral ischemia. The emopamil binding protein (EBP) (Hanner *et al*., 1995; Silve *et al*., 1996), a 26 kD protein containing four putative Tm segments and probably resident in the endoplasmic reticulum, has been demonstrated to encode a functional sterol C8-C7 isomerase that catalyzes the penultimate step in the synthesis of cholesterol (Silve *et al*., 1996a,b; Hanner *et al*., 1996; Moebius *et al*., 1996). Expression of human EBP in mutant yeast permitted functional reconstitution of cholesterol synthesis while rendering the yeast susceptible to growth inhibition by trifluoperazine (Silve *et al*., 1996b). In addition to inhibition by 7-ketocholestanol (Paul *et al*., 1998), the protein displays surprisingly high affinity for the non-steroidal anti-estrogen tamoxifen (Moebius *et al*., 1997; Paul *et al*., 1998). Deficiency in EBP/sterol isomerase has been associated with lipid abnormabilities and skin and limb defects (CHILD syndrome; Grange *et al*., 2000) and with X-linked chondrodysplasia (Conradi-Hunermann syndrome) (Derry *et al*., 1999; Braverman *et al*., 1999).

The relationship between sigma and EBP remains unclear (Moebius *et al*., 1997). Although dissimilar in primary sequence, both bind some "sigma" ligands such as tridemorph with high affinity, and whereas haloperidol targets the mammalian sigma-1 receptor and not EBP, haloperidol inhibits cholesterol synthesis in yeast by targeting the endogenous EBP/sterol isomerase (Moebius *et al*., 1996). The absence of apparent enzymatic activity associated with the sigma-1 polypeptide in yeast may be for technical reasons. As discussed (Moebius *et al*., 1997), sigma-1 could be a sterol-binding regulatory protein or catalyses a novel conversion. At least some pharmacologic sigma ligands (e.g., SR31747) inhibit cholesterol synthesis and stop cell proliferation; the block can be overcome by exogenous cholesterol (Labit-Le Bouteiller *et al*., 1998).

Are sterol (and steroid) targets generic sigma sites?

The endogenous ligand of the pharmacologic binding site on sigma-1 is not known. Although neuropeptide Y has been discussed (reviewed by Wan and Lau, 1995), other evidence has argued that the endogenous ligand could be steroidal in nature (Su *et al*., 1998). Corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al*., 1988, 1990), and manipulation of systemic steroids affects behavioral changes mediated through the sigma-1 receptor (Phan *et al*., 1999). There is every likelihood that neurosteroids can target the sigma-1 receptor (and probably EBP) in addition to their well-documented actions on cell surface ion channels. For instance (and as discussed further below), DHEA (and pentazocine) appears to improve memory function in mice by acting as a sigma agonist, while progesterone (and haloperidol) appears to be an antagonist and reduces cognitive performance (Maurice *et al*., 1998). Similar conclusions were reached for DHEA by Bergeron *et al*. (1996) and Urani *et al*. (1998); in both cases the effects of DHEA were reversed by sigma antagonists (see

also Monnet *et al*., 1995). These studies must be interpreted with caution in view of possible pharmacologic overlap between sigma sites (sigma-1, -2) and EBP.

Extending the discussion, Jbilo *et al*. (1997) put forward the intriguing suggestion that sterol binding sites may constitute targets for sigma ligands. This would argue that other enzymes metabolizing, transporting or responding to sterols might furnish novel "sigma" sites. Among candidate polypeptides one might consider the four sterol sensing proteins discussed by Lange and Steck (1998) (Ptc, signal transduction; HMGCoA reductase, cholesterol sythesis; NPC1, Niemann-Pick disease type 1 and sterol transport; and the SREBP-cleavage protein, SCAP). Also, the cholesterol sythesis enzymes listed by Fitzky *et al*. (1999), diverse sterol-metabolizing enzymes (including P450s and dehydrogenases), and diverse nuclear receptors (see below) including CPF (Nitta *et al*., 1999). Transmembrane domains of cell-surface receptors interact with cholesterol and could afford further sigma sites. No pharmacologic evidence for sigma ligand targeting at such sites has yet been presented, but this situation seems ripe for change.

STEROL SYNTHESIS AND METABOLISM IS CRUCIAL TO BRAIN FUNCTION

Targets for anxiolytics and anticonvulsants (e.g., benzodiazepines) and neuroleptics (e.g., haloperidol) include enzymes and transporters involved in sterol transport and metabolism. This would suggest that sterol metabolism can play a regulatory role. This is confirmed by other genetic and pharmacologic evidence.

Different alleles at the locus encoding cholesterol delivery protein ApoE can in part determine the onset of Alzheimer's disease (Strittmatter *et al*., 1993; Corder *et al*., 1993; Saunders *et al*., 1993), although the linkage is by no means perfect (Lannfelt *et al*., 1994). ApoE deficient mice are cognitively impaired (Gordon *et al*., 1995) while transgenic mice overexpressing ApoE4 show age-dependent learning and memory deficits, but only significantly in females (Raber *et al*., 1998). Cholesterol uptake receptor genes are also involved; loci encoding low-density lipoprotein (LDL) receptor related protein, LRP, or the very low-density lipoprotein (VLDL) receptor, have also been linked with late-onset Alzheimer's (Kang *et al*., 1997; Okuizumi *et al*., 1995; but see Okulzumi *et al*., 1996). LRP-deficient mice are cognitively impaired (Van Uden *et al*., 1999). Mutations affecting *de novo* cholesterol synthesis also have dramatic effects on cognition. Deficiency of 7-dehydrocholesterol reductase causes Smith-Lemli-Opitz syndrome (SLOS), characterized by developmental abnormalities, adrenal insufficiency and mental retardation (Waterham *et al*., 1998; Wassif *et al*., 1998; Moebius *et al*., 1998). Inhibition of 7-dehydrocholesterol synthesis in weanling rats impairs later learning abilities (Xu *et al*., 1998) while cholesterol synthesis inhibition during gestation produces developmental deficits reminiscent of SLOS (Lanoue *et al*., 1997). Niemann-Pick disease type C ("NPC"), a condition associated with marked neurological deterioration (reviewed by Vanier and Suzuki, 1998), affects intracellular sterol transport mediated by the NPC1 gene product (Carstea *et al*., 1991). Finally, deficiency of cholesterol 27-hydroxylation (CYP27) causes cerebrotendinous xanthomatosis,

a disease characterized by mental retardation and atherosclerosis (Cali *et al*., 1991; see also Leitersdorf *et al*., 1993; Bjorkhem, 1994). However a similar deficency in mice seems to have rather less pronounced consequences (Rosen *et al*., 1998).

Some evidence argues that the potent neuroprotective drug FK506 (Dawson *et al*., 1993; Sharkey and Butcher, 1994) may operate in cholesterol trafficking, in addition to its well-known action on calcineurin. Transport of cholesterol "rafts" and/or caveolae, cholesterol-rich domains in the cell membrane, is mediated by the FK506 binding protein FKBP52 (Gold *et al*., 1999; also known as HSP56) in a complex with cholesterol, the cholesterol binding protein caveolin, and cyclophilins A and 40 (Uittenbogaard *et al*., 1998). The immunosuppressive drug cyclosporin, that also targets cyclophilins, also seems to modulate cholesterol delivery to mitochondria (Dahlback-Sjoberg *et al*., 1993) and has neurotoxic effects on chronic administration (Hauben, 1996; Gijtenbeek *et al*., 1999). Significantly, the sigma receptor may bind tightly to cyclophilin A, as demonstrated by copurification of a cyclophilin fragment with sigma (Schuster *et al*., 1994).

Circumstantially, cholesterol levels change in Alzheimer's disease and during FK506 treatment; blood cholesterol may in turn influence behavior (Fawcett *et al*., 1997; Kaplan *et al*., 1997). Circumstantially, cholesterol-derived vitamin D may subvert brain function (Stumpf, 1988; Holick, 1995; Lansdowne and Provost, 1998; Keddie, 1987); note that 25-hydroxylation of vitamins D is performed by the mitochondrial sterol 27-hydroxylase, CYP27 (Usui *et al*., 1990; Guo *et al*., 1993).

