Inhibitory Gain Control by 5α-Reduced Neurosteroids and Their Cellular Distribution in the Rat Brain

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This thesis is my own original work.
Abstract

Endogenous $5\alpha$-reduced neurosteroids act allosterically to increase the efficacy of the inhibitory $\gamma$-aminobutyric acid-type A (GABA-A) receptor. The $3\alpha,5\alpha$-reduced steroids are some of the most potent positive modulators of GABA-ergic inhibition. While it is known that $5\alpha$-reduced neurosteroid levels change during stress or depression, and over the oestrus cycle, a basic physiological role consistent with their pharmacological action remains elusive. We have utilised the unique architecture of the auditory midbrain to reveal a role for $5\alpha$-reduced neurosteroids in regulating inhibitory efficacy, thereby influencing the balance of excitatory and inhibitory inputs in a complex neural circuit. Permanent removal of a subset (ipsilateral-ear-dominated) of GABA-ergic inputs to the adult rat auditory midbrain induced an increased effectiveness of the remaining (contralateral-ear-dominated) inhibitory inputs, with a delay of around 20 minutes. Using electrophysiological recordings, antagonism of $5\alpha$-reductase (by finasteride) and immunohistochemical assays, it was shown that this counteracting plasticity depends upon local upregulation of $5\alpha$-reduced neurosteroids. This is consistent with their known role in potentiating the action of GABA at GABA-A receptors.

As neurosteroid synthetic enzymes, neurosteroid-sensitive receptors and physiologically relevant neurosteroid concentrations have been reported for a number of brain areas, this demonstration of neurosteroid-mediated
gain control is likely to have a broader applicability across the brain. This proposal is consistent with the observation of widespread 3α,5α-reduced steroid immunoreactivity from the brainstem through to the forebrain of the adult rat. The highest density of immunolabelling was found in the olfactory bulb, striatum and cerebral cortex, and the lowest density of labelling in the brainstem reticular formation, in accordance with the regional distribution of 3α,5α-reduced steroids determined by gas chromatography-mass fragmentography and radioimmunoassays in other laboratories. However, in many brain regions, immunoreactivity concentrated in specific neuronal populations, including pyramidal cells of the hippocampus and Purkinje cells in the cerebellum. It was once assumed that neurosteroidogenesis occurred primarily in glial cells, however synthetic roles for neurons have been emerging in recent years. This is consistent with the observation that the cell bodies and thick dendrites of neurons were labelled. Putative glial cells were not found to be immunoreactive. In most brain areas, the location and morphology of labelled cells showed a distinct bias to shapes and sizes of putative excitatory neurons. Although local circuit GABA-ergic neurons did not appear to be labelled, GABA-ergic neurons providing long-range projections were immunoreactive, for example, cerebellar Purkinje cells, reticular thalamic neurons and cells of the basal ganglia. The cellular distribution of 3α,5α-reduced steroids suggests that sensory, motor, limbic and homeostatic systems can be influenced by neurosteroids at multiple stages of processing.
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Abbreviations

ac anterior commissure
Ach acetylcholine
AH anterior hypothalamic area
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA analysis of variance
AOB accessory olfactory bulb
Arc arcuate nucleus
AVCN anteroventral cochlear nucleus
BL basolateral amygdaloid nucleus
BSA bovine serum albumin
CA cornu ammonis
cAMP adenosine 3',5'-cyclic monophosphate
Ce central amygdaloid nucleus
CN cochlear nuclei
CNS central nervous system
Co cortical amygdaloid nucleus
CPu caudate-putamen
DA dark agouti
DAB 3,3'-diaminobenzidine
DBI diazepam binding inhibitor
DCN dorsal cochlear nucleus
DG dentate gyrus
DHDOC dihydrodeoxycorticosterone
DHP dihydroprogesterone
DL deep layer
DLG dorsal lateral geniculate
DNLL dorsal nucleus of the lateral lemniscus
DS donkey serum
EI contralateral ear excitatory, ipsilateral ear inhibitory
EP evoked potential
EPL external plexiform layer
EPSC excitatory post-synaptic current
EPSP excitatory post-synaptic potential
f fornix
GABA γ-aminobutyric acid
GCL granule cell layer
GL glomerular layer
GC-MF gas chromatography-mass fragmentography
Gr granular layer
HiF hippocampal fissure
HPβCD 2-hydroxypropyl-β-cyclodextrin
3αHSD 3α-hydroxysteroid dehydrogenase
5-HT 5-hydroxytryptamine
IC inferior colliculus
ICC central nucleus of the inferior colliculus
ICD dorsal cortex of the inferior colliculus
ICX  external cortex of the inferior colliculus
ILD  interaural level difference
In   intermediate layers
INLL intermediate nucleus of the lateral lemniscus
Ins  insular cortex
IPD  interaural phase difference
IPSC inhibitory post-synaptic current
IPSP inhibitory post-synaptic potentials
ITD  interaural time difference
KA   kainic acid
La   lateral amygdaloid nucleus
LD   laterodorsal nucleus
LH   lateral hypothalamic area
LL   lateral lemniscus
LNTB lateral nucleus of the trapezoid body
LP   lateral posterior nucleus
LSD  lateral septal division, dorsal
LSI  lateral septal division, intermediate
LSO  lateral superior olive nucleus
LTD  long-term depression
LTP  long-term potentiation
LVe  lateral vestibular nucleus
M1   primary motor cortex
M2   secondary motor cortex
MBR  mitochondrial benzodiazepine receptor
MCL  mitral cell layer
MD   mediodorsal thalamic nucleus
Me   medial amygdaloid nucleus
Mo   molecular layer
Mo5  motor trigeminal nucleus
MOB  main olfactory bulb
mRNA messenger ribonucleic acid
MS   medial septal nucleus
MSO  medial superior olive nucleus
NMDA N-methyl-D-aspartate
nACh nicotinic acetylcholine
op   optic tract
Op   optic layer
Or   stratum oriens
OTD  ongoing time difference
P450scc cytochrome P450 side chain cleavage enzyme
Pa   paraventricular nucleus
PAG  periaqueductal grey
PB   phosphate buffer
PBN  parabrachial nucleus
PCL  principal cell layer
PCRt parvocellular reticular field
Pe   periventricular nucleus
PFA  paraformaldehyde
Pir  piriform cortex
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PL</td>
<td>plexiform layer</td>
</tr>
<tr>
<td>Po</td>
<td>posterior nuclear group</td>
</tr>
<tr>
<td>PoL</td>
<td>polymorphic layer</td>
</tr>
<tr>
<td>PVCN</td>
<td>posteroventral cochlear nucleus</td>
</tr>
<tr>
<td>Py</td>
<td>pyramidal cell layer</td>
</tr>
<tr>
<td>Ra</td>
<td>stratum radiatum</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>Rt</td>
<td>reticular nucleus</td>
</tr>
<tr>
<td>S1</td>
<td>primary somatosensory cortex</td>
</tr>
<tr>
<td>S1BF</td>
<td>barrel field in primary somatosensory cortex</td>
</tr>
<tr>
<td>S1DZ</td>
<td>dysgranular zone in primary somatosensory cortex</td>
</tr>
<tr>
<td>S1Tr</td>
<td>trunk representation in primary somatosensory cortex</td>
</tr>
<tr>
<td>scp</td>
<td>superior cerebellar peduncle</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SF-1</td>
<td>steroidogenic factor-1</td>
</tr>
<tr>
<td>SNR</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SO4</td>
<td>sulfate ester</td>
</tr>
<tr>
<td>SPO</td>
<td>superior paraolivary nucleus</td>
</tr>
<tr>
<td>SpVe</td>
<td>spinal vestibular nucleus</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>StAR</td>
<td>steroid acute regulatory protein</td>
</tr>
<tr>
<td>Su</td>
<td>superficial grey</td>
</tr>
<tr>
<td>SuVe</td>
<td>superior vestibular nucleus</td>
</tr>
<tr>
<td>TC</td>
<td>tuber cinereum</td>
</tr>
<tr>
<td>THDOC</td>
<td>tetrahydrodeoxycorticosterone</td>
</tr>
<tr>
<td>THP</td>
<td>tetrahydroprogesterone</td>
</tr>
<tr>
<td>TTD</td>
<td>transient time difference</td>
</tr>
<tr>
<td>3V</td>
<td>3rd ventricle</td>
</tr>
<tr>
<td>VDB</td>
<td>nucleus of the diagonal band, vertical limb</td>
</tr>
<tr>
<td>VLG</td>
<td>ventral lateral geniculate</td>
</tr>
<tr>
<td>VMH</td>
<td>ventromedial hypothalamic nucleus</td>
</tr>
<tr>
<td>VNLL</td>
<td>ventral nucleus of the lateral lemniscus</td>
</tr>
<tr>
<td>VNTB</td>
<td>ventral nucleus of the trapezoid body</td>
</tr>
<tr>
<td>VP</td>
<td>ventroposterior thalamic nuclei</td>
</tr>
<tr>
<td>VPL</td>
<td>ventral posterolateral nucleus</td>
</tr>
<tr>
<td>VPM</td>
<td>ventral posteromedial nucleus</td>
</tr>
<tr>
<td>Zo</td>
<td>zonal layer</td>
</tr>
</tbody>
</table>
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And to my wife, Leah, you have been a source of inspiration. I love you.
1 BACKGROUND

1.1 Overview

In this doctoral thesis, neurosteroid-mediated gain control is demonstrated within an auditory midbrain model. Furthermore, the distribution of neurosteroids shown across and within brain areas suggests neurosteroid action is not limited to the midbrain. Prior to presenting the experimental results, the biochemistry and pharmacology of neurosteroids will be introduced, with a focus on the 5α-reduced derivatives of progesterone and deoxycorticosterone and their effect upon the γ-aminobutyric acid (GABA) type A receptor. This will be followed by the anatomy and physiology of the auditory midbrain, leading to an explanation for the choice of the rat auditory midbrain as a model system to examine the role of neurosteroid modulation of inhibitory gain control in vivo.

1.2 Neurosteroids

1.2.1 Neurosteroid Introduction

Steroids, such as glucocorticoids, mineralocorticoids and sex hormones, bind to intracellular receptors, which then interact with distinct nucleotide sequences to alter gene transcription (review: Gronemeyer, 1992).
Resulting effects on protein synthesis generally lag steroidal entry to cells (and steroid-receptor complexation) by more than one hour. In contrast, steroids also have non-genomic effects which are evident within seconds to minutes. Steroid hormones, apparently largely via membrane-bound receptors, activate G-proteins, adenylyl cyclase and protein kinases (review: Losel and Wehling, 2003). Furthermore, neuroactive steroids act upon ligand-gated ion channels and G-protein coupled receptors to rapidly change neuronal excitability (review: Rupprecht and Holsboer, 1999). Neuroactive steroids are synthesised in endocrine organs and the nervous system, with steroids derived from the latter called ‘neurosteroids’ (review: Baulieu, 1998). Hormones of the hypothalamus (gonadotropin-releasing hormone, corticotropin-releasing hormone) and pituitary (luteinising hormone, adrenocorticotrophic hormone) control the production of neuroactive steroids by the reproductive and adrenal glands (Genazzani et al., 1998). However, neurosteroid levels can also be locally regulated and synthesized de novo or from circulating precursors within the brain (see section 1.2.7).

Various steroids interact with the major classes of ionotropic receptors on neurons including GABA-A, glycine, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, N-methyl-D-aspartate (NMDA), nicotinic acetylcholine (nACh), 5-hydroxytryptamine 3 (5-HT₃), sigma 1 and/or oxytocin receptors (Table 1). Typically, GABA-ergic function is potentiated by 3α-hydroxysteroids but negatively modulated by steroid sulfates and 3β-hydroxysteroids (Fig. 1). Of the many steroid-receptor
interactions determined pharmacologically, those that manifest at concentrations well above $10^{-6}$ molar are unlikely to be physiologically relevant, as brain levels of steroids are generally submicromolar (rat: Jo et al., 1989; Zwain and Yen, 1999; human: Lanthier and Patwardhan, 1986; Schumacher et al., 2003; see section 1.2.5). Nevertheless, the 3α-hydroxysteroids (and in particular the 3α,5α-tetrahydro derivatives), which are central to this thesis, are efficacious at concentrations above a few nanomolar, with levels in the brain of male and female rats inducible to between 10 and 100 nM (Paul and Purdy, 1992).

Figure 1. Steroid Structural Reference. Steroids are comprised of 4 carbon rings (A-D), with carbon atoms numbered as shown. A chemical group below the plane of the ring system is designated the $\alpha$ configuration (dashes), while $\beta$ corresponds to above the plane. The compound illustrated is 3α-hydroxy-5α-pregnan-20-one, also known as allopregnanolone.
<table>
<thead>
<tr>
<th>Steroid</th>
<th>Receptor</th>
<th>Action</th>
<th>Dose M</th>
<th>Reference</th>
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<tr>
<td>3α,5α-THDOC</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Majewska et al. (1986)</td>
</tr>
<tr>
<td>p1/GABA-C</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Morris et al. (1999)</td>
</tr>
<tr>
<td>3α,5β-THDOC</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-7}$-$10^{-8}$</td>
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</tr>
<tr>
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<tr>
<td>DOC</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-4}$-$10^{-5}$</td>
<td>Wu et al. (1990)</td>
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<td>Glycine</td>
<td>GABA-A</td>
<td>+ modulation</td>
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<td>- modulation</td>
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<td>- modulation</td>
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<td>Park-Chung et al. (1997)</td>
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<td>Park-Chung et al. (1999)</td>
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<td>3β,5α-THP SO4</td>
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<td>- modulation</td>
<td>$10^{-4}$-$10^{-5}$</td>
<td>Park-Chung et al. (1997)</td>
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<td>5β-DHP</td>
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<td>+ modulation</td>
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<td>progesterone</td>
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<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wu et al. (1990)</td>
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<td>Kainate</td>
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<td>+ modulation</td>
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<td>GABA-A</td>
<td>- modulation</td>
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<td>5-HT3</td>
<td>GABA-A</td>
<td>- modulation</td>
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<td>sigma 1 antagonist*</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wetzel et al. (1998)</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Grazzini et al. (1998)</td>
</tr>
<tr>
<td>pregnenolone SO4</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Majewska et al. (1988)</td>
</tr>
<tr>
<td>Glycine</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wu et al. (1990)</td>
</tr>
<tr>
<td>AMPA</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wu et al. (1991)</td>
</tr>
<tr>
<td>Kainate</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wu et al. (1991)</td>
</tr>
<tr>
<td>NMDA</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wu et al. (1991)</td>
</tr>
<tr>
<td>sigma 1</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Monnet et al. (1995)</td>
</tr>
<tr>
<td>DHEA</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Demirgoren et al. (1991)</td>
</tr>
<tr>
<td>DHEA SO4</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Demirgoren et al. (1991)</td>
</tr>
<tr>
<td>androsterone</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Park-Chung et al. (1999)</td>
</tr>
<tr>
<td>androsterone SO4</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Park-Chung et al. (1999)</td>
</tr>
<tr>
<td>testosterone</td>
<td>GABA-A</td>
<td>+ modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Park-Chung et al. (1999)</td>
</tr>
<tr>
<td>NMDA</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Valera et al. (1992)</td>
</tr>
<tr>
<td>5-HT3</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Wetzel et al. (1998)</td>
</tr>
<tr>
<td>17β-oestradiol SO4</td>
<td>GABA-A</td>
<td>- modulation</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>Weaver et al. (1997)</td>
</tr>
</tbody>
</table>

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Table 1. The Pharmacology of Neurosteroids. DHEA, dehydroepiandrosterone; 5ß-DHP, 5ß-dihydroprogesterone; DOC, deoxycorticosterone; SO₄, sulfate ester; 3α,5α-THDOC, 3α,5α-tetrahydrodeoxycorticosterone (alloTHDOC); 3α,5α-THP, 3α,5α-tetrahydroprogesterone (allopregnanolone); 3α,5β-THP, pregnanolone; 3β,5β-THP, epipregnanolone; 3β,5α-THP, isopregnanolone. # DHEA SO₄ and pregnenolone SO₄ antagonism. Dose M refers to the estimated range of concentration (molarity) required to have a physiologically relevant effect.

1.2.2 5α-Reduced Neurosteroids

Progesterone and deoxycorticosterone are converted by 5α-reductase into 5α-dihydroprogesterone (5α-DHP) and 5α-dihydrodeoxycorticosterone (5α-DHDHC) respectively (Fig. 2). These dihydro derivatives regulate gene expression through progesterone receptors (Rupprecht et al., 1993). A possible influence of 5α-DHP and 5α-DHDHC over GABA-A receptors should also be noted, but this has not been consistently demonstrated (Majewska et al., 1986; Paul and Purdy, 1992; Rupprecht et al., 1993). 5α-DHP and 5α-DHDHC can be further reduced to 3α-hydroxy-5α-pregnane-20-one (also known as 3α,5α-tetrahydroprogesterone or allopregnanolone; Fig. 1) and 3α,21-dihydroxy-5α-pregnane-20-one (also known as 3α,5α-TetraHydroDeOxyCorticosterone or alloTHDOC) respectively. These tetrahydro derivatives are amongst the most potent known ligands of GABA-A receptors (review: Lambert et al., 2001).
Allopregnanolone and alloTHDOC, as positive allosteric modulators of the GABA-A receptor, enhance neuronal inhibition (Majewska et al., 1986; review: Lambert et al., 2001). Although the 5α-reduced neurosteroids can reach effective physiological levels within minutes (Purdy et al., 1991), there appear to be no readily releasable pools of these neurosteroids (Corpechot et al., 1993; Korneyev et al., 1993b). Rather, their synthesis (Fig. 2) is upregulated on demand (for example, Reddy and Rogawski, 2002). Altered brain levels of 5α-reduced neurosteroids have been demonstrated during stress (Purdy et al., 1991) and depression (Uzunova et al., 1998), over the oestrus cycle (Palumbo et al., 1995), and following ethanol ingestion (VanDoren et al., 2000); however, the mediating physiological determinant in the rise and fall of neurosteroid levels under these different conditions remains to be determined.

5ß-reduction in the rat only occurs to a significant extent in the liver (Onishi et al., 1991). Consequently, the concentration of 5ß-reduced steroids in rat brain is unlikely to be of physiological importance, dissimilar to a number of other species (human: Klak et al., 2003; dog: Kawahara et al., 1975; bird: Hutchison and Steimer, 1981). In light of insufficient brain levels of 5ß-reduced steroids and androstane derivatives to potentiate GABA (Jo et al., 1989; Zwain and Yen, 1999), the neurosteroids primarily responsible for positive modulation of GABA-A receptors in the rat are the 5α-reduced steroids. Until very recently (Mennerick et al., 2004), no (pure) competitive antagonist of the binding site(s) of the 5α-reduced steroids on the GABA-A receptor was available. Instead, neurosteroid enhancement of GABA-ergic
responses can be effectively blocked in the rat by inhibitors of 5α-reductase, such as the synthetic steroid finasteride (17β-N-(2-methyl-2-propyl)carbamoyl-4-aza-5α-androst-1-en-3-one; Azzolina et al., 1997).

1.2.3 Neurosteroid Synthesis and Metabolism

Neurosteroids, like all steroids, are derived from cholesterol (Fig. 2). The putative rate-limiting step in neurosteroid synthesis is the conversion of cholesterol to pregnenolone by the cytochrome P450 side chain cleavage

Figure 2. The Synthesis Pathway from Cholesterol to Neurosteroids that Enhance GABAergic Inhibition. The 5α-reduced neurosteroids are boxed, and enzymes are in italics. We used finasteride, an inhibitor of 5α-reductase, to block the synthesis of 5α-reduced neurosteroids. Abbreviations: DHP, dihydroprogesterone; THP, tetrahydroprogesterone; DHDOC, dihydrodeoxycorticosterone; THDOC, tetrahydrodeoxycorticosterone.
enzyme (P450scc; review: Warner and Gustafsson, 1995). This is dependent upon the steroid acute regulatory protein (StAR), in cooperation with the diazepam binding inhibitor (DBI) and the mitochondrial benzodiazepine receptor (MBR), facilitating cholesterol transport to P450scc in the inner mitochondrial membrane (Bose et al., 2002; West et al., 2001). The aforementioned neurosteroid synthetic machinery, and the enzymes downstream in the pathway leading to 5α-reduced neurosteroid production, is present within neurons and glia within the central nervous system (CNS; StAR and P450scc: King et al., 2002; DBI: Alho et al., 1985; MBR: Anholt et al., 1984; P450scc and 3β-hydroxysteroid dehydrogenase: Sanne and Krueger, 1995; 21β-hydroxylase: Iwahashi et al., 1993; Kishimoto et al., 2004; 5α-reductase and 3α-hydroxysteroid oxidoreductase: Li et al., 1997). With the removal of peripheral sources of neuromodulatory steroids, physiologically relevant levels of neurosteroids persist within the CNS (for 5α-reduced neurosteroids, see Cheney et al., 1995). Even taking into account the possibility of a slow turnover of neurosteroids, this persistence is in a large part attributable to the de novo synthesis of neurosteroids in the brain (for discussion, see Baulieu, 1998).

The reactions from cholesterol to the 5α-reduced dihydro-derivatives of progesterone and deoxycorticosterone are effectively irreversible (Celotti et al., 1992). However, the enzymatic step involving 3α-hydroxysteroid oxidoreductase can work in both the reductive and oxidative directions. This means that allopregnanolone and alloTHDOC can be converted back
into their 5α-reduced dihydro-precursors (Rupprecht et al., 1993), and in doing so, attenuate GABA agonistic effects. In addition, the modulatory effects of neurosteroids can be altered by the action of sulfotransferases upon the steroid. For instance, the sulfate ester of allopregnanolone has been reported to show reduced potentiation of the GABA-A receptor, in comparison with the unsulfated form of this steroid (El-Etr et al., 1998), or display negatively modulation of the GABA-A receptor (Park-Chung et al., 1999). The cycling between sulfated and unsulfated steroids and between dihydro- and tetrahydro-5α-reduced neurosteroids allows, at least in principle, rapid control over the duration and magnitude of neurosteroid action.

1.2.4 Neurosteroid and Synthetic Enzymes Distribution Across the Brain

Physiologically relevant 5α-reduced neurosteroid levels, reaching more than 100 nM (Corpechot et al., 1993), are present in numerous regions of the brain. Cheney et al. (1995), using gas chromatography-mass fragmentography, reported that the highest concentration of 5α-reduced neurosteroids in rat brain are in the olfactory bulb, followed by the striatum, then the cerebellum, cerebral cortex and hippocampus (control/resting condition of adrenalectomised/castrated rats). The heterogenous regional distribution of 5α-reduced neurosteroids in the brain is unlikely attributable to circulating steroids, as 3H-tagged 5α-reduced neurosteroids and their precursors distribute uniformly through the brain when administered into
the tail vein (Cheney et al., 1995). For a number of brain areas, there is evidence that 5α-reduced neurosteroids and the necessary synthetic enzymes are present together (regional neurosteroid concentrations – Corpechot et al., 1993; Cheney et al., 1995; synthetic enzyme localisations – StAR and P450scc: King et al., 2002; DBI: Alho et al., 1985; MBR: Anholt et al., 1984; P450scc and 3β-hydroxysteroid dehydrogenase: Sanne and Krueger, 1995; 21β-hydroxylase: Iwahashi et al., 1993; Kishimoto et al., 2004; 5α-reductase and 3α-hydroxysteroid oxidoreductase: Li et al., 1997). Local administration of enzyme inhibitors such as finasteride, which is an antagonist of 5α-reductase and thereby blocks 5α-reduced neurosteroid production, decreases the neurosteroid level in the targeted brain area (Frye and Vongher, 2001). The preceding results suggest that neurosteroid levels can be locally regulated (also, see section 1.2.7).