OXSTEROL AND STEROID SIGNALING INTERACTIONS

Oxysterol metabolism in brain

It has been suggested that only low levels of *de novo* steroid synthesis occur in brain, but agents modifying sterol synthesis and metabolism can have profound effects on brain function. This underlines the importance of sterol metabolism in brain.

A large proportion of cholesterol delivered to mitochondria is converted to oxysterols: hydroxycholesterols and cerebrosterols (Pedersen *et al*., 1989; Bjorkhem *et al*., 1997). Two alternative pathways seem to operate. In one, hydroxylation by a P450 enzyme selectively expressed in brain, CYP46 (Lund *et al*., 1999), produces the brain-enriched 24(S)-hydroxycholesterol or "cerebrosterol". This molecule is preferentially exported from the brain (Bjorkhem *et al*., 1997, 1998). 24(S)-hydroxycholesterol is not a substrate for brain CYP7B, and is instead processed, in liver, by a dedicated hepatic 7α-hydroxylase for 24(S)-hydroxycholesterol (CYP39A1; Li-Hawkins *et al*., 2000), this enzyme appearing to be absent from brain. In the other, hydroxylation at the 25, 26/27 positions produces hydroxycholesterols that are, most likely, further hydroxylated at the 7α position to provide a series of di- (or tri-) hydroxycholesterols; these may be further metabolised to 3-oxo steroids (see Zhang *et al*., 1997; Vatassery *et al*., 1997). Note that the 27-hydroxylase (CYP27) is, like P450scc, mitochrondrial in location (e.g., Okuda, 1994; Cali, 1999a), while the 24(S) hydroxylase is inferred to be

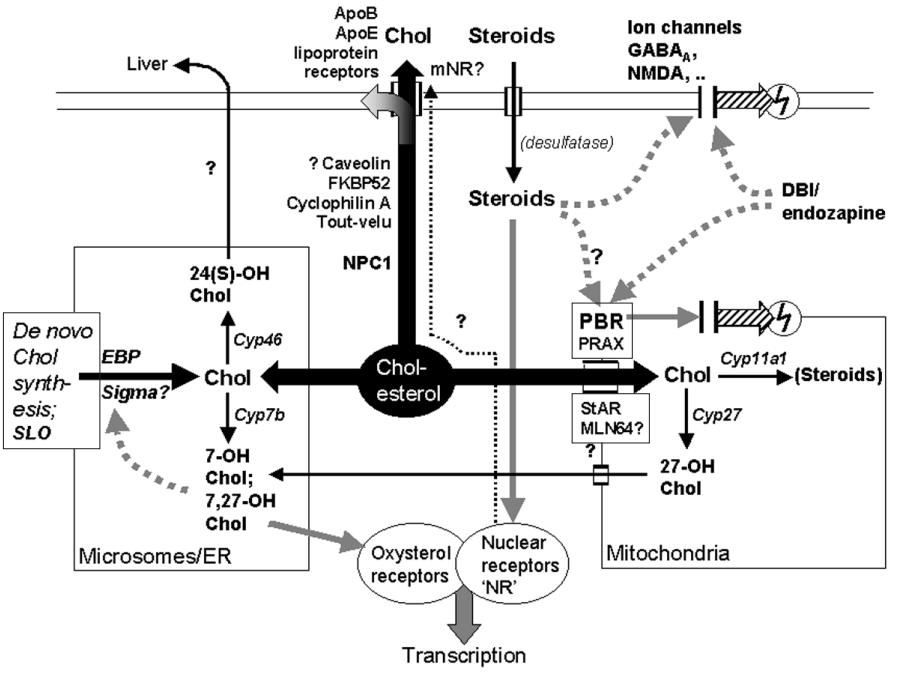


Figure 15.2 Pathways of sterol and steroid signaling in brain. CHOL, cholesterol; DBI, diazepam binding inhibitor; EBP, emopamil binding protein; ER, endoplasmic reticulum; PBR, peripheral benzodiazepine receptor; (Z), membrane depolarization.

microsomal (Lund *et al*., 1999). Signaling pathways related to oxysterol metabolism are presented schematically in Figure 15.2.

Do steroids target oxysterol sites?

The possibility that sigma targets might define steroid binding sites (Jbilo *et al*., 1997) was discussed earlier. Equally, some sterol targets might interact with steroids. Corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al*., 1990) while effects of glucocorticoids on immunity could be, in part, via lymphocyte sigma targets (Deroca *et al*., 1995). DHEA may also target sigma (Bergeron *et al*., 1996; Debonnel *et al*., 1996); Monnet *et al*. (1995) argued that DHEAsulfate is a sigma agonist, pregnenolone is an inverse agonist, while progesterone is an antagonist.

Is there cross-talk between sterol synthesis and surface ion-channels activity?

While some pharmacologic agents act both at cell surface channels (e.g., $GABA_A$) and intracellular sites, cross-talk may be more extensive. $GABA_A$ agonists, like PBR ligands, are reported to increase cholesterol conversion to pregnenolone in

isolated rat retina $GABA_A$ agonists (Guarneri *et al*., 1995). GABA channel activity can regulate intracellular sterol metabolism. Conversely, intracellular oxysterol sites can can alter surface channel activity.

NMDA channels are modulated by DHEA. Potentiation of NMDA currents was reversed by either haloperidol or the selective sigma ligand NE100, arguing strongly that sigma occupancy can govern the NMDA channel activity (Bergeron *et al*., 1996; Debonnel *et al*., 1996). The link between sigma ligands (including sigma-2 ligands; Couture and Debonnel, 1998) and NMDA currents raises the possibility that the effects of the important estrogen replacement analog equilin on NMDA currents (Brinton *et al*., 1997) might be mediated, in part, through this pathway. Sigma ligands have also been reported to prevent depolarization neurotoxicity of retinal cells (Senda *et al*., 1998) while, conversely, the sigma ligand trifluoperazine inhibits glutamate-induced mitochondrial depolarization (Hoyt *et al*., 1997), reminiscent of the association of PBR with voltage-dependent anion channels of the outer mitochondrial membrane (McEnery *et al*., 1992, 1993). In addition, sigma ligands can inhibit voltage-activated K+ channels in intact neurons (Nguyen *et al*., 1998) and in a neuropeptide positive tumor cell (Wilke *et al*., 1999).

How might sterols modulate neuronal activity?

There are four obvious routes. First, by direct interactions with cell surface channels. Neurotransmitter receptors contain steroid binding sites that modulate their activity, though it is unclear whether all such sites are structurally distinct from the transmembrane segment that may interact with membrane cholesterol. Sterols could exert important influences via direct interactions with steroid and/or cholesterol sites on neurotransmitter receptors.

Second, via DBI/enzapine. Modified cholesterols generally feed back on the enzymes involved in cholesterol synthesis, possibly including PBR/sigma. Feedback upon the production of the endogenous PBR ligand, DBI/endozapine has not been excluded. This polypeptide targets $GABA_A$ in addition to PBR, and changes in DBI/endozapine activity or expression could govern the activity of $GABA_A$ and other DBI-responsive channels.

Third, via neurotransmitter release. Ligands targeting the sigma site and/or PBR are reported to modulate Ca^{2+} release from intracellular stores (Vilner and Bowen, 2000; Hayashi *et al*., 2000) and Ca^{2+} fluxes (Church and Fletcher, 1995; Klette *et al*., 1995; Brent *et al*., 1996; Ramp and Triggle, 1987) and could alter synaptic firing properties.

Fourth, electrical coupling. Mitochondrial membrane polarization is tied to sterol metabolism, exemplified by the copurification of PBR with outer membrane porins (McEnery *et al*., 1992, 1993). It is possible that cell-surface and intracellular membrane depolarization are electrically coupled.