1.2.5 Neurosteroid Enhancement of GABA-ergic Inhibition

Fast inhibitory synaptic transmission in the CNS is largely mediated by chloride conductances through receptor-mediated chloride channel complexes. At higher levels the GABA-A receptor dominates, while at brainstem levels the glycine receptor is the major fast inhibitory receptor-iontophore. At the GABA-A channel, typically, a fast rising current is observed followed by a decay that is often best modelled biphasically (with a fast and slow component; for example, Edwards et al., 1990). 5α-
reduced neurosteroids have been shown to prolong the decay of these inhibitory post-synaptic currents (IPSCs), both evoked and spontaneous, recorded from hippocampal (Spigelman et al., 2003), cerebellar (Cooper et al., 1999) and hypothalamic (Brussaard et al., 1997) neurons. According to a study by Zhu and Vicini (1997) in which high concentrations of GABA were applied in pulses to patched cerebellar granule cells mimicking vesicular release, the second phase of the inhibitory current decay with a slow time constant, thought to reflect movements in and out of desensitised states of GABA-A receptors (Jones and Westbrook, 1996), is affected by alloTHDOC (Zhu and Vicini, 1997). The 5α-reduced neurosteroid increased late channel openings and the probability of a channel open state.

When low, non-saturating levels of GABA are maintained, which may be relevant to situations where a tonic GABA concentration influences neuronal excitability (such as at extra-synaptic GABA-A receptors), progesterone metabolites can increase mean channel open time, due to an increased probability of longer duration channel openings and an increased frequency of single channel openings (Twyman and MacDonald, 1992). In addition, at concentrations (micromolar) much higher than that required for potentiating GABA-evoked currents, a direct effect of progesterone metabolites upon GABA-A receptor-mediated chloride flux has been reported (Callachan et al., 1987). For the sake of completeness, it should be mentioned that 5α-reduced neurosteroids have been shown to modulate nicotinic acetylcholine (Bullock et al., 1997) and 5-HT₃ receptors.
(Wetzel et al., 1998). However, as the concentrations required in these instances were in the high micromolar range and unlikely to be of physiological relevance, one concludes that the 5α-reduced neurosteroids are highly selective for the GABA-A receptor.

1.2.6 GABA-A Receptor Subunits Conferring Neurosteroid Sensitivity

The GABA-A receptor is formed from a combination of five of the following subunits: α1-6, β1-3, γ1-3, δ, ε, π, θ and ρ1-3 (Barnard et al., 1998). The subunit composition of the GABA-A receptor determines its pharmacological profile, including sensitivity to neurosteroids (Table 2). The most common brain receptor type, consisting of α1β2γ2 subunits, is sensitive to neurosteroid action, with 5α-reduced neurosteroid modulation of GABA-ergic function in the nanomolar range (review: Lambert et al., 2003). However, GABA-A receptor subunit make-up varies across the brain, suggesting regional neurosteroid sensitivity differences.

With regard to neurosteroid binding sites upon the GABA-A receptor, precise locations are yet to be identified. Strict neurosteroid structure-activity requirements (Harrison et al., 1987; Gee et al., 1988) are consistent with the proposal of a steroid recognition site on the receptor. It has been suggested that neurosteroids interact with the N-terminal region of the middle of the second transmembrane domain of the GABA-A receptor α2 and/or β1 subunits (Rick et al., 1998). However, (to my
knowledge) there has been no demonstration of displacement of bound (labelled) steroids (by unlabelled steroids) nor saturation of binding sites on receptors. At the current time, one is limited to the statement that steroids bind somewhere at the interface between the cell membrane and the receptor (for discussion, see Wetzel et al., 1998).

<table>
<thead>
<tr>
<th>Receptor Constitution</th>
<th>EC$_{50}$ (nM)</th>
<th>Maximum Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1\beta_1$</td>
<td>380 ± 10</td>
<td>143 ± 2</td>
</tr>
<tr>
<td>$\alpha_1\beta_1\gamma_1$</td>
<td>559 ± 22</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>$\alpha_1\beta_1\gamma_2\alpha$</td>
<td>89 ± 6</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>$\alpha_1\beta_1\gamma_2$</td>
<td>294 ± 36</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>$\alpha_1\beta_2\gamma_2\alpha$</td>
<td>177 ± 2</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>$\alpha_1\beta_3\gamma_2\alpha$</td>
<td>195 ± 36</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>$\alpha_2\beta_1\gamma_2\alpha$</td>
<td>146 ± 11</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>$\alpha_3\beta_1\gamma_2\alpha$</td>
<td>74 ± 1</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>$\alpha_4\beta_1\gamma_2\alpha$</td>
<td>317 ± 25</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>$\alpha_4\beta_3\gamma_2\alpha$</td>
<td>322 ± 27</td>
<td>80 ± 13</td>
</tr>
<tr>
<td>$\alpha_5\beta_1\gamma_2\alpha$</td>
<td>302 ± 38</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>$\alpha_6\beta_1\gamma_2\alpha$</td>
<td>220 ± 12</td>
<td>131 ± 6</td>
</tr>
<tr>
<td>$\alpha_6\beta_2\gamma_2\alpha$</td>
<td>350 ± 29</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>$\alpha_6\beta_3\gamma_2\alpha$</td>
<td>264 ± 33</td>
<td>90 ± 9</td>
</tr>
<tr>
<td>$\alpha_4\beta_3\delta$</td>
<td>241 ± 21</td>
<td>167 ± 4</td>
</tr>
<tr>
<td>$\alpha_1\beta_1\epsilon$</td>
<td>-</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

Table 2. The Sensitivity of GABA-A Receptors to Allopregnanolone Is Dependent upon Receptor Subunit Make-up. EC$_{50}$ is the concentration of neurosteroid that produces half-maximal potentiation of GABA-evoked current. The maximum effect of the steroid is reported as the percentage increase above the current evoked by GABA alone. Values ± standard errors are displayed. The sources of the data are Lambert et al. (2001) and Belelli et al. (2002).
1.2.7 Conditions in Which Neurosteroid Levels Are Modulated

5α-reduced steroid levels in the brain fluctuate in correspondence with progesterone during the oestrus cycle (Corpechot et al., 1993). Due to the physicochemical nature of the steroids, they can cross the blood-brain barrier after production from a peripheral source (Corpechot et al., 1993). However, under acute stressful conditions, elevated neurosteroid concentrations have been demonstrated in the brain and still manifest after adrenalectomy, pointing to a central origin in this case (Purdy et al., 1991). In relation to the environment at a cellular level that leads to increased neurosteroid synthesis, acute stress initially (within 5 minutes) changes GABA-ergic inhibition in the cerebral cortex, cerebellum and striatum by reducing the receptor binding of GABA (Biggio et al., 1981; 1984). In the hippocampus, increased production of pregnenolone (and oestradiol) has been reported to occur after stimulation of NMDA receptors (Kimoto et al., 2001; Hojo et al., 2004). These studies suggest that in response to raised neuronal excitability, neurosteroid production can be up-regulated.

1.2.8 Clinical Relevance of Neurosteroids

5α-reduced neurosteroids have been shown to possess sedative (Arafat et al., 1988), anaesthetic (Norberg et al., 1987), anxiolytic (Bitran et al., 1991), neuroprotective (Brinton, 1994) and anticonvulsant (Belelli et al.,
1989) properties. Altered levels of neurosteroids can be detected in the cerebral spinal fluid of patients experiencing depressive episodes (Uzunova et al., 1998). Moreover, neurosteroids are thought to contribute to the symptoms of pre-menstrual syndrome (Smith et al., 1998) and post-partum depression (Nappi et al., 2001). With the demonstration that fluoxetine affects the function of 3α-hydroxysteroid oxidoreductase (Griffin and Mellon, 1999), the antidepressant action of this drug may in part be attributable to changing the equilibrium between dihydro- and tetrahydro-5α-reduced neurosteroids species. In the condition of perimenstrual catamenial epilepsy, women have a greater susceptibility to seizures on days of their cycle in which progesterone levels are falling (Foldvary-Schaefer and Falcone, 2003). The corresponding decline in 5α-reduced neurosteroids may well underlie the increased likelihood of seizures (Reddy et al., 2001). Recently, 5α-reduced neurosteroid levels in the cerebral cortex have been reported to correlate with the sedation brought about by ethanol ingestion (VanDoren et al., 2000); for alcohol-dependent patients, ethanol withdrawal is marked by reduced levels of 5α-reduced neurosteroids (Romeo et al., 1996). The above examples illustrate that changes in 5α-reduced neurosteroid status can have profound consequences for the individual. It is clear that the recognised actions of these steroids on increasing efficacy of the GABA-A receptor allows for parsimonious explanation of most of these clinically-relevant effects.
1.3 Auditory Midbrain

1.3.1 Auditory Midbrain Introduction

With neurosteroids introduced, I now present the anatomical substrate of the electrophysiological experiments that were performed during my doctoral work. The review of the auditory midbrain that follows in this chapter focuses on the advantages that the ascending auditory pathway connectivity provided for our investigation of neurosteroid-mediated gain control and the neuronal response characteristics which served as a metric of change in cell excitability. There is much similarity in the anatomy and physiology of the auditory midbrain across mammalian species (Irvine, 1986), with many of the consistent features first described in the cat. Nevertheless, as the rat predominantly served as experimental animal in the present study, the material reviewed below derives from studies of the rat when available (data collected from other species will be annotated accordingly).

The inferior colliculus (IC) is located on the dorsal surface of caudal midbrain. It receives input from nearly all major brainstem auditory nuclei and feedback from auditory cortical areas. The IC can be divided into a large central volume, the central nucleus (ICC), and two surrounding cortices: the dorsal cortex (ICD) in the dorsal third of the IC, and the external cortex (ICX) in the ventral two-thirds of the IC. The extensively studied ICC of the cat contains disc-shaped/principal cells with planar
dendritic fields (Rockel and Jones, 1973; Oliver and Morest, 1984); these cell bodies, their dendrites and ascending afferent fibres tend to form laminae. Multipolar/stellate neurons are also present within the cat ICC and have spherical dendritic fields (Rockel and Jones, 1973; Oliver and Morest, 1984). With regard to the rat ICC, 'flat' and 'less-flat' neurons have been described (Malmierca et al., 1993; 1995). The flat cells essentially correspond to the disc-shaped/principal cells in cat, but the less-flat cells do not extend across laminae and so appear to differ from stellate/multipolar cells. In addition to the above features, the ICC is conspicuous in having a high cell density relative to the cortices and a relatively small variation in cell size. Nonetheless, within the rat ICC there is a dorsal-to-ventral gradient of neuron density and size: the highest density is found dorsally, and the average diameter of neurons is 7-12 μm in dorsal ICC and 9-20 μm in ventral ICC. With respect to the cortices, the ICD can be divided into three layers. The most superficial layer, layer 1, consists of flattened cells, around 7 μm in mean diameter. Layer 2 contains mainly multipolar neurons, measuring 7-12 μm in diameter, whilst layer 3 also has larger multipolar neurons, up to 23 μm in diameter. Like the ICD, the ICX can be thought of as comprising 3 layers. Cells with an average diameter of 7-9 μm are dispersed in layer 1, the outermost layer. Clusters of neurons, with neurons between 7 and 12 μm in diameter, are present in layer 2. Cells ranging from 9-28 μm in diameter constitute layer 3, which is marked by large multipolar cells with rough Nissl bodies (Faye-Lund and Osen, 1985).
1.3.2 Inputs to the Auditory Midbrain

The ICC receives input from the dorsal, anteroventral and posteroventral cochlear nuclei (CN), lateral superior olivary nucleus (LSO), lateral and ventral nuclei of the trapezoid body, superior paraolivary nucleus, and dorsal nucleus of the lateral lemniscus (DNLL), all bilaterally; in addition, the ipsilateral medial superior olivary nucleus (MSO), ipsilateral intermediate and ventral nuclei of the lateral lemniscus, contralateral IC, and the ipsilateral primary auditory cortex send projections to the ICC (Beyerl, 1978; Druga and Syka, 1984; Tokunaga et al., 1984; Saldana et al., 1996; Fig. 3). Ascending fibres from the CN, LSO and DNLL have been demonstrated to terminate mostly within alternating sublayers of nearby fibrodendritic laminae of the ICC, producing a pattern of ‘afferent bands’ (CN: Oliver, 1987; Oliver et al., 1997; LSO: Shneiderman and Henkel, 1987; DNLL: Shneiderman et al., 1988, Bajo et al., 1993). It appears that inputs driven by the same ear, and deriving from areas of nuclei with corresponding frequency representation, target the same sublayer of an ICC lamina (Oliver et al., 1997). However, within the sublayer, inputs seem to be largely segregated according to their nucleus of origin, forming ‘afferent patches’. It has been shown that the endings of contralateral and ipsilateral projections to the ICC, from the CN, LSO and DNLL, sort into interdigitating laminar sublayers (CN and LSO: Oliver et al., 1997; LSO: Shneiderman and Henkel, 1987; DNLL: Gabriele et al., 2000a). This termination pattern — aural dominance bands — has been
compared to ocular dominance columns in visual cortex (Gabriele et al., 2000b).

Figure 3. Ascending Inputs to the Central Nucleus of the Inferior Colliculus. Projections (lines) from brainstem auditory nuclei (boxes) bilaterally to one ICC are shown. AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; DNLL, dorsal nucleus of the lateral lemniscus; ICC, central nucleus of the inferior colliculus; INLL, intermediate nucleus of the lateral lemniscus; LNTB, lateral nucleus of the trapezoid body; LSO, lateral superior olivary nucleus; MSO, medial superior olivary nucleus; PVCN, posteroventral cochlear nucleus; SPO, superior paraolivary nucleus; VNLL, ventral nucleus of the lateral lemniscus; VNTB, ventral nucleus of the trapezoid body.

1.3.3 Frequency Tuning and Tonotopic Organisation

When the sound level required to elicit a designated neuronal response (investigator-set threshold) is plotted against the frequency of that stimulus, over a range of frequencies, ICC neurons generally exhibit sharp frequency tuning (for example, Kelly et al., 1991). The lowest point of the tuning curve, corresponding to that frequency at which the threshold response is achieved with the minimum sound level, specifies the ‘characteristic frequency’. Neurons with similar characteristic frequencies
form layers in the ICC, with these frequency-band layers inclined along a
dorsocaudal-to-ventrorostral plane (Clopton and Winfield, 1973). There is
a correspondence between frequency-band layers and fibrodendritic
laminae (linking function and structure; cat: Schreiner and Langner, 1997);
the ordering of layers maintains the frequency organisation of the cochlea.
As an electrode is lowered from dorsal-to-ventral ICC, the characteristic
frequency of neurons encountered increases (Clopton and Winfield, 1973).
In the rat, characteristic frequencies range from approximately 500 Hz to
50 kHz, with the median reported between 10 and 15 kHz (Sprague-
Dawley: Syka et al., 1981; Wistar: Kelly et al., 1991; Fischer 344: Palombi
and Caspary, 1996a). Neuronal response thresholds at characteristic
frequency seem to be lowest for those frequencies from 3 to 10 kHz (Syka
et al., 1981; Kelly et al., 1991; Palombi and Caspary, 1996a).

1.3.4 Monaural Response Characteristics

The majority of ICC neurons, at least in the anaesthetised preparation,
have a very low level of spontaneous/maintained activity (less than 2
spikes/second) (Syka et al., 1983; Palombi and Caspary, 1996a).
Nonetheless, nearly all ICC neurons are excited when appropriate sounds
are presented to the contralateral ear (Syka et al., 1983; Kelly et al., 1991;
Palombi and Caspary, 1996a) – this subsection will refer to those
responses to contralateral ear stimulation only (see subsection 1.3.5 for
ipsilateral ear-evoked responses). From the point of view of extracellular
recordings, neurons have been observed to respond at the onset or offset
of an auditory stimulus, over a sustained period during the stimulus, or some combination of the above. The most common temporal pattern is the onset response (quoted at over half of ICC neurons), followed, in prevalence, by the combined onset and sustained response (with or without a pause between response components; Syka et al., 1983; Palombi and Caspary, 1996a). However, different stimuli, varying in either frequency or sound level, can elicit different temporal response patterns from the same neuron (see discussion in Irvine, 1986).

When sound level is increased from threshold, if neuronal response rate only rises with elevations in sound level, or plateaus after an intermediate sound level, then the resultant rate/level function is usually classified as monotonic (although the saturating response profile has also been described as a 'plateau'); if neuronal response reaches a maximum at an intermediate sound level and decreases at higher sound levels, then the resultant rate/level function is classified as nonmonotonic. As the criteria employed in the classification of nonmonotonicity has varied amongst studies, and the fact that deeper anaesthesia may be associated with a greater incidence of nonmonotonic functions (cat: compare Bock et al., 1972, with, for instance, Erulkar, 1959), the proportion of ICC neurons associated with a particular function type is difficult to specify – this notwithstanding, a number of studies have found approximately half of their sample to be describable as nonmonotonic (Faingold et al., 1991a; Palombi and Caspary, 1996a).
In addition, when increasing sound level, first-spike latency, the interval from stimulus onset to the first action potential generated by the neuron in profile, decreases (Syka et al., 1983). The (mean minimum) latency for ICC neurons has been reported to range from 6 to 15 ms in response to clicks (Syka et al., 1983) and from 6.2 to 77.2 ms (median 11.8 ms, mean 14.1 ms) to tones at characteristic frequency, 30 dB above threshold (Palombi and Caspary, 1996a); also, Rees and Möller (1983) have reported a mean latency of 9 ms in response to tones 25 to 30 dB above threshold.

1.3.5 Binaural Response Characteristics

According to the scheme of Goldberg and Brown (dog: 1968; 1969), a two letter designation is commonly used for classifying binaural neurons: the first letter corresponds to the effect of monaural stimulation of the contralateral ear, and the second letter to that of the ipsilateral ear. The effect is indicated as either 'E' for excitatory, 'I' for inhibitory, or 'O' for null. Special mention should be made of the fact that E, I and O represent the net effect of sound presentation. In actuality, stimulation of either ear drives excitatory and inhibitory inputs to the majority of ICC neurons. To describe any binaural interaction, the two letter designation is refined by some by adding a forward slash followed by an additional letter corresponding to the effect of binaural stimulation: if the binaural response is less than that to contralateral ear stimulation only, then the effect is indicated as ‘I’ for inhibitory; if the binaural response is larger than that to
each ear, then the effect is indicated as ‘F’ for facilitatory. As spontaneous/maintained activity is generally low for ICC neurons, it is difficult to gauge inhibition during monaural (ipsilateral ear) stimulation. Consequently, the EO/I and EI designations tend to be applied interchangeably.

Over 50% of ICC neurons are reported to belong to the EI class (Silverman and Clopton, 1977; Syka et al., 1983; Kelly et al., 1991; Palombi and Caspary, 1996b); EO/monaural and EE are the only other classes represented by larger than 10% of ICC neurons. It should be noted that there is a relationship between binaural class and characteristic frequency. Neurons with higher characteristic frequency are more often EI or EO/monaural than those with lower characteristic frequency, and the converse is true regarding the EE class (Kelly et al., 1991). In relation to ipsilateral ear-evoked excitation, the derived characteristic frequency may be slightly lower, and the threshold higher, than that obtained with contralateral ear stimulation (gerbil: Semple and Kitzes, 1985).

Interaural disparities are exploited for the localisation of sounds (as are monaural aspects such as spectral transformations due to the pinna), with contralateral space represented in an IC (review: Konishi, 2003). Sound from a source off the median plane will travel different distances to both ears resulting in interaural time differences (ITDs) and, due to diffraction about the head, the sound reaching the far ear will have been attenuated (relative to near ear) giving interaural level differences (ILDs). ITDs can
take the form of transient (or sound onset) time differences (TTDs) or ongoing time differences (OTDs), which induces interaural phase differences (IPDs). Due to simple considerations of the physics of sound, at low frequencies, IPDs are the main cues for azimuth, whereas at high frequencies, ILDs provide the most useful cue (for example, cat: Delgutte et al., 1999; guinea pig: McAlpine et al., 2001). Most ILD-sensitive neurons, and TTD-sensitive neurons, are EI (Flammino and Clopton, 1975), and as IPD-sensitive cells can exhibit facilitation at favourable delays and suppression at other delays, they are commonly referred to as 'delay-sensitive' (rather than by a two letter designation). As may be expected with its high frequency hearing, the rat principally uses ILDs for azimuthal determination (Syka et al., 1983).

1.3.6 Neurotransmitter and Receptor Contributions to Response Characteristics

Excitatory synaptic transmission in the ICC is largely served by the action of glutamate (review: Kelly and Zhang, 2002). In a study involving slice preparations of the auditory midbrain, stimulation (current pulse) of the lateral lemniscus (LL) – ascending fibres from lower auditory nuclei – typically elicited from post-synaptic ICC neurons an early and a late excitatory response, the former AMPA receptor-mediated and the latter NMDA receptor-mediated (Ma et al., 2002a). Consistent with this finding, extracellular recordings from single neurons in vivo during acoustic stimulation show that onset responses and the early part of sustained
responses are predominantly mediated by the AMPA receptor, whilst the latter part of sustained responses are principally NMDA receptor-mediated (Zhang and Kelly, 2001).

GABA and glycine are the main inhibitory transmitters in the ICC, with GABA and glycine reducing the excitability of nearly all ICC neurons (review: Faingold et al., 1991b). GABA-ergic inhibition in the ICC appears to rely upon the activation of GABA-A receptors, with GABA-B receptors mainly involved in regulating transmitter release (see below). Inhibitory post-synaptic potentials (IPSPs) recorded from ICC neurons are reduced or abolished when GABA-A receptor-mediated inhibition is blocked by the application of bicuculline (mouse: Wagner, 1996). Considering extracellular recordings, bicuculline causes higher discharge rates of greater than 90% of neurons in the ICC and can affect different components of temporal response patterns (Faingold et al., 1991a). Faingold et al. (1991a) have shown increased onset and sustained responses, reduced duration of pauses between combined onset and sustained response patterns, and the appearance of sustained or offset components (in formerly onset only or sustained only response patterns respectively). In a number of ICC neurons, bicuculline had little effect on contralateral-ear-evoked inhibition but reduced ipsilateral-ear-evoked inhibition (Faingold et al., 1989).

GABA-B receptors outside the ICC can be located pre- or post-synaptically, with receptors at each position interacting with different ion
channels. Post-synaptic GABA-B receptors cause membrane hyperpolarisation through activation of inwardly rectifying potassium channels, whilst pre-synaptic GABA-B receptors reduce transmitter release by inactivation of voltage-gated calcium channels (review: Misgeld et al., 1995). In the ICC, the limited evidence available suggests that GABA-B receptors be primarily involved in regulating excitatory and inhibitory transmitter release. The GABA-B receptor agonist baclofen has little effect on the membrane conductance of patched neurons in brain slice preparations of the ICC (Zhang and Wu, 2000; Ma et al., 2002b), pointing to a pre-synaptic localisation of GABA-B receptors. Using this interpretation, the activation of GABA-B receptors, which can reduce (Vaughn et al., 1996) or increase (Ma et al., 2002b) neuronal excitability in the ICC, may reduce glutamate or GABA release, respectively.