Local versus systemic control

Cholesterol delivery and intracellular transport is crucial to the adrenal and gonadal synthesis of glucocorticoids, estrogens and androgens, and body

homeostasis. Responsive changes in adrenal steroid levels may modulate memory encoding (Lathe, 2001). Corticosteroid abnormalities contribute to depression (Murphy, 1997) and estradiol promotes neuronal maintance and can be beneficial in Alzheimer's disease (Honjo *et al*., 1989; Haskell *et al*., 1997; Asthana *et al*., 1999).

Neuronal life and death: does local sterol metabolism govern apoptosis and neurodegeneration?

Cholesterol depletion threatens cell integrity. It is not surprising, therefore, that there may be coupling between cholesterol synthesis and metabolism in the CNS, on the one hand, and cell growth and survival on the other. Further, imbalance between cholesterol and cell growth, such as may occur during disease states, seems to predispose to an active cell death process (e.g., apoptosis). Importantly, it seems likely that brain could exploit cholesterol/apoptosis signaling mechanisms to achieve selective neuronal elimination during development; such processes may fail in ageing. Importantly, apoptosis may be governed at the local level by sterols, and at the systemic level by steroids. The different aspects are discussed separately below.

Cholesterol metabolism and cell death

Inhibition of cholesterol synthesis blocks DNA replication in primary brain-derived glial cultures (Langan and Volpe, 1987). This could not be overcome by exogenous cholesterol, suggesting an obligate requirement for intermediates in the cholesterol pathway (e.g., isoprenoids). Conversely, Michikawa and Yanagisawa (1999) report that blockade of cholesterol production, but not that of isoprenoids, could result not merely in impaired cell division but in an active neuronal cell death process, such as apoptosis or necrosis.

As might be expected, agents targeting the intracellular sites discussed above (peripheral benzodiazepine receptor, PBR; the sigma/sterol isomerase site; and FK506 binding proteins involved in intracellular cholesterol trafficking) have all been linked to the regulation of apoptosis. PBR ligands can induce apoptosis in thymocytes (Tamimoto *et al*., 1999; see also Hirsch *et al*., 1998) but may be potent inhibitors of apoptosis in a human lymphoblastoid cell line (Bono *et al*., 1999).

Blockade of cholesterol synthesis by the sigma ligand haloperidol is cytotoxic to glioma cells (Vilner *et al*., 1995) and to hippocampal neurons, possibly by necrosis rather than via apoptosis (Behl *et al*., 1995). Conversely, benzodiazepines are protective for hippocampal neurons, though both $GABA_A$ and PBR were targeted (Schwartz-Bloom *et al*., 2000). Haloperidol is also a dopamine receptor ligand, but specific dopamine agonists/antagonists were without effect (Vilner *et al*., 1995). Protoporphyrin IX, a natural sigma ligand, was found to potentiate cell death in energy stressed hepatocytes, whereas potentiation was blocked by cyclosporin A (Pastorino *et al*., 1994). In lymphocytes, sigma ligands can exert immunosuppressive actions by preventing cholesterol synthesis and cell division

(Jbilo *et al*., 1997; Labit-Le Bouteiller *et al*., 1998), possibly inducing apoptotic mechanisms (Brent *et al*., 1996).

FK506 and cyclophilins may collaborate in cholesterol delivery to mitochondria and to cell surface microdomains (Dahlback-Sjoberg *et al*., 1993; Uittenbogaard *et al*., 1998). FK506 is protective against glutamate-mediated toxicity (Dawson *et al*., 1993) and in a model of stroke (Sharkey and Butcher, 1994), while FK506 but not cyclosporin can block neuronal apoptosis in response to serum deprivation (Yardin *et al*., 1998). The reverse was reported during hypoglycemic challenge (Ferrand-Drake *et al*., 1999). Cyclosporin neurotoxicity on chronic administration (Hauben, 1996; Gijtenbeek *et al*., 1999) may involve apoptotic mechanisms (McDonald *et al*., 1996).

Together these reports argue that intracellular cholesterol isomerization, trafficking and mitochondrial delivery can govern the induction of apoptosis or necrosis. The major pathways involve the release, from mitochondria, of cytochrome C and apoptosis-inducing factor (AIF); these activate downstream death processes including caspase-mediated proteolysis (reviewed by Waters and Lavin, 1999). Agents interfering with mitochondrial integrity promote apoptosis – we should not forget that mitochondrial function is crucial to sterol/steroid synthesis.

Steroids and cell death

One of the best-studied apoptotic systems comprises the induction of apoptosis by glucocorticoids. In lymphocytes, glucocorticoids are powerful inducers of apoptosis and produce classical DNA fragmentation in CD4/CD8 lymphocytes that requires macromolecular synthesis. The process is inhibited by classical glucococorticoid antagonists such as RU486 and is increased by dexamethasone (Caron-Leslie *et al*., 1991; LaVoie and Witorsch, 1995). However, different glucocorticoids differ markedly in their ability to promote apoptosis (Hofmann *et al*., 1988) and the effect may not be mediated exclusively through a direct interaction with the nuclear glucocorticoid receptor GR.

In the brain, massive levels of apoptosis accompany developmental modeling during fetal growth (Blaschke *et al*., 1996). In the adult, where neuronal division is restricted to proliferative regions in the dentate gyrus, olfactory bulb, and to a lesser extent in the cerebellum (Altman and Das, 1965), continuing proliferation is regulated systemically by adrenal steroids. Here both glucocorticoid excess and insufficiency can cause neuronal loss. In the rat, adrenalectomy results in cell death, particularly in the dentate gyrus, which can be prevented by glucocorticoid replacement (Sloviter *et al*., 1989; Gould *et al*., 1990). Neuronal loss is most pronounced in young animals but also takes place in later life (Sapolsky *et al*., 1991) though only a proportion of animals showed cell loss (Jaarsma *et al*., 1992). Conversely, excess glucocorticoids suppress cell proliferation (Gould *et al*., 1992) and increase neuronal death; neurotoxic death in the hippocampus is reduced by adrenalectomy and aggravated by corticosterone (Sapolsky 1985; reviewed by Sapolsky *et al*., 1986; McEwen, 1999). Note, however, that glucocorticoid-driven neuronal cell death in the brain may not involve the classical apototic pathway (Masters *et al*., 1989).

Intracellular targets for apoptosis induction

Both cholesterol depletion and glucocorticoid excess can promote cell death in the CNS. Is it possible that they operate through a common intracellular pathway? As noted previously, the sigma/EBP site related to cholesterol isomerase may have a steroidal ligand because corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al*., 1990). The immunosuppressive effects of glucocorticoids may be mediated, in part, through an interaction with the sigma receptor (e.g., Derocq *et al*., 1995) while glucocorticoids and oxysterols (see below) synergise to promote lymphocyte apoptosis (Thompson *et al*., 1999).

Is apoptosis positively regulated? In the above it has been suggested that apoptosis is brought about passively, by disruption of orderly cholesterol trafficking and metabolism in the cell. A contrasting view is emerging, in which apoptotic mechanisms may be deliberately activated *in vivo* by steroidal molecules. Specifically, some oxidised derivatives of cholesterol are extremely potent inducers of cell death.

Cholesterol itself is largely inert, but when oxidised at the 25-position, and more particularly at the 7-position, it gives rise to a series of highly efficient inducers of apoptosis in the immune system, endothelial cells, and in the CNS. The 7-position modification is intriguing, particularly in view of the fact that neurosteroids can be selectively modified at the 7-position in brain extracts (see Box 15.6) and 7-modified cholesterols (and 7-oxo molecules) are detected in primary brain extracts (Vatassery *et al*., 1997). Note, however, that the data are often confusing, not only because cholesterols may be further metabolised during assay, but also because, at least in theory, either excess or depletion could cause similar effects if there is significant feedback regulation.