In relation to glycine, Moore et al. (gerbil: 1998) found that only half of their sample of voltage-clamped neurons in the ICC was sensitive to strychnine (a glycine receptor antagonist), and the IPSCs of these cells were reduced but never abolished by strychnine. Turning our attention to extracellular recordings, increased discharge rates of around two-thirds of ICC neurons has been observed with local strychnine administration (Faingold et al., 1989). Unlike bicuculline, strychnine has not been shown to selectively affect ipsilateral-ear-evoked inhibition.
1.3.7 Evoked-Potentials

A field potential evoked by an auditory stimulus or by electrical stimulation (known as an evoked-potential or EP) can be recorded, using a low impedance electrode (and low-pass filter), which reflects the activity of the surrounding neural structure. So EP waveforms recorded from the ICC are dependent upon the position of the electrode within the nucleus. Nevertheless, waveforms can be thought of as having two parts in common, whether evoked by sound or electrical stimulation of an input pathway. The early part of the EP from the ICC, elicited by electrical stimulation of the dorsal cochlear nucleus for example, is insensitive to stimulus rate (up to 200 Hz), and the late part is rate-sensitive (Semple and Aitkin, 1980). In response to acoustic stimulation, the early part (as a guide, the first 6-7 ms after stimulus onset) of the EP from the ICC is unaffected by local lignocaine injection, whereas the late part is abolished (Szczepaniak and Möller, 1993). Taking into account the aforesaid results and latencies of waveform parts, the early part of the EP represents input to the ICC from lower auditory centres (that is, the activity of axons entering the ICC), and the late part is attributable to post-synaptic events generated in the ICC. Usually the late part consists of a negative-going wave (negative peak around 7-10 ms after stimulus onset) followed by a positive-going wave. In addition, component waves may be resolvable in the early part of the EP, associated with either second-, third- or fourth-order neurons from the CN, superior olivary complex or nuclei of the lateral lemniscus respectively (Szczepaniak and Möller, 1993).
1.4 Plasticity

1.4.1 Plasticity Introduction

In the broadest sense, plasticity is change in an operation of the nervous system when viewed across time. Change can be observed at levels of functional organisation from the molecular to the behavioural and throughout the lifespan of an organism. The concept of plasticity encompasses an extensive range of phenomena, biological substrates and causative factors. During development, the basic architecture of the nervous system is established, dependent upon critical temporal windows, genetic coordination and sensory input. Sensory deprivation within the putative sensitive periods and various manipulations of gene expression produce abnormal brain structuring (for example, plasticity of developing visual cortex – review: Berardi et al., 2003). Through maturation and beyond, learning and memory formation are supported by changes in synaptic weights and relations within and between neural networks (reviews: Horn, 2004; Lynch, 2004); these plastic events are modulated by mechanisms of attention, emotion and reward (Horn, 2004; Lynch, 2004). Within this wide construct of plasticity also fits the abnormal alterations in cellular processes manifesting as disease states and the counteractions and reorganisations for homeostasis (review: Turrigiano and Nelson,
2004), in addition to the patterning associated with biorhythms and aging (review: Hedden and Gabrieli, 2004).

From the perspective of electrophysiological investigation, which forms the core of this doctoral thesis, the key feature of the central nervous system serving plasticity is the adaptability of neuronal responses. When the evoked-response differs from prior responses to the same stimulus, the underlying modification represents a form of plasticity. This refined interpretation of plasticity affords greater utility in gauging change within the context of the ensuing neurophysiology.

1.4.2 Demonstrated Forms of Auditory Midbrain Plasticity

Changes in neuronal excitability within the ICC can result after tetanic stimulation of the LL under conditions of reduced inhibition. Long-term potentiation (LTP) of field potentials recorded from the ICC post-tetanus (50 Hz for 20 s) has been reported by Hosomi et al. (1995) with GABA-A receptors blocked and by Zhang and Wu (2000) with GABA-A receptor-mediated and glycinergic inhibition suppressed. In the latter study, increased pre-synaptic GABA-B receptor activation, possibly reducing GABA release, facilitated LTP. In experiments performed by Wu et al. (2002), high frequency tetanic stimulation of the LL (100 Hz for 10 s or 200 Hz for 5 s usually being more effective in eliciting LTP than 50 Hz for 20 s) could induce LTP of excitatory post-synaptic potentials (EPSPs) from whole-cell patch clamp recordings in the ICC. LTP in the aforementioned
demonstrations took the form of an increase in response to more than 120% of the pre-tetanus level, had duration of at least 30 minutes and was NMDA receptor-dependent. Besides LTP, other forms of plasticity were observed by Wu et al. (2002). Long-term depression (LTD) could be generated by low frequency tetanic stimulation of the LL (50 Hz at 20 s being the lowest frequency used by Wu et al.). EPSPs decreased to more than 70% of the pre-tetanus level and remained depressed for at least 30 minutes. Regardless of whether LTP or LTD was expressed, a short-term enhancement of excitability could occur post-tetanus. In these instances, EPSP amplitude more than doubled within a minute after tetanus and then declined 2-3 minutes later.

In addition to changes in gain, the frequency response characteristics of neurons in the ICC can be altered. The characteristic frequency of ICC neurons and their tuning curves may shift toward the frequency of a repeatedly presented auditory stimulus, the frequency of a conditioned sound, or the characteristic frequency of cortical neurons that have been electrically stimulated (big brown bat: Gao and Suga, 1998; Yan and Suga, 1998). The result of such a shift in the characteristic frequency of ICC neurons is a reorganisation of the tonotopic map of the ICC. This plasticity requires feedback from the auditory cortex and is dependent upon acetylcholine (Ji et al., 2001). Other neuromodulators, such as 5-HT, can also affect the processing of behaviourally relevant acoustic stimuli (Mexican free-tailed bat: Hurley and Pollak, 2001).
1.4.3 Neurosteroid-Mediated Plasticity in the Auditory Midbrain?

Based on their pharmacological action, 5α-reduced neurosteroids would appear to have the capacity to modulate inhibition, facilitating a regulated balance between excitatory and inhibitory influences. In order to test this hypothesis, we manipulated a subset of GABA-ergic inputs to an auditory midbrain area, allowing reactive synaptic plasticity to occur at the other GABA-ergic inputs. Unlike most brain areas, the ICC receives GABA-ergic input from a number of external sources, in addition to local interneurons.

**Figure 4. Schematic Depiction of the Experimental Paradigm.** Recordings were made from the ICC before and after kainic-acid-induced deactivation of the contralateral DNLL (contra- and ipsilateral designations are with reference to the ICC from which recordings were made). The major inhibitory input to the ICC, that derived from the contralateral DNLL, is activated by stimulation of the ipsilateral ear. We investigated whether the remaining sources of ICC inhibition, predominantly activated by stimulation of the contralateral ear, were upregulated to counteract the effect of contralateral DNLL deactivation. Brainstem and pontine auditory pathway nuclei are not shown, but the effective binaural differentiation of the output of this processing is represented by arrows indicating left-ear- and right-ear-dominated inputs to the midbrain.
(Roberts and Ribak, 1987; Gonzalez-Hernandez et al., 1996; Zhang et al., 1998). For this reason, we chose to adopt the ICC as our model (Fig. 4) and, in an acute physiological experiment, permanently deactivate its major GABA-ergic projection from the contralateral DNLL. This intervention leaves viable sources of GABA-ergic inhibition within the ICC, with the potential to be upregulated as a compensatory response to removal of inputs from the DNLL. The remaining inhibition in the ICC is largely elicited by stimulation of the contralateral ear (Faingold et al., 1991), whereas the inhibitory input from the contralateral DNLL is activated by ipsilateral ear stimulation (Li and Kelly, 1992). Hence, an additional advantage of this model is that the contribution of each set of inhibitory inputs to ICC neurons is separable by sound stimulation of the appropriate ear.

Within this paradigm, the immediate consequence of DNLL deactivation would be a state of elevated excitability within the contralateral ICC. After this initial effect, enhanced GABA-ergic inhibition consistent with increased levels of 5α-reduced neurosteroids would lead to a subsequent reduction in excitability. Such reactive increase in neurosteroid synthesis would presumably be triggered by changes occurring locally in the ICC, where 5α-reductase and 3α-hydroxysteroid oxidoreductase, the enzymes which produce the various 5α-reduced neurosteroids found in the rat (Fig. 2), have been demonstrated (Li et al., 1997).
1.5 Thesis Organisation

The remainder of this doctoral thesis is organised as a methodology chapter (2), four results chapters (3-6) and a discussion chapter (7). Chapter 2 contains the experimental procedures pertaining to the collection of the data presented in chapters 3-6, in addition to research directions that, for a variety of reasons (stated in chapter 2), were only partially explored. Chapters 3 and 4 present the results of two different electrophysiological recording methods, extracellular single-neuron and EP respectively, that show an enhancement of inhibition within the ICC in response to an experimentally induced increase in ICC excitability. Furthermore, the mechanism of the aforesaid inhibitory synaptic plasticity is consistent with gain control mediated by 5α-reduced neurosteroids. This mechanism is confirmed in chapter 5, where a relationship is demonstrated between the level of 5α-reduced neurosteroids determined by immunohistochemistry and the inhibitory gain observed in electrophysiological recordings. Chapter 6 shows the distribution of 5α-reduced neurosteroids across brain areas and cell types from immunohistochemical experiments, suggesting that neurosteroid-mediated inhibitory gain control may not be limited to the auditory midbrain. The data of the results chapters are discussed in chapter 7, in which the model of inhibitory synaptic plasticity, where neurosteroids increase the efficacy of GABA-A receptor-mediated inhibition following an increase in neuronal excitability (the trigger), is elaborated. In addition, a plausible biochemical pathway from the trigger to increased neurosteroid synthesis is presented,
as is the relevance of neurosteroids in normal neural processing and pathological conditions.
2 EXPERIMENTAL PROCEDURES

2.1 Electrophysiology

Two recording methods were used to monitor the effects of deactivation of the contralateral DNLL upon responsiveness of cells in the ICC: extracellular single-neuron recording and locally generated evoked potential (EP) recording (subdivision of the rat inferior colliculus according to Faye-Lund and Osen, 1985). Experiments were approved by the Australian National University Animal Experimentation Ethics Committee and conformed to the Animal Welfare Act 1992, the Animal Welfare Regulations (ACT) (1993) and the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Anaesthesia was induced in adult (8-14 wk) male Dark Agouti (DA) rats (170-300 g; all animals regularly handled prior to the day of the experiment) with ketamine (100 mg/kg im; Troy Laboratories, Smithfield, Australia) and acepromazine (10 mg/kg im; Troy Laboratories), then maintained with ketamine as required. Local EP or single-neuron recordings were made with glass-coated tungsten microelectrodes in response to pure-tone stimulation of either ear. The frequency of a tone and the sound level presented to each ear were controlled via the on-line interface of the Macintosh program MALab (Kaiser Instruments, Irvine, CA). The tone duration was set at 100 ms, with 5 ms cosine-shaped rise and fall periods. Stimuli were delivered with Beyer DT48 transducers.
(Audio Telex, Silverwater, Australia) for single-unit recording experiments or with Motorola piezoelectric drivers (Sydney, Australia) for EP recording experiments. The output of each transducer through a hollow ear-bar was first calibrated using a Brüel and Kjaer 4165 microphone (Naerum, Denmark) fitted to the end of the hollow ear-bar with tygon tubing, providing an approximation of the output close to the tympanic membrane. The electrode signal was amplified (A-M Systems 1800 microelectrode amplifier; SDR, Sydney, Australia), filtered (AudioControl C-131 one-third octave equaliser, MountlakeTerrace, WA), and processed: neuronal activity was displayed on line in a computer window and recorded (Kaiser Instruments: MALab running on a Macintosh 7300, G3 or G4). EPs represent the average of 23 individual responses, with bandpass filtering at 25 Hz to 1.6 kHz, and a sampling frequency of 20 kHz. EP amplitude was measured between the negative and positive evoked-peaks of the post-synaptic wave generated within the ICC following the method of Szczepaniak and Möller (1993). The zero crossing of this wave was used to determine latency. For single-neuron recordings, spike counts were accumulated over 20 stimulus repetitions. Latency, the interval to the first evoked-spike, was determined for each stimulus presentation, with the mean calculated from 20 stimulus repetitions. In order to gauge spontaneous (maintained) activity, recordings were summed over 400 ms periods for 20 presentations of a sham stimulus (sub-threshold, typically – 20 dB SPL).
2.2 Treatments

Baseline responses from the ICC were monitored for approximately one hour, after which kainic acid (KA; Sigma-Aldrich, Sydney, Australia) was infused over 1 minute into the contralateral DNLL (14 mM, 0.5-1.5 μL). KA was dissolved in an isotonic saline solution, to which pontamine sky blue was added in order to reveal the spread of the injected volume (average radius 0.5 mm). A pipette with a tip diameter of 20 μm was affixed to a microsyringe, and the injection site confirmed after each experiment by reconstructing the microsyringe track from cresyl-violet- or thionin-stained sagittal brain sections. A recording electrode was glued to the pipette, with a tip separation of around 100 μm. Recorded spontaneous and evoked activity was used to confirm the efficacy of the KA injection, which typically took around 10 minutes to fully deactivate sound-induced activity in the DNLL. KA induced excitotoxic cell death. It is unlikely that any release of GABA within the ICC, which might have been produced as an initial effect of KA excitation of DNLL neurons, was significant, as an initial increase in ICC excitability was monitored after KA delivery. As the DNLL projects tonotopically to the contralateral ICC, best frequencies were matched between the recording sites in each nucleus to ensure that the DNLL-derived inhibitory input to the contralateral ICC neuron(s) being studied had been deactivated (best frequency of profiled ICC neurons > 4 kHz). Recordings from the ICC continued at regular intervals following the KA injection into the contralateral DNLL. Animals were randomly assigned to one of three treatment groups: DNLL LESION, CONTROL or
FINASTERIDE + DNLL LESION (EP and single-neuron data were collected from separate animals). For CONTROL experiments, the full experimental procedure was followed except that KA was injected into the region rostral of the DNLL and ventral of the intermediate white layer of the superior colliculus. FINASTERIDE + DNLL LESION experiments differed from DNLL LESION studies only in the additional step of finasteride administration (60 mg/kg ip; Sigma-Aldrich) approximately 2 hours before recordings commenced (an average of 3 hours prior to the KA injection). ICC ‘mapping’ experiments were also conducted in unlesioned animals, normal and finasteride-treated (neuronal activity was recorded between 1.5 and 5 hours after finasteride delivery), to determine the effect of finasteride on baseline electrophysiology. Finasteride is a specific inhibitor of 5α-reductase (for example, Steckelbroeck et al., 1999). 6 mg of finasteride (17β-N-(2-methyl-2-propyl)carbamoyl-4-aza-5α-androst-1-en-3-one; Steraloids Inc., Newport, RI) were added per mL of 30% w/v 2-hydroxypropyl-β-cyclodextrin (HPβCD; Sigma-Aldrich) in isotonic saline solution. Cyclodextrins show limited transfer across the blood-brain barrier (Camargo et al., 2001), and finasteride was administered via the intra-peritoneal route. In the experiments presented here, intra-peritoneal HPβCD and isotonic saline did not alter baseline neuronal responses within the ICC (see also Disney and Calford, 2001).
2.3 Antisera Production

An immunisation programme (adapted from Pow and Crook, 1993) involving 2 young-adult female rabbits was conducted in order to raise antibodies against 5α-reduced neurosteroids. The immunogen used was allopregnanolone 3-hemisuccinate conjugated to bovine serum albumin (BSA; Steraloids Inc.). Prior to the first immunisation, 5 mL of blood was collected from the marginal ear vein of the rabbits to provide pre-immune serum for testing the specificity of the final antisera. Each rabbit was then given a set of injections every 3 weeks, for a total of 4 sets per animal. An injection set consisted of 2 different treatments. The first was 4 mg immunogen in 0.25 mL distilled water emulsified with 0.25 mL Freund's adjuvant, injected subcutaneously at two sites around the flexure of the thigh and abdomen. Freund's complete adjuvant (Sigma-Aldrich) was used for the first set and incomplete adjuvant (Sigma-Aldrich) for the remaining sets. The second treatment was 4 mg immunogen in 0.25 mL distilled water incorporated with 0.75 mL of a colloidal gold-adjuvant mix, administered into the marginal ear vein. The gold-adjuvant mix consisted of 1 mg/mL adjuvant peptide (Sigma-Aldrich) in colloidal gold solution: 0.1% gold chloride (Sigma-Aldrich), 1% tannic acid (Sigma-Aldrich) and 1% sodium citrate (Sigma-Aldrich) in distilled water, with the pH adjusted to 8.0 with sodium hydroxide. Seven days after each of the first 3 immunisations, test bleeds (2-5 mL) were taken from the marginal ear vein for the purpose of monitoring the antibody response. However seven days after the final immunisation, the animal was deeply anaesthetised with
pentobarbitone sodium (60 mg/kg iv; Merial, Sydney, Australia) and terminally bled from the eye orbit. The blood (about 100 mL) was allowed to clot for 30 minutes and, after ringing, refrigerated overnight. The sample minus the clot was then centrifuged at 10000g for 10 minutes, following which the serum was removed and frozen at -20°C.

2.4 Antisera Specificity Tests

We purchased a commercial sheep antibody raised against allopregnanolone carboxymethyl ether coupled to BSA (Dr. R. Purdy, Department of Psychiatry, University of California San Diego) that became available, or at least came to my attention, around the time of our antisera harvest. With 3 polyclonal antisera at our disposal, 2 in-house and 1 commercial, each was characterised in pilot studies. Working dilutions (from 1:500 to 1:62500 tested) and staining patterns were evaluated in slices from the brain and adrenal glands of DA rats. However, the staining pattern obtained with each antiserum was not identical to the others. As the commercial anti-allopregnanolone antibody had shown minimal cross-reactivity (less than 1%) with compounds present in rat, determined by radioimmunoassay (Bernardi et al., 1998), we further investigated the specificity of the commercial antibody using competitive binding assays and immunoblots (dot blots; only successful methods are presented below); the reactivity of the in-house antibodies is currently being clarified and will not be reported here. The final staining protocol for the competition assays is presented in section 2.5, and this protocol was
accelerated for the dot blots (with the following final changes: no blocking, overnight primary antibody incubation; 1 hour secondary antibody incubation; 1 hour peroxidase complex incubation). For competition, the primary antibody was combined with allopregnanolone, alloTHDOC, 5α-pregnane-3,20-dione (also known as 5α-DiHydroProgesterone or 5α-DHP) or 21-hydroxy-5α-pregnane-3,20-dione (5α-DiHydroDeOxyCorticosterone or 5α-DHDOC) at 0.17 mg/mL of 45% propylene glycol/10% absolute ethanol/45% 0.1 M phosphate buffer (PB) and 1 hour later brain tissue was added. The substrate for dot blots was Immobilon-P polyvinylidene fluoride microporous membranes (Millipore, Sydney, Australia) to which allopregnanolone conjugated to BSA (our immunogen) and BSA (control) at 0.1 mg, 1 μg and 10 ng in 10 μL distilled water were applied. Also blotted were allopregnanolone, alloTHDOC, 5α-DHP and 5α-DHDOC at 0.1 mg, 10 μg and 1 μg in 10 μL absolute ethanol.

2.5 Immunohistochemistry

Further DNLL LESION and FINASTERIDE + DNLL LESION experiments were conducted, but, different from those used for physiology investigation, at 30 minutes post-KA, animals were perfused transcardially with saline followed by 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M PB. For the purpose of establishing baseline staining, additional animals were anaesthetised with ketamine (100 mg/kg im)/acepromazine (10 mg/kg im) and perfused when pedal and corneal reflexes were no longer
present. Brains were post-fixed for 48 hours in 4% PFA/0.1 M PB, to which sucrose was added (30%) for cryoprotection. Brain tissue was sliced using a freezing microtome into 50 µm parasagittal sections. The ICC generally spanned 1500 µm in the mediolateral direction, equivalent to 30 sections. We considered the ICC represented by 10 possible samples of 3 sections spaced 10 sections apart (that is, sample 1 = sections 1, 11 and 21 of the ICC; sample 2 = sections 2, 12 and 22...). One sample (that is, 3 equally spaced sections) was chosen at random (West, 1999) and immunoassayed. The substantia nigra pars reticulata (SNR) was sampled similarly and immunoassayed. Free-floating sections were blocked using 1% donkey serum (DS; Sigma-Aldrich) in 0.1 M PB for 1 hour. They were then incubated in 1:2500 commercial primary antibody (purchased from Dr. R. Purdy) in 1% DS/0.1 M PB, for 48 hours at 4°C on an orbital shaker (used throughout the immunoassay). After 3 x 10 minute rinses in 0.1 M PB, sections were incubated overnight in 1:200 anti-sheep/goat immunoglobulin biotinylated secondary antibody (from donkey; Amersham Biosciences, Sydney, Australia) in 0.1 M PB at 4°C. Following further rinses, sections were placed in 1:200 streptavidin biotinylated horseradish peroxidase complex (Amersham-Biosciences) in 0.1 M PB for 5-6 hours at room temperature. They were subsequently rinsed again, before being placed in 0.05% 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) in 0.1 M PB for 10 minutes and then transferred into a solution of 0.015% hydrogen peroxide/0.05% DAB/0.1 M PB for around 3 minutes. Finally, tissue was thionin counterstained. Sections were air-dried on gelatinised slides, mounted in DPX and coverslipped.
2.6 Cell Counts

The number of labelled cells was quantified using a 3-dimensional counting method (see Williams and Rakic, 1988; for discussion of applicability to frozen sections, see Williams' revision of the aforesaid paper at www.nervenet.org/papers/3DCounting.html). Sections were viewed using a Leitz Orthoplan microscope (Wetzlar, Germany), with a 100x oil-immersion objective (Leitz, Pl Apo Oel, NA 1.32) at a final magnification of 1000x, under Köhler illumination. Labelled cells (defined by dark staining and clear cellular shape/features) were counted if they were either (i) wholly within a rectangular prism (70 μm x 35 μm x 50 μm (idealised section thickness)), marked out by an eyepiece reticle as the section was moved vertically or (ii) partially within the prism, but not in contact with the base, front or left sides, nor their defined extensions (base extends forward-back, left-right and down; front extends forwards, but not above, nor to the right, of the prism; left side extends forward-back, left, but not above the prism). Five such counting windows were applied per section, at ICC sites chosen at random under low power, from which the mean count was calculated for each treatment group. The same was done for the SNR. A positive correlation has been demonstrated between the level of neurosteroids measured by radioimmunoassay and the density of neurosteroid-immunoreactive cells (Frye and Vongher, 2001). The images presented in this thesis were acquired using a Zeiss Axioplan 2: Imaging and Axiocam and Axiovision software (Sydney, Australia).
The counting procedure was repeated completely independently by an experienced histologist (Shannon Waldron) using a 100x oil-immersion objective (Zeiss, Plano Neofluar, NA 1.30) fitted to an Olympus BX60 light microscope. The aforementioned 3 dimensional-counting method was applied.

2.7 Cellular Distribution of Neurosteroids

The cellular distribution of 5α-reduced neurosteroids was examined in coronal and sagittal sections of brain tissue from 12 male DA rats (6 brains sectioned in each plane). Tissue was prepared and stained according to section 2.5. Sections were viewed using a Leitz Orthoplan microscope, or an Olympus BX60 light microscope, under Köhler illumination. Brain areas were identified using the rat brain atlas of Paxinos and Watson (1998). Two investigators (Matthew Kirkcaldie and I) independently evaluated the immunoreactivity, with data for each area derived from at least 5 animals. The density of stained cell bodies ranged from very low to very high and the density of labelled processes varied from little visible extension to a dense network. The images presented in this thesis were acquired using a Zeiss Axioplan 2: Imaging and Axiocam and Axiovision software or a Nikon Coolpix 995 high resolution digital camera with a C-mount adapter attached to the camera tube of the Olympus microscope.
3 RESULTS

Neurosteroid-Mediated Gain in the Auditory Midbrain: Single-Neuron Recordings

3.1 Electrophysiology Introduction

A test of the overall hypothesis (see section 1.4.3) first required a demonstration that loss of the major GABA-ergic input to the ICC from the contralateral DNLL was effective in reducing ipsilateral ear-dominated inhibition and increasing excitability – as previously demonstrated with a short-acting blockade of DNLL activity by Li and Kelly (1992). We then needed to show that this loss was effective in stimulating increased efficacy of the remaining, mostly interneuron-mediated and contralateral-ear-dominated, GABA-ergic inhibition. Finally, we postulated that the increased inhibitory efficacy would not occur if the effect of 5α-reduced neurosteroids at the GABA-A receptor was blocked. Given that no effective competitive antagonist for the 5α-reduced neurosteroid binding site was available until very recently (Mennerick et al., 2004), we addressed this hypothesis with the use of the synthetic steroid finasteride, which blocks 5α-reduced neurosteroid production. Single-neuron and local EP electrophysiology in response to dichotic sound stimulation were used to examine the outcome. Electrophysiological responses were compared between animals in three treatment groups. The first group comprised rats
subjected to KA-induced unilateral DNLL deactivation (designated DNLL LESION), while the second group acted as a control for the deactivation procedure, with KA delivered outside the DNLL (CONTROL). Animals in the third group (FINASTERIDE + DNLL LESION) underwent DNLL deactivation, but also had the synthesis of 5α-reduced neurosteroids blocked by pre-treatment with finasteride (Fig. 2). Finasteride is a reversible inhibitor of the type 1 isozyme of 5α-reductase, the purported predominant brain type, and a time-dependent inhibitor of the type 2 isozyme in rat (Azzolina et al., 1997). The finasteride dosing schedule that we used has previously been shown to prevent increases in neurosteroid synthesis in rat brain (for example, Concas et al., 1998).