In lymphocytes, 25-hydroxycholesterol and 7β,25-dihydroxycholesterol diminish mitogen-induced proliferation (Richert *et al*., 1986), block HIV replication (Moog *et al*., 1998) and induce the death of lymphoma cells and thymocytes (Christ *et al*., 1993). 7β-hydroxy and 7-oxocholesterols induce apoptosis in lymphocytes (Lizard *et al*., 1997, 1998) and synergise with glucocorticoids for immunosuppression and apoptosis induction (Johnson *et al*., 1997). 7-oxocholesterol causes typical DNA fragmentation in human and bovine endothelial cells (Lizard *et al*., 1997) while in murine tumor lines cell-death mediated by either 7-oxocholesterol or 25-hydroxycholesterol was prevented by inhibitors of macromolecular synthesis (Hwang, 1992) suggestive of classic apoptosis. 7β- and 7-oxocholesterols elicit necrosis in fibroblasts (Lizard *et al*., 1999) while, in the brain, 24-hydroxycholesterol and cholestanol (5α-cholestan-3β-ol) are neurotoxic (Kolsch *et al*., 1999) and 7β- and 7-oxocholesterols are reported to kill cultured cerebellar granule cells (Chiang and Liu, 1998).

The nature and stereochemistry of the 7-position modification appears to be of critical importance. Cell killing mediated by 25-hydroxycholesterol can be abrogated by upregulation of 7α-hydroxylase systems (Leighton *et al*., 1991) suggesting that 7α-hydroxysterols are inert or protective. 7β-hydroxy and 7-oxo molecules

efficiently induce apoptosis in endothelial cells (Lemaire *et al*., 1998) and lymphocytes (Zhang *et al*., 1997), but the 7α-hydroxy molecules failed to produce cell death and may be protective against apoptosis (Zhang *et al*., 1997). In a particularly interesting study, Chisholm and colleagues purified a cholesterol derivative that appears to be an exceptionally potent inducer of apoptosis. Low density lipoprotein (LDL) circulates in the bloodstream as a cholesterol complex and oxidised LDL is itself a potent inducer of apoptosis (Nishio *et al*., 1996; Sata *et al*., 1998). Chisolm *et al*. (1994) extracted and purified the toxic activity associated with oxidized LDL. Perhaps surprisingly, the primary active molecule was 7β-hydroperoxy cholesterol (7β-OOH-cholesterol). This molecule is a major non-enzymic oxidation product of cholesterol (see Chisholm *et al*., 1994; Beriozov *et al*., 1990) though oxidation at other positions (e.g., 25-hydroxylation) is also likely to occur. 7β-OOH-cholesterol has been observed *in vivo* and concentrations increase with age (Ozawa *et al*., 1991). One potentially confusing factor is that a proportion of 7β-OOH-cholesterol is likely to derive from the diet, possibly arguing against a specific *in vivo* signaling role, though a possible role of 7α derivatives produced *in vivo* could be to compete with environmental or damage-induced 7β-modified cholesterols.

Receptors for apoptotic oxysterols

As remarked earlier, passive interference with cholesterol metabolism predisposes to apoptotic processes. This we feel contrasts with the potent induction of apoptosis by molecules such as 7β-OOH-cholesterol. Although it is possible that 7β-OOH-cholesterol and related molecules such as 7β-hydroxycholesterol merely inhibit cholesterol metabolism and trafficking, they may also have dedicated receptors.

At the level of cholesterol synthesis, 7α-hydroxylation of cholesterol was required for repression of cholesterol synthesis in one study (Axelson *et al*., 1996) although 3-oxo and 7-oxo molecules were also effective. In contrast, Martin *et al*. (1997) report that 7α-hydroxycholesterol fails to inhibit cholesterol synthesis, perhaps suggesting that, as for apoptosis induction, 7β-hydroxycholesterol is the active molecule that blocks cholesterol synthesis and ultimately elicits cell death. However, the identity of the cholesterol derivatives that feedback on the enzymes involved in cholesterol synthesis remains to be established.

Oxysterols are also likely to target nuclear receptors to repress transcription of genes involved in cholesterol synthesis. In addition to reducing proteolytic activation of the sterol response element (SRE) binding protein SREBP (Brown and Goldstein, 1997; Lund *et al*., 1998), there may be dedicated nuclear receptors responding to 7-modified cholesterols and bile acids. LXRα responds principally to oxysterols (Janowski *et al*., 1996), particularly 24(S)-hydroxycholesterols (Lehmann *et al*., 1997), as does the inhibitory receptor CAR-β (Forman *et al*., 1998), possibly FXR (Makashima *et al*., 1999; Parks *et al*., 1999) and potentially PPAR, PXR, SXR and CPF, nuclear receptors regulating peroxisome proliferation and xenobiotic metabolism (Steir *et al*., 1998; Waxman, 1999; Blumberg *et al*., 1998) or the gene encoding hepatic cholesterol 7α-hydroxylase (Nitta *et al*., 1999).

At the cell surface, they may target a receptor that modulates cAMP production (Moog *et al*., 1991), potentially similar to the brain adenyl cyclase that is regulated by calcineurin, FK506 and cyclosporin (Antoni *et al*., 1998).

The field remains confused. That cholesterol metabolism is crucial to mitochondrial integrity, surface neurotransmitter receptor activity, and cell survival, is beyond any reasonable doubt. That 7-modified cholesterols can be either protective (e.g., 7α molecules) or toxic (7β-hydroxy- and 7β-hydroperoxy-cholesterols) is also well-established. But we have little idea at what level this regulation occurs, or at which receptor the protective and destructive oxysterols compete for binding (if indeed they do). Are there intracellular or cell surface ion channels that are directly modulated by these molecules? Is mitochondrial energy generation subject to sterol regulation? The pathways might converge on the endogenous benzodiazepine, DBI/endozapine, to link, for example, GABA receptor function with mitochondrial steroid transport and metabolism. Mitochondrial membrane potential and levels of free Ca^{2+} are important regulators of apoptosis (Crompton *et al*., 1999). Apoptosis induction may operate, in part, by sterol-regulation of Ca^{2+} levels, evinced by the fact that ligands targeting sigma (Church and Fletcher, 1995; Klette *et al*., 1995; Brent *et al*., 1996) or PBR (e.g., Ramp and Triggle, 1987) can affect Ca^{2+} flux. PBR appears to bind to ion channels of the mitochondrial membrane (McEnery *et al*., 1992, 1993); mitochondrial membrane potential and apoptosis initiation may be subservient to sterol binding at PBR (Figure 15.3).

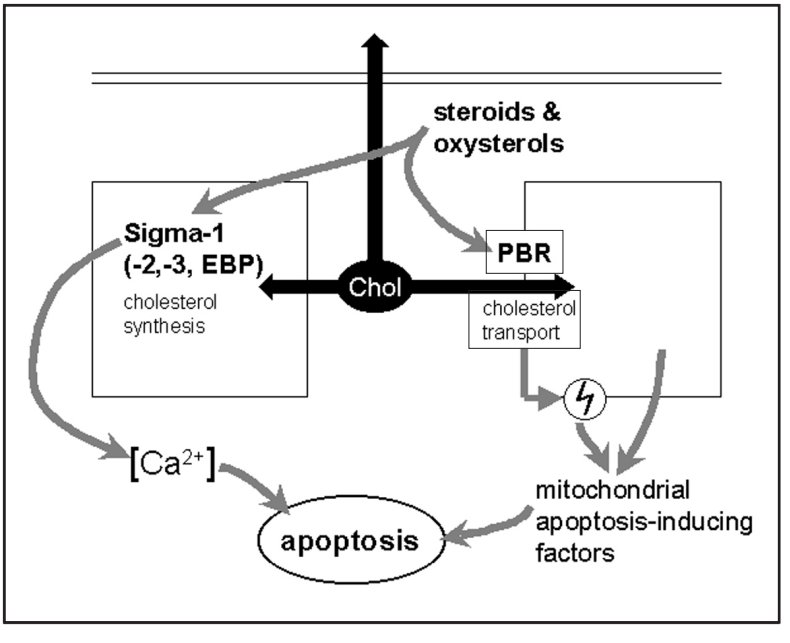


Figure 15.3 Possible convergence of oxysterol and steroid signaling pathways on apoptosis induction.