### 3.2 Treatment Group Baselines

There was little difference in baseline neuronal responses between DNLL LESION, CONTROL and FINASTERIDE + DNLL LESION animals. Surveys of the ICC prior to the KA injection showed that ICC neurons were excited by contralateral ear stimulation. Binaural stimulation mainly resulted in responses less than or equal to those obtained with contralateral ear stimulation alone. ICC neurons generally responded at stimulus onset, with or without a sustained component, and predominantly yielded monotonic spike count versus sound level profiles. First-spike latencies (minimum mean first-spike latency range of 8.7-21.2 ms; no significant difference between treatment groups) fell into previously quoted ranges for ICC neurons (Palombi and Caspary, 1996a).
Approximately half of ICC neurons in intact animals were excited by stimulation of the contralateral ear and inhibited by ipsilateral ear stimulation (EI neurons). We demonstrated ipsilateral-ear-evoked inhibition by fixing the sound level at the contralateral ear and then incrementally increasing the level at the ipsilateral ear; this resulted in successive reductions of the response of EI neurons. As the contralateral DNLL is known to provide the bulk of ipsilateral-ear-evoked inhibition (Li and Kelly, 1992), we focused our attention on EI neurons, as their responses would immediately reflect the effects of DNLL deactivation. After a stable baseline response from the ICC was demonstrated, a neurotoxic dose of KA was injected into the contralateral DNLL. There was no difference in the time taken for DNLL deactivation between DNLL LESION and FINASTERIDE + DNLL LESION groups. KA was selected as the lesioning tool because its effect is non-reversible, circumventing any possible difficulties in interpretation related to deactivation time course. Confirmation of DNLL deactivation was provided by (i) electrophysiological responses consistent with a DNLL locus upon implantation of the microsyringe, (ii) suppression of tone-evoked responses within the DNLL following the KA injection, (iii) ICC neuronal responses showing a reduction in ipsilateral-ear-evoked inhibition post-KA, and (iv) histological identification of the DNLL injection site.
3.3 DNLL LESION Group

Within 10 to 15 minutes of the KA injection (8 animals), ipsilateral-ear-activated inhibition declined within the ICC and did not recover within the time frame of our experiments (Figs. 5A, 5B and 6A), corresponding with the decline in activity within the contralateral DNLL. Furthermore, there was a small increase in the response of ICC neurons to stimulation through the contralateral ear alone (Figs. 5B and 6A), suggesting a reduction in tonic inhibitory input to the ICC with deactivation of the contralateral DNLL. The induced increase in responsiveness of ICC neurons was followed by a reduction in neuronal excitability within the ICC (Figs. 5C and 6A). Spike counts started to decrease by 20 to 50 minutes post-KA, reaching a minimum – equal to or less than the baseline – between 30 and 90 minutes post-KA. The reduction in spike counts at low sound levels occurred before that at high sound levels. Onset and sustained response components were similarly affected.

The reduction in ICC excitability, after the initial increased activity following DNLL deactivation, was also apparent from mean first-spike latency determinations (Figs. 7A and 8). Significant increases in latency were observed at all sound levels by 20 minutes post-KA, reaching a maximum between 30 and 90 minutes post-KA.
Figure 5. The Removal of a Subset of GABA-ergic Inputs to the ICC Was Countered by an Enhancement of the Other Sources of Inhibition in the ICC. (A-C) Single-neuron recording demonstration that an induced reduction in GABA-ergic inhibition within the ICC was quickly followed by a counteracting increase in remaining inhibition. Prior to KA delivery, this ICC neuron from a DNLL LESION animal was inhibited by ipsilateral ear stimulation – evident from the lower response to binaural stimulation with an 80 dB sound level at the ipsilateral ear in comparison with that evoked by contralateral ear stimulation only (A). Within 15 min of KA delivery, the ipsilateral-ear-activated inhibition had disappeared (and never recovered). In addition, loss of the tonic component of this inhibition was reflected in elevated spike counts to contralateral ear stimulation (B). By 50 min after KA delivery, spike counts were declining, and at 80 min post-KA (shown here), the response to contralateral ear stimulation was close to baseline (C). (D-F) Cell recordings in FINASTERIDE + DNLL LESION animals revealed a similar initial effect (D and E) after the KA delivery but failed to show the later decrease to baseline.
responsiveness. In the example shown here, the response to contralateral ear stimulation continued to increase for 30 minutes (remaining elevated thereafter), and an increase in spontaneous activity was evident (F). (G-I) Neurons studied under the CONTROL paradigm displayed no changes in excitability or in the effect of ipsilateral-ear-stimulated inhibition.

3.4 CONTROL Group

There was no change in ICC responses (CONTROL group studies involved single-neuron recordings only; 4 animals) following KA injections outside the contralateral auditory nuclei (Figs. 5G, 5H, 5I, 6C, 7C and 8). In one animal, a control KA injection was made, and 90 minutes later, KA was also injected into the DNLL. Although the control injection produced no change in ICC neuronal responses, the second injection brought about the plasticity response typically observed in the DNLL LESION group. This suggests that reduced inhibition within the ICC, due to the loss of the contralateral DNLL, triggers the reduction in ICC excitability observed in DNLL LESION animals, and that it is not due to a non-specific effect of KA injection.
Figure 6. ICC Neurons Showed Ipsilateral-Ear-Evoked Excitation and Elevated Spontaneous Activity After Contralateral DNLL Deactivation when Neurosteroid Synthesis Was Blocked. (A-C) Onset responses recorded from three ICC neurons, one from a DNLL LESION (A), one from a FINASTERIDE + DNLL LESION (B) and one from a CONTROL (C) animal, plotted against time relative to KA delivery. Prior to KA delivery, the ICC neuron from a DNLL LESION animal was inhibited by ipsilateral ear stimulation. Initially after KA delivery, the ipsilateral-ear-activated inhibition disappeared and tonic inhibition was reduced. From 20 min to 50 min post-KA, spike counts decreased, and thereafter remained just below the baseline level (A). In contrast, there was no reduction in ICC excitability following the initial effect of KA in finasteride-treated animals. Although reduced inhibition in the ICC was observed after deactivation of the contralateral DNLL, the raised spike counts remained well above the baseline level at all times after KA delivery. At 30 min post-KA, ipsilateral-ear-evoked excitation and spontaneous activity were recorded (the spontaneous count represents those spikes that occurred during the window of the onset response); at 60 min post-KA, the excitatory response to ipsilateral ear stimulation matched that to contralateral ear stimulation (B). For the CONTROL case, spike counts remained stable throughout the experiment (C).
Figure 7. The increase in ICC inhibition which countered the deactivation of the GABA-ergic input from the contralateral DNLL also manifested as increased discharge latency. (A-C) Mean first-spike latency recorded from three ICC neurons, representative of each treatment group, plotted against the level of the sound presented at the contralateral ear. Latency contours at four important experimental time points are displayed – kainic acid delivery at 0 minutes. The neuron from the DNLL LESION animal exhibited a significant latency shift (across sound level) from baseline (-15 minutes) at 15 minutes post-kainate (p < 0.001, two-way analysis of variance) and after (A). However, finasteride treatment prevented the increase in latency, with a significant latency shift downward at 30 minutes (p < 0.01) and 60 minutes (p < 0.001) post-kainate (B). The discharge latency of the control neuron was stable for the duration of the experiment (C).
Figure 8. The Effect of Neurosteroids Was Evident from Changes in Latency in Addition to Spike Count. (A and B) Changes in mean minimum first-spike latency (compared with baseline) plotted against time relative to KA delivery. For each treatment, the latency at a particular time was compared with the initial value using a t test: stars, $p < 0.005$; hashes, $0.01 > p > 0.005$. Three cells are featured in A, one from each treatment group. For the DNLL LESION case (best frequency 15.3 kHz), latency was significantly elevated at 20 min post-KA and beyond. In contrast, the FINASTERIDE + DNLL LESION case (best frequency 6.5 kHz) showed a significant reduction in latency after 30 min post-KA. For the CONTROL case (best frequency 28.5 kHz), latency was stable throughout. Treatment group averages are illustrated in B. Finasteride prevented the elevation in latency that occurred after KA delivery in DNLL LESION animals.
3.5 **FINASTERIDE + DNLL LESION Group**

There was a qualitative difference in the behaviour of ICC neurons, in response to deactivation of the contralateral DNLL, for the FINASTERIDE + DNLL LESION group (8 animals) in comparison with the DNLL LESION group. Following the disappearance from the ICC of inhibition activated by the ipsilateral ear (Figs. 5D, 5E and 6B), ipsilateral ear stimulation elicited excitatory responses (Figs. 5F and 6B). Ipsilateral-ear-evoked excitation appeared in El ICC neurons by 15 to 35 minutes post-KA, with maximum ipsilateral ear drive between 30 and 80 minutes post-KA (7 of 8 animals, compared with 0 of 8 animals in the DNLL LESION group: p < 0.001, Fisher's exact test). This generally manifested as onset responses (6 of 7 animals), with over half of these cases also presenting with sustained components (4 of 7 animals). Ipsilateral ear drive manifested as an offset response in one animal. These responses were strong, and in 4 out of 7 animals, the maximum ICC spike count achieved after KA administration was produced in response to ipsilateral stimulation alone. An unmasking of ipsilateral-ear-evoked excitation in the ICC has previously been reported after ablation of the contralateral cochlea (Kitzes and Semple, 1985; McAlpine et al., 1997; Mossop et al., 2000).

In addition to the ‘unmasking’ of ipsilateral-ear-evoked excitation, a large elevation in spontaneous activity occurred within the ICC of finasteride-treated animals following deactivation of the contralateral DNLL (Figs. 5F and 6B). Spontaneous activity within the ICC, prior to deactivation of the
contralateral DNLL, was very low in all experimental groups (usually 5 spikes or less, summed over 20 periods of 400 ms). However, after DNLL deactivation in finasteride-treated animals, ICC spontaneous activity increased from 15 to 40 minutes post-KA (7 of 8 animals), reaching a maximum 30 to 70 minutes post-KA (20 to 350 spikes, with 4 animals recording between 275 and 350 spikes, summed over 20 400 ms periods). This contrasts with the DNLL LESION group, in which only one animal showed an increase in spontaneous activity (from 30 minutes post-KA, reaching a maximum of 225 spikes 85 minutes post-KA).

Although the overall excitability of ICC neurons was permanently raised post-KA, contralateral-ear-evoked responses, minus spontaneous activity, tended to vary from animal to animal in the FINASTERIDE + DNLL LESION group. Onset, sustained and offset response components were differentially affected, and in half of the cases, differences were apparent at high versus low sound levels. One neuron presented with an increased onset response, but reduced sustained component, post-KA. Another showed a reduced onset component at high sound levels and an increased onset at low levels post-KA. Two cells responded with a larger onset component at high sound levels and a smaller onset response at low levels post-KA. One of these cells, plus another neuron, gave sustained responses that showed a similar dependency on sound level post-KA. There were two examples of reduced onset responses, and in both cases offset responses manifested post-KA. The remaining neuron exhibited little
change in its onset response, despite the appearance of ipsilateral-ear-
evoked excitation and high spontaneous activity post-KA.

Furthermore, with contralateral-ear-evoked excitation, ICC neurons showed reduced latency (3 animals) or no change (2 animals) following the deactivation of the contralateral DNLL in the presence of finasteride (Figs. 7B and 8). The DNLL LESION group showed increased latency. For the remaining animals (3), the stimuli that elicited onset responses from ICC neurons post-KA were not effective pre-KA, so there was no baseline latency for comparison.