Intracellular regulation of oxysterol metabolism and apoptosis, both centrally dependent on the mitochondrion, are paralleled at the systemic level by the regulation of apoptosis by glucocorticoids – the synthesis of glucocorticoids (and mineralocorticoids) only takes place in mitochondria. Is it possible that steroids (acting systemically), and hydroxycholesterols (local intracellular action), once targeted the same intracellular receptor or receptors? In which case one must consider the possibility that neurosteroids may be evolutionary relics, performing local intercellular signaling but targeting intracellular receptors.

Box 15.6 Steroid/sterol 6 and 7 hydroxylation

There is an ongoing debate concerning the enzymology and role of steroid and sterol hydroxylation in brain. Pregnenolone and DHEA, either produced by local synthesis or derived from the circulation, have alternative metabolic routes. On the one hand, via 3β-HSD to progesterone and androstenedione; on the other, hydroxylation at the 7 (or 6) positions is perhaps the major metabolic route for these molecules in brain extracts (Akwa *et al*., 1991). In rat and mouse brain, 3β-hydroxy steroids including pregnenolone, DHEA and 5α-androstane-3β,17β- diol (A/ane-diol) are hydroxylated at the 6α, 7α and 7β positions, while 7-hydroxylated cholesterols are also produced (see text). Steroid and sterol hydroxylation at the 7 position has profound effects on receptor activation. 7α-hydroxylation of DHEA reduces its affinity for the estrogen receptor (Li *et al*., 1978) while other 7α-substituted steroids are particularly potent modulators of androgen, estrogen or aldosterone action (Weier and Hofmann, 1975; Bullock *et al*., 1978; deFriend *et al*., 1994; Beri *et al*., 1997). 7α-hydroxypregnenolone is a major steroid in some teleost fish (Ponthier *et al*., 1998). These reports point to early evolutionary ancestry and conservation of a modulatory site with 7α-stereochemistry.

Enzymology. It is so far unclear whether one or several enzymes are responsible for 6- and 7-hydroxylation of steroids, including DHEA, A/ane diol and A/ene diol, and cholesterols. Akwa *et al*. (1991, 1992) reported a brain activity converting pregnenolone and DHEA to 7α-hydroxy derivatives. Little 7β-hydroxy steroid was produced and the reaction was sensitive to estradiol. Warner *et al*. (1989) reported that brain and prostate catalyze the conversion of 5α-androstane-3β,17β-diol to the triol compound, with most hydroxylation at the 7β-position, significant modification at 7α, and lesser conversion at 6α. Stromstedt *et al*. (1993) argued in favor of a single enzyme activity, and that this brain enzyme also participates in the hydroxylation of 3β-hydroxy-5α-pregnan-20-one (Figure 15.1: Isopregnanolone; 3β-hydroxy-5α-dihydroprogesterone). 3α versions were not modified. Gemzik *et al*. (1992a,b,c) report that conversion of 5α-androstane-3β, 17β-diol to the 7β-, 7α- and 6α-hydroxylated molecules by rodent prostate is equally reduced by P450 inhibitors, arguing that one enzyme catalyzes all three hydroxylations in this tissue. Parallel studies in primates indicate that different enzymes

Box 15.6 (*Continued*)

mediate 6- and 7- hydroxylation (Gemzik *et al*., 1992b). In contrast, on the basis of inhibitor studies, Morfin and colleagues (Doostzadeh and Morfin; 1997; Doostzadeh *et al*., 1997) suggest that distinct brain enzymes are responsible for 7α- and 7β-hydroxylation. These authors suggest that 7α-hydroxylation of pregnenolone and Δ^5-androstenediol in brain may be performed by one enzyme, while DHEA is hydroxylated by another.

We reported a P450 cDNA, CYP7B, encoding an enzyme that selectively 7α-hydroxylates DHEA, pregnenolone and Δ^5-androstenediol (Stapleton *et al*., 1995; Rose *et al*., 1997). The enzyme is also active against hydroxycholesterol and is likely to be identical to hepatic oxysterol-selective 7α-hydroxylase (Schwarz *et al*., 1997), an enzyme distinct from the major hepatic cholesterol 7α-hydroxylase (CYP7A). The CYP7B enzyme thus most resembles the activity reported by Akwa (1992) while the rat prostate activity could represent a third enzyme. Breast tumor and ovarian cells are particularly rich in an oxysterol 7-hydroxylation activity (Couch *et al*., 1975; Li *et al*., 1978; Payne *et al*., 1995) although the enzyme described by Payne *et al*. (1995) may be distinct from both CYP7A and 7B as it is not competitively inhibited by DHEA. Note that CYP46, which hydroxylates cholesterol at 24(S), is abundantly expressed in brain (Lund *et al*., 1999). Activity on non-cholesterol substrates has not yet been evaluated.

7-hydroxylation of cholesterols and oxysterols. In liver, cholesterol hydroxylation at the 7α position (by CYP7A) is a major route for metabolic elimination via conversion to bile acids and subsequent secretion. In brain, however, the major route for cholesterol metabolism is to 24(S)-hydroxycholesterol that is secreted from brain, while 25- and 27-hydroxycholesterols are hydroxylated in brain, as in liver, at the 7α position (see text). Hepatic cholesterol 7α-hydroxylase CYP7A is not expressed in brain, and oxysterol 7α-hydroxylation is likely to be mediated by CYP7B (Rose *et al*., 1997; Schwarz *et al*., 1997; Martin *et al*., 1997). Hydroxylation is unlikely to be the end of the metabolic pathway, for a distinct hepatic 7α-hydroxycholesterol dehydrogenase/oxidoreductase has been reported with significant homology to 11β-hydroxysteroid dehydrogenases (Song *et al*., 1998; see Box 15.5). A patient deficient in CYP7B activity has been reported (Setchell *et al*., 1998); the genetic lesion precipitated the accumulation of hepatotoxic unsaturated monohydroxy bile acids and oxysterols, and liver failure. The severity of the disease precluded brain function analysis.

Does 7-hydroxylation gate receptor access? Cholesterols, and particularly 7β-oxidised cholesterols, are inducers of apoptosis (see text), 7α-oxidised cholesterols are most probably protective, and although the specific receptors responsible are not known, sigma/EBP and PBR furnish likely candidates. In other words, 7α versus 7β modification gates sterol access to the pertinent target. Steroid signaling is thought to have evolved from sterol signaling, and we suggest (see text) that DHEA and its 7α-hydroxylated

derivatives might modulate glucocorticoid action at cognate receptors. However, whether the action is via GR, via another nuclear receptor type (LXR, FXR, PXR), or (perhaps more likely) at intracellular sites also targeted by glucocorticoids, remains to be determined. Recent reports suggest that oxysterols may have one or more dedicated receptors distinct from the classic hormone-binding nuclear receptors (Janowski *et al*., 1996; Forman *et al*., 1998; Parks *et al*., 1999; Makishima *et al*., 1999); the same may be true for 6 or 7-hydroxlated pregnansteroids. There is some evidence that 7-modification may gate activation of the estrogen receptor (and perhaps the androgen receptor). 5α-androstane-3β,17β-diol (see Figure 15.1) is a breakdown product of testosterone and is a significant agonist of estradiol and androgen action at their cognate receptors (ER and AR) in breast cancer cells (Hackenberg *et al*., 1993). Hydroxylation of this molecule, by an activity that modifies both DHEA and Δ^5-androstenediol (Li *et al*., 1978), and catalyzed by CYP7B (Rose *et al*., 1987), abolishes activity at ER (Li *et al*., 1978). Similarly, 5α-androstane-3β, 17β-diol and DHEA were both active in stimulating AR in prostate cancer cells, but 7-oxoDHEA (potentially interconverting with 7-hydroxy derivatives) was ineffective (Miyamoto *et al*., 1998). Note the effect of 5α-androstane-3β, 17β-diol may involve separate stimulatory and inhibitory components (Boccuzzi *et al*., 1994). 7-hydroxylated derivatives of DHEA have further potential as local modulators of aromatase.

In support of a regulatory role for 7-hydroxylation, Akwa *et al*. (1993) report that alternative metabolism of pregnenolone and DHEA to either progesterone and androstenedione (by 3β-HSD) or to 7α-hydroxylated derivatives (possibly by CYP7B) is dependent on the plating density of purified type 1 astrocytes *in vitro*. At low plating density 3β-HSD metabolites predominated and only 1% of substrate was converted to 7α derivatives. In marked contrast, at high plating density 50% of substrate was converted to 7α-hydroxy products while 3β-HSD metabolites were scarce (Akwa *et al*., 1993). There may be active switching between the two pathways.

OXYSTEROLS AND NEUROSTEROIDS: IS THERE A LINK WITH AGEING?

Steroids and sterols contribute to the regulation of neuronal activity and cell death in the CNS. This opens the possibility that deregulated steroid/sterol signaling might accompany or cause cognitive decline with ageing. Sapolsky and colleagues (1986) proposed that the slow rise in glucocorticoids that accompanies ageing might precipitate a cascade of neurodegeneration, further amplified through increasing failure of the brain to repress adrenal output. In its simplest form the hypothesis has not been fully corroborated, giving way to a reciprocal contention that the effects of glucocorticoids might be exacerbated by concominant decline in another adrenal steroid, dehydroepiandrosterone (DHEA).

DHEA is the major adrenal steroid in primates, though not in rodents (see earlier). As widely discussed, plasma levels of free DHEA in human decay asymptotically but variably over the lifetime of human individuals, while similar declines are observed in other primates, paralleling their lifespans (see Orentreich *et al*., 1992; Sapolsky *et al*., 1993). The possibility that DHEA decline contributes to age-related diseases has been debated (Morales *et al*., 1994; Nestler, 1996; Hinson and Raven, 1999); encouraging effects of high dose DHEA replacement therapy have been recorded in early old age (Morales *et al*., 1994) but not in the more elderly (Wolf *et al*., 1997, 1998). DHEA had at best marginal efficacy in an extended trial (Baulieu *et al*., 2000) and there was no correlation between cognitive status or decline with steroid levels (Moffat *et al*., 2000).

Other related molecules also decline markedly with age, and possibly both in rodents and humans. These include the precursor molecule 7-dehydrocholesterol in both sciatic nerve and skin (Bourré *et al*., 1990; De Groot, 1989; MacLaughlin and Holick, 1985), plasma levels of 24(S)-hydroxycholesterol (Lütjohann *et al*., 1996, 1998) and skin vitamins D_3 (MacLaughlin and Holick, 1985), though others have suggested that 24(S)-hydroxycholesterol levels in brain (rather than plasma) may increase with age, at least in mice (Lund *et al*., 1999). Perhaps more likely is that conversion and transport at the sterol/steroid frontier display general age-related loss of function, leading one to suspect that elevated blood DHEA in primates might reach intracellular targets to offset this decline.

The reciprocal decline in DHEA and slow increase in glucocorticoid levels does seem to correlate with age-related cognitive dysfunction, but the relationship between cortisol/DHEA (and pregnenolone) levels and ageing is not as clear-cut as has sometimes been asserted (e.g., Kalmijn *et al*., 1986). A confounding factor is that DHEA and pregnenolone levels may be significantly elevated or reduced in brain disease, including anxiety and chronic fatigue syndrome (George *et al*., 1994; Takebayashi *et al*., 1998; Heuser *et al*., 1998; Scott *et al*., 1999; De Becker *et al*., 1999) in addition to the well-documented increases in cortisol production that can accompany depression (reviewed by Murphy, 1991; Sapse, 1997). What also emerges from these studies is that the absolute level of DHEA is perhaps less important than the DHEA/cortisol ratio, contributing to the idea that DHEA might be a "natural" antiglucocorticoid.

Circumstantially the evidence is strong. Excess glucocorticoids impair learning and memory, while DHEA and pregnenolone improve post-training memory when injected into limbic structures of the mouse brain (Flood and Roberts, 1988; Flood *et al*., 1992, 1995), and enhance synaptic plasticity (e.g., Yoo *et al*., 1996). DHEA can have antiglucocorticoid actions both *in vitro* (Blauer *et al*., 1991; May *et al*., 1990; Araneo *et al*., 1990, 1995) and *in vivo* (Daynes *et al*., 1990), including antiglucocorticoid effects in the CNS (Singh *et al*., 1994; Fleshner *et al*., 1995; reviewed by Kalimi *et al*., 1994). Inhibition of astrocyte proliferation by the synthetic glucocorticoid dexamethasone was reversed by DHEA as well as progesterone (Crossin *et al*., 1997) and DHEA enhancement of hippocampal primed-burst potentiation was blocked by stress (Diamond *et al*., 1999). Kimonides *et al*. (1999) report that DHEA can protect hippocampal neurons against corticosterone-mediated neurotoxicity while corticosterone-induced nuclear accumulation

of GR in a mouse hippocampal cell line was blocked by DHEA (Cardounel *et al*., 1999). DHEA is also reported to block corticosterone inhibition of synaptic potentiation in dentate gyrus (Kaminska *et al*., 2000). In other tissues, McIntosh *et al*. (1999) report systemic anti-glucocorticoid effects of DHEA on hepatic lipogenesis, thymic and spleen weight reduction and serum IGF-1. Nevertheless, a direct effect on GR seems unlikely, although changes in the subcellular distribution of GR in response to DHEA have been noted (Cardounel *et al*., 1999). The antiglucocorticoid activity of DHEA could not be reconstituted in an *in vitro* system (Rupprecht *et al*., 1996) and we have confirmed that DHEA is, at best, a poor inhibitor of human GR (unpublished observations). Two explanations may be offered.

First, DHEA may act systemically. DHEA can reduce cortisol levels in healthy adult volunteers (Wolf *et al*., 1997) and serum corticosterone levels in rats, possibly by altering peripheral glucocorticoid metabolism (Homma *et al*., 1998). DHEA can also act at central $GABA_A$ and possibly PBR and sigma sites; free DHEA could thereby feed back to the brain to alter adrenal output. In support, the central $GABA_A$ agonist alpralozam decreased plasma cortisol but increased DHEA (Krobeth *et al*., 1999) and systemic sigma agents affect glucocorticoid (and no doubt DHEA) release from the adrenal (Pechnick and Poland, 1989; Matheson *et al*., 1991; Gudelsky and Nash, 1992; Eaton *et al*., 1992).

Second, local DHEA metabolism may be required for activity (Shealy *et al*., 1995), particularly in the immune system (Morfin and Courchay, 1994). As we discussed (Box 15.6), 7-hydroxylation is the major route for the metabolism of DHEA in the CNS. In lymphocytes, 7β-hydroxylated steroids (including androstenetriol: 5-androstene-3β,7β,17β-triol) are reported to activate the immune response in mice (Padgett and Loria, 1994; Loria *et al*., 1996) and 7β-androstenetriol molecules may counteract corticosteroid-induced immunosuppression *in vitro* (Loria, 1997). Others have argued that DHEA exerts its immunomodulatory activity not via 7β-modified derivatives but instead through 7α-hydroxylation (Lafaye *et al*., 1999), a conversion taking place in brain (Rose *et al*., 1997) (though binding of hydroxylated derivatives of androstenediol and DHEA to the same cellular targets has not been formally demonstrated). 7α-hydroxyDHEA was more effective than DHEA in preventing dexamethasone-induced thymic cell loss (Chmielewski *et al*., 2000); 7-oxoDHEA had benefial effects on long-term memory retention in old (22 month) mice, as did 7α-hydroxyDHEA, while DHEA was less effective (Shi *et al*., 2000). 7-oxoDHEA may though interconvert with 7-hydroxylated derivatives.

Does DHEA act as an antiglucocorticoid at the level of steroidogensis and/or apoptosis? Glucocorticoids (corticosterone and deoxycorticosterone) and related steroids are active at the sigma/sterol site (Su *et al*., 1990; Derocq *et al*., 1995) as are DHEA and pregnenolone (Monnet *et al*., 1995; Debonnel *et al*., 1996), with EBP and PBR affording further promising co-targets. DHEA was effective in preventing dexamethasone-induced apoptosis in mouse thymocytes (Chmielewski *et al*., 2000). However, a specific antagonism between DHEA and glucocorticoids at sigma, EBP or PBR has not been investigated. Notwithstanding, one might reasonably speculate that DHEA and its derivatives, particularly 7α-hydroxylated DHEA, might act as protective oxysterol analogues by protecting, e.g., sigma or EBP from glucocorticoid inhibition. Steroids like DHEA and its derivatives could have an

advantage over endogenous sterols because, unlike native oxysterols, they cannot be side-chain hydroxylated – this could prevent further metabolism and export.

This challenges the assumption that glucocorticoids and antiglucocorticoids principally act through the nuclear glucocorticoid receptors (GR and MR), and emphasizes the importance of non-nuclear targets including sigma, EBP and PBR. One must however avoid the impression that there is an either/or choice to be made between nuclear receptors (GR/MR) and sterol metabolism sites (PBR/sigma/EBP). On the one hand, the nuclear receptors are very likely to play a prominent role outwith the nucleus, and possibly at the cell surface (see mGR, earlier). On the other, there are many other nuclear receptors that may respond to steroids important in this discussion (e.g., glucocorticoids and DHEA). This latter possibility is amply illustrated by studies on the bioactivity of rifampicin.

A CONFUSING DIVERSITY OF NUCLEAR RECEPTORS: THE RIFAMPICIN EXAMPLE

Rifampicin (rifampin) is a powerful antibacterial antibiotic used in peripheral and brain infections because the drug enters the brain. However, it interacts adversely with corticosteroid therapy (e.g., Jubiz and Meikle, 1979), attributed to rifampicin-induced alterations in hepatic corticosteroid metabolism (Edwards *et al*., 1974) and expression of P450s including those metabolising bile acids (Weitholz *et al*., 1996). Recently, rifampicin was reported to activate directly the human glucocorticoid receptor (GR) *in vivo* (Calleja *et al*., 1998). This was contradicted by another study (Jaffuel *et al*., 1999) and it seems that rifampicin may operate through a different receptor.

Hepatic CYP expression is controlled by a group of nuclear receptors that include PXR (pregnan-X receptor), CARβ, PPAR (peroxisome proliferator receptors), LXR (liver-X (oxysterol) receptor), FXR (farnesol/oxysterol receptor), and the probable oxysterol receptors SXR and CPF (reviewed, in part, by Waxman, 1999). Many of these (if not all) are well expressed in brain. Although an interaction with the vitamin D receptor was suggested (Mellon, 1984), it transpires that PXR mediates rifampicin induction of liver P450 CYP3A4 (Bertilsson *et al*., 1998; Goodwin *et al*., 1999) as well as induction by other xenobiotics. Confusingly, all these receptors and the pathways they govern interact in diverse ways. For instance, PPAR can activate an estradiol-responsive gene (Nunez *et al*., 1997). The glucocorticoid agonist dexamethasone increases PXR-1 mRNA in rat (Zhang *et al*., 1999) and, depending on species, PXR is potently activated by glucocorticoids and antiglucocorticoids including the powerful glucocorticoid antagonist pregnenolone-16α-carbonitrile (Kliewer *et al*., 1998). It is reasonable to suppose that modulation of gene action by neurosteroids including pregnenolone, DHEA and their derivatives may be partly mediated through these nuclear receptors, in addition to other direct actions on cell-surface, cytoplasmic and mitochondrial receptors. Finally, if both ER and GR have cell-surface forms, this may be a common feature of nuclear receptors; PXR for example could also have a non-nuclear isoform that can respond to glucocorticoids and antiglucocorticoids.

DEVELOPMENT, STEROL SIGNALING AND SUBCELLULAR TARGETING

Brain function is critically reliant on sterol metabolism. It comes as no surprise that sterol metabolism in the brain, an organ whose signature is plastic remodeling in response to experience, is also critical for brain development. Abnormalities of sterol metabolism predispose to developmental deficits and there is a strong association between fetal malformations and cholesterol synthesis perturbations during pregancy. Inborn errors in early cholesterol metabolism, including Smith-Lemli-Opitz syndrome (7-dehydrocholesterol reductase), Niemann-Pick disease type C (NPC1-mediated sterol transport) and cerebrotendinous xanthomatosis (cholesterol 27-hydroxylase), discussed earlier, induce pronounced developmental abnormalities in addition to mental and neurologic complications. Knockout mice for ApoB have defects in brain development (Farese *et al*., 1995; Huang *et al*., 1995; Homanics *et al*., 1995), while mice lacking the ApoB receptor megalin, a member of the LDLR superfamily, show defects akin to Smith-Lemli-Optiz (Willnow *et al*., 1996).

One may infer that cholesterol traffic is critical for both structural and informational processes. Structurally, in the synthesis of brain membranes (for instance during postnatal myelination of brain neurons), and informationally, for orderly developmental selection of neuronal circuits (expoiting oxysterol/apoptosis pathways to eliminate inappropriately wired neurons). However, recent evidence suggests that sterols (and possibly steroids) may play a far more direct role in pattern formation during brain development. This is suggested by two discoveries – that the product of the key developmental regulatory gene *Hedgehog/Sonic hedgehog* (*Hh/Shh*) is covalently linked to cholesterol, and that the mammalian homologue of the developmental gene *Fushi tarazu* of *Drosophila melanogaster* is, intriguingly, a regulator of sterol metabolism.

The fruitfly Hh protein undergoes autocatalytic cleavage resulting in the addition of cholesterol, via its 3β-OH group, to the C-terminus of the N-terminal fragment (Porter *et al*., 1996), very substantially increasing its effectiveness as a signaling molecule. Cholesterol linkage is not restricted to Hh, and it is not clear whether cholesterol is the only adduct (Porter *et al*., 1996). In *Drosophila*, unlike mammals, yeast-derived ergosterol is the major membrane sterol. Studies with the mammalian Hh homolog (*Sonic hedgehog*, *Shh*) have shown that Shh protein can be coupled efficiently to other sterols including desmosterol and 7-dehydrocholesterol *in vitro* (Cooper *et al*., 1998). 3β-hydroxy steroids were not tested. In flies, cholesterol-linked Hh protein modulates gene expression via *Patched* (*Ptc*). This protein has similarities to NPC1 protein implicated in Niemann-Pick disease type C and contains a sterol sensing domain. Hh (and by inference Shh) may therefore be involved in sterol signaling (Johnson and Scott, 1998).

Disruption of Shh in mammals is responsible for holopresencephaly (Belloni *et al*., 1996; Roessler *et al*., 1996), a usually lethal disease of variable penetrance whose consequences range from microcephaly to failure of hemispheric separation and craniofacial abnormalities. A similar condition is produced by disruption of the mouse *Shh* gene (Chiang *et al*., 1996). It is of note that Smith-Lemli-Opitz, discussed

earlier, is accompanied by malformations similar to holoprosencephaly. Together these observations argue that sterol signaling mediated by Shh is critical to brain development. NPC1, a vertebrate analog of the Hh-reponsive Ptc (Johnson and Scott, 1998), is involved in sterol trafficking to and from cell surface lipoprotein receptors, particularly the LDL receptor, and it is intriguing that a novel LDL-receptor family member shows homology to the "neuropeptide head regulator" of Hydra (Hermans-Borgmeyer *et al*., 1998). Control of development by sterol (rather than steroid) signaling between cells may have arisen early in evolution.

A clue to the nature of this sterol signaling was provided by studies on the *Drosophila* mutant *Fushi tarazu* (*Ftz*). The Ftz gene encodes an important homeobox gene that determines segmentation patterns during fly development. Molecular cloning of the fruitfly receptor for Ftz protein identified FtzF1 (Guichet *et al*., 1997; Yu *et al*., 1997), an orphan receptor of the steroid nuclear receptor superfamily. This would be purely circumstantial, except that, most remarkably, CPF (a human homolog of Ftz-F1) has been demonstrated to regulate cholesterol 7α-hydroxylase (CYP7A) expression in liver (Nitta *et al*., 1999).

From an evolutionary perspective, it has been argued that the Ftz system was first required for nervous system development (where sterol regulation would make sense given the enormous brain demand for structural cholesterol) and only more recently for developmental specification, as exemplified by its role in *Drosophila* segmentation (Brown *et al*., 1994).

Analysis of *Drosophila* signaling has provided a final insight into the mammalian system. Signaling by cholesterol-bound Hh can extend over several cell diameters, arguing that there must be a mechanism that facilitates transport of Hh protein to more distant sites. Such a function is provided by the product of the *Drosophila* gene *Tout-velu*, the mammalian homolog of which is a transmembrane protein of the endoplasmic reticulum (reviewed by Strigini and Cohen, 1999). While not proven, these reports begin to suggest that Hh protein, and by inference Shh, can traffic from the interior of the cell to sites some distance away, and in particular may concentrate in cholesterol-rich rafts in distal membranes (Rietvald *et al*., 1999). These are principal sites for GPI-linked protein accumulation and for signal transduction, and it is possible that local transport of Hh exploits a sterol transport mechanism (e.g., that comprising caveolin and FK506- and cyclosporin-binding proteins; Uittenbogaard *et al*., 1998).

If sterol ligands modulate CPF activity, as seems likely in view of the sterol-responsiveness of the CYP7A gene, we have a remarkable situation in which a sterol-binding nuclear receptor interacts with a key homeobox developmental regulator to control gene expression. Some homeobox proteins, e.g., Antennapedia, can penetrate the cell surface by an unusual receptor and temperature-independent mechanism to reach the nucleus (Joliot *et al*., 1991; Derossi *et al*., 1996). Because homeobox protein-mediated transport cotransfers ligands bound to the homeoprotein (Theodore *et al*., 1995) it is plausible to suggest that some nuclear receptors, at least, traffic from the cell surface to the nucleus, and perhaps (like Shh) even between cells (discussed by Prochiantz and Theodore, 1995). Whether similar overlaps in developmental and physiological regulation exist for other nuclear

hormone receptors (GR? ER?) remains to be elucidated, but the appearance of immunologically related versions of GR and ER at the cell surface (Gametchu *et al*., 1999; Monje and Boland, 1999) might be consistent with such a process. The intimate involvement of the homeo domain proteins Pbx and Oct-1 in glucocorticoid repression via a glucocorticoid response element (Subramaniam *et al*., 1998) is most suggestive, particularly because it has been suggested that Pbx1 and GR proteins may form a tight complex (see Subramaniam *et al*., 1999) and both GR and the progesterone receptor have been shown to associate tightly with FKBP52 implicated in intracellular transport (Schmitt *et al*., 1993; Smith *et al*., 1993; Silverstein *et al*., 1999). Could interactions with homeobox proteins and FKBP52 permit nuclear receptors such as GR to appear at the cell surface, and even translocate between cells? If so, steroid signaling would take on an entirely new demension.

CONCLUSIONS AND OBSERVATIONS

The primary objective of this review was to overview what is known of neurosteroids and their mechanism of action. We have been inextricably drawn towards the notion that intracellular and extracellular signaling may have a common origin, and steroids are likely to have intracellular targets operating at the level of sterol metabolism. But it would be a mistake to think that neurosteroids can only act in this way. Brain steroids can have acute actions, exemplified by the anxiogenic and proconvulsant effects of "natural" withdrawal of progesterone (Morau *et al*., 1998). Testosterone elevations may contribute to elation following transient stress, while glucocorticoids have been postulated to play a role in memory selection, facilitating the long-term storage of memory traces coincident with adrenal activation (Lathe, 2000). Other steroids whose levels vary according to time of day, such as DHEA, are likely to modulate mood, alertness and attention, while the onset, maintenance and termination of sleep in humans is likely to be governed, at least partly, by steroid signaling.

By way of conclusion, we have attempted to crystallize our thoughts into a shortlist of observations, some of which may stand the test of time, others of which may be less robust but we hope may point the way to new avenues of exploration. There can be no doubt that the field offers many new and exciting opportunities for future research.

1 The evidence argues against major levels of neurosteroid synthesis in brain; most brain steroids appear to derive from the circulation. At the same time, the brain is largely self-sufficient in cholesterol, and exploits a unique 24(S) hydroxylation and export pathway.
2 In contrast to local *de novo* steroid sythesis, local steroid transformations in brain are likely to be of importance. For instance, metabolism can regulate steroid access to receptors, exemplified by inactivation of corticosterone by 11B-HSD, and potentially could regulate the type of interaction (activation versus inhibition).

3 Local steroid metabolism may play a significant role, but the synthesis and metabolism of cholesterol and its oxysterol derivatives is of crucial importance to brain development and function. This is demonstrated by:

   (a) the major brain effects of anxiolytics, anticonvulsants and neuroleptics that target intracellular sites regulating sterol transport and metabolism. In addition to more classic actions on neurotransmitter receptors (e.g., $GABA_A$ and dopamine receptors), benzodiazepines potently target the peripheral benzodiazepine receptor, PBR (sterol transport) while neuroleptics such as haloperidol target the sigma site and/or emapomil binding protein, EBP (involved in sterol synthesis);
   (b) developmental and functional deficits of the brain due to abnormalities of cholesterol synthesis and metabolism, exemplified by Smith-Lemli-Opitz syndrome (cholesterol synthesis), Niemann-Pick disease type C (intracellular sterol transport), and cerebrotendinous xanthamatosis (cholesterol 27-hydroxylation).

4 Local intracellular sterol signaling includes:

   (a) regulation of cholesterol synthesis and cell division;
   (b) onset of apoptosis, and, potentially;
   (c) neurotransmitter release processes.

5 Systemic steroid signaling operates not only at classical nuclear "genomic" targets, but also at non-genomic sites including cell-surface neurotransmitter receptors (e.g., $GABA_A$, NMDA and others) and intracellular sites including sigma/EBP (sterol synthesis) and PBR (sterol transport). From an evolutionary perspective, intracellular regulation of sterol synthesis and metabolism predates systemic signaling; intracellular (and enzymatically-active) sites may be the earliest (and most important?) targets of steroid action (Stoka, 1999).

6 Local sterol signaling could potentially extend between cells by taking advantage of a reverse transport pathway from the interior to the cell surface, and uptake pathways to adjacent cells. This could contribute to developmental wiring in the nervous system, including synaptogenesis.

7 Steroid signaling may also operate via "nuclear" receptors stationed at or near the cell surface. These may traffic to the cell surface via the same reverse transport system.

8 There is extensive cross-talk between activity of neurotransmitter receptors at the cell surface (e.g., $GABA_A$) and pathways operating within the cell (e.g., sigma, EBP, PBR). Both can be targets for (a) steroids and possibly sterols (b) the diazepam-binding inhibitor, DBI/endozapine. For instance, GABA-active agents can affect cholesterol synthesis. Cross-talk may also operate through second-messenger cascades and by coupling between cell membrane and intracellular (including mitochondrial) ion fluxes.

9 Intracellular sterol metabolism, particularly in the mitochondrion, may decline with age. This could have consequences both at the systemic level (excess glucocorticoid production by the adrenal) and locally (neuronal loss). The abundant secretion of the steroid dehydroepiandrosterone (DHEA), in primates but not in rodents, could act to brake the age-related decline in sterol metabolism by operating at intracellular sterol sites.

10 Finally, glimpses are emerging that selective metabolism of steroids and sterols may be localized within specialized cell regions, and possibly to active regions such as synapses. Potentially, this could permit fast steroid interconversions to govern brain activity at immediate (1 sec – 1 min) time intervals.

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