3.6 Finasteride Effect on Unlesioned Animals – ICC ‘Mapping’ Experiments

Finasteride had little effect on neuronal activity in the ICC of unlesioned animals. ICC ‘mapping’ experiments revealed that the tonotopic organisation of the ICC and sharp neuronal frequency tuning were maintained in finasteride-treated animals. Similar proportions of cell types, as defined by response characteristics, were observed in finasteride-treated (El neurons: 47.8% of 69 cells) and normal (El neurons: 45.8% of 118 cells) animals. Response latency of single neurons (median of cell population: 11.4 ms, finasteride-treated; 11.35 ms, normal) and spontaneous activity (mean of cell population: 4.4 spikes summed over 20 periods of 400 ms, finasteride-treated; 5.0 spikes, normal) were not changed by finasteride treatment. Similarly, VanDoren et al. (2000)
reported that finasteride did not have an effect on baseline neuronal activity in the septum. It should be noted that glycine is the major inhibitory transmitter within the cochlear and pontine nuclei that provide ascending afferents to the ICC (Kotak et al., 1998; Godfrey et al., 2000). As 5α-reduced neurosteroids have not been shown to modulate glycinergic inhibition, this limits the influence of finasteride on the baseline state of the ICC. Furthermore, the basal turnover of neurosteroids may occur on a time scale greater than that of our experiments.
4 RESULTS

Neurosteroid-Mediated Gain in the Auditory Midbrain:
Evoked-Potential Recordings

4.1 Baseline

EPs were recorded from the ICC under the same experimental conditions as those through single-neuron recordings. The effect of unilateral DNLL deactivation upon the contralateral ICC was compared between DNLL LESION and FINASTERIDE + DNLL LESION animals. Prior to KA delivery, there was little variation in waveform shape or component latency at similar ICC depths across treatment groups (see also section 3.2). We primarily studied responses to contralateral ear stimulation, as the contralateral ear would drive the ICC inhibition remaining after DNLL deactivation, allowing us to observe any upregulation of inhibitory inputs.

4.2 DNLL LESION Group

EP recordings (6 animals) showed a similar time course for the reduction in ICC excitability post-KA to that obtained in the single-neuron studies (Figs. 9A, 9B, 10A and 11A). Consistent with the fact that the DNLL to ICC input provides inhibition dominated by the ipsilateral ear pathway, there was little immediate effect of DNLL deactivation upon the EP recorded in
Figure 9. Neurosteroids Reduced EP Amplitude in the ICC Following Deactivation of the Contralateral DNLL. (A and B) EP recording demonstration (waveforms and % amplitude change shown) that an induced reduction in GABA-ergic inhibition within the ICC was quickly followed by a counteracting increase in remaining inhibition. After a small increase in ICC excitability with the deactivation of the contralateral DNLL (5 and 10 min post-KA values), EP amplitude fell, reaching a minimum around 30 min post-KA (A and B). With neurosteroid synthesis blocked, the state of increased ICC excitability as a result of contralateral DNLL deactivation persisted. There was no reduction in ICC EP amplitude following deactivation of the contralateral DNLL in C. While in D, ICC excitability was elevated until 40 min post-KA, after which the change in EP amplitude was less than that observed in DNLL LESION animals.
Figure 10. The Effect of Neurosteroids Was Evident Across Sound Levels. (A and B) The EP amplitude recorded from the ICC of a DNLL LESION (A) and a FINASTERIDE + DNLL LESION animal (B) plotted against the level of the sound presented at the contralateral ear. Amplitude contours at four experimental time points – 15 minutes before kainic acid delivery, and 10, 30 and 60 minutes post-kainate – are displayed. There was a significant effect of time on the ICC EP amplitude of the DNLL LESION animal (p < 0.005, two-factor, time and amplitude, analysis of variance) but not the FINASTERIDE + DNLL LESION animal (p > 0.05).

response to contralateral ear stimulation. However, after approximately 10 minutes a slight decline was apparent and EPs recorded from the ICC in response to contralateral stimulation fell to a minimum amplitude between 25 and 40 minutes post-KA (for the group, p < 0.001, significant effect of time, one-way repeated measures analysis of variance (ANOVA)). Latency increased, reaching a maximum 25 to 60 minutes post-KA. At 30 minutes post-KA, the group average amplitude fell by 25.4% from the pre-KA level for the ICC response evoked by an 80 dB stimulus presented to the contralateral ear. At the same time, latency increased by 0.47 ms (Fig. 11B).
Figure 11. Finasteride Attenuated and Delayed the Changes in ICC EP Amplitude and Latency that Followed Contralateral DNLL Deactivation in Normal Animals. (A) Group mean EP amplitude (80 dB stimulus presented to the contralateral ear at the frequency evoking the largest response at the ICC site for each animal) as a percentage of the baseline established prior to KA delivery, plotted against time relative to KA delivery. There was a significant difference in ICC EP amplitude between the groups after deactivation of the contralateral DNLL – treatments at a particular time were compared using Tukey’s Honestly Significant Difference (after two-factor, treatment and time, analysis of variance, with repeated measures on time): two stars, p < 0.05; one star, 0.1 > p > 0.05. (B) For the 30 min post-KA versus baseline comparison, the percentage change in peak-to-peak EP amplitude plotted against the change in EP latency (measured from stimulus onset to the negative-to-positive zero crossing of the ICC wave), for each DNLL LESION animal and FINASTERIDE + DNLL LESION animal. There was a significant difference (p < 0.01, Hotelling’s $T^2$ test) between the amplitude and latency at 30 min post-KA versus the pre-KA level.
4.3 FINASTERIDE + DNLL LESION Group

Finasteride pre-treatment (6 animals) attenuated the reduction in ICC excitability observed in the DNLL LESION group following contralateral DNLL deactivation (Figs. 9C, 9D, 10B and 11A). At 30 minutes post-KA (Fig. 11B), the group average change in amplitude from the pre-KA level was −5.1% (compared with −25.4% for the DNLL LESION group: p < 0.05, t test independent) for the ICC response evoked by an 80 dB stimulus presented to the contralateral ear. At the same time, latency was only altered by 0.125 ms (compared with 0.47 ms for the DNLL LESION group: p < 0.05, t test independent).
5 RESULTS

Neurosteroid Levels in the Auditory Midbrain: Immunohistochemistry

5.1 Immunohistochemistry Introduction

The manipulation of the physiological consequences of DNLL deactivation by finasteride points to an involvement of 5α-reduced neurosteroids. Immunohistochemical staining for cells containing 5α-reduced neurosteroids was used to confirm such involvement. The commercially available anti-allopregnanolone antibody that was employed shows minimal cross-reactivity (less than 1%) with compounds present in rat, as determined by radioimmunoassay (Bernardi et al., 1998). We further tested the specificity of the antibody with immunoblots and competitive binding studies in brain slices, the latter shedding light on the interaction of the antibody with compounds in situ, prior to commencing assays of neurosteroids within the auditory midbrain. Whereas the commercial antibody has been found useful in a number of radioimmunoassay investigations (Bernardi et al., 1998; Genazzani et al., 1998; Monteleone et al., 2000), only one study has applied the antibody to immunohistochemistry (Frye and Vongher, 1999). This study (of the ventral tegmental area) did not characterise the specificity of the antibody
for 5α-reduced steroid immunohistochemistry. Hence, it is important to conduct a full characterisation.

5.2 Immunoblots

Standard immunoblot methodology was not found to be very useful for characterising the antibody. Inconsistent labelling of allopregnanolone-BSA (BSA alone did not stain) from blot-to-blot with the commercial antibody, which was not solved by manipulating the staining protocol, prompted different approaches for coupling steroids to the blotting membrane (the in-house antibodies had also inconsistently labelled allopregnanolone-BSA). 'Steroid sandwich' configurations, in which one of the in-house antibodies was membrane-fixed in order to present steroids to the commercial antibody, yielded negative reactions. Two possible explanations are that the steroids did not contain a second epitope (or a first) or that geometrical constraints prevented antibody-antigen-antibody complex formation. The attempts at coupling steroids to the blotting membrane via proteins were followed by the simpler method of directly applying steroid solutions. The membrane, designed to bind proteins, is hydrophobic, offering a potential weak interaction with steroids. Steroids in ethanol produced positive immunoblots, with some low-level staining of allopregnanolone and alloTHDOC at the highest concentration applied; the dihydro precursors of allopregnanolone and alloTHDOC did not stain (nor did steroids solubilised using cyclodextrins – see section 5.3 for comment).
5.3 Competitive Binding Studies

The reactivity of the primary antibody for a particular compound also was tested by the degree to which the compound (added to the primary antibody solution) reduced immunolabelling of brain tissue. Reduced labelling results from the competition for the antibody between the test compound and endogenous antigen(s). As our test compounds were highly lipophilic steroids, this posed the challenge of finding a solvent (to be used instead of phosphate buffer in the primary antibody incubation step of the immunoassay; see section 2.5) that would not only be suitable for the steroids, but also the antibody – antibody function must be unaltered. A further consideration was that the solvent should not affect the structure of the tissue, especially the location/presentation of endogenous antigen(s). Initially, test compounds were solubilised using cyclodextrins in phosphate buffer. Cyclodextrins are cyclic oligosaccharides, with lipophilic interiors, which have high affinity for fatty molecules such as steroids, and hydrophilic exteriors, allowing incorporation into aqueous solutions (Williams et al., 1998). As a steroid would be partially contained within the central cavity of a cyclodextrin, the question raised is whether antibody access to the test compound is impeded. This is a possible interpretation of the minimal competition observed in our binding experiments utilising cyclodextrins. Next, we adopted a solvent system consisting of propylene glycol (45% v/v) and absolute ethanol (10% v/v) in phosphate buffer (0.1M), for which there should be no accessibility question. Competition binding studies testing
allopregnanolone (in the propylene glycol/ethanol solution) showed near abolition of immunolabelling in brain slices (Fig. 12). Exogenous alloTHDOC also greatly reduced immunoreactivity. In contrast, 5α-dihydroprogesterone and 5α-dihydrodeoxycorticosterone, the precursors of allopregnanolone and alloTHDOC respectively, left labelling intact, as did the solvent. However, solvent-induced tissue damage was evident. When the concentration of propylene glycol in the solvent was reduced, in an attempt to avoid the unwanted effect, the resulting poor steroid solubility rendered the solvent of little use. Nevertheless, the pattern of labelling was essentially the same with the propylene glycol (45%)/ethanol (10%) solvent employed for the primary antibody incubation step (solvent control binding experiments) as that with phosphate buffer (immunoassays in sections 5.4 and 5.5 and chapter 6). So antibody function and the location/presentation of endogenous antigen(s) were not significantly different under the two solvent conditions. In the assays that follow, it should be considered that allopregnanolone and/or alloTHDOC are the endogenous antigen(s) being immunolabelled.
Figure 12. **Allopregnanolone and AlloTHDOC Were the Immunolabelled Endogenous Compounds.** When alloTHDOC (B) or allopregnanolone (C), in contrast with their dihydro precursors (D and E), were added with the primary antisera onto brain slices, there was little staining of the tissue – parietal cortex in the cases here. Although the solvent for the steroids (F) caused some tissue damage, the labelling pattern was not different from that obtained with the usual assay buffer (A). Abbreviations: DHP, dihydroprogesterone; THP, tetrahydroprogesterone; DHDOC, dihydrodeoxycorticosterone; THDOC, tetrahydrodeoxycorticosterone (scale bar = 100 μm).

5.4 **Neurosteroid Immunoreactivity in the ICC**

Cell bodies and large dendrites of neurons in the ICC, including disc-shaped/principal neurons, were immunolabelled with anti-allopregnanolone/alloTHDOC antiserum. Glial-like labelling was not observed. Using an unbiased tissue sampling and counting procedure
(see sections 2.5 and 2.6), we established the baseline in the ICC as 18500 ± 1620 labelled cells/mm³ (mean ± standard error (rounded to the nearest ten) of counts from 5 animals; Fig. 13A and 13D; Table 3). This increased significantly (p < 0.0001, t test after single factor, treatment, ANOVA) to 31020 ± 1730 labelled cells/mm³ in DNLL LESION rats at 30 minutes post-KA (n=5; Fig. 13B and 13E). At the same experimental time point, the labelled cell density of 14690 ± 1490 cells/mm³ in FINASTERIDE + DNLL LESION rats (n=5) was not significantly different from baseline (experiment-wise error rate of 0.05; Fig. 13C and 13F).

Figure 13. The Number of Neurosteroid-Immunoreactive Cells in the ICC Increased Following Deactivation of the Contralateral DNLL. (A-C) Corresponding regions of the ICC (400X) from one normal, one DNLL LESION and one FINASTERIDE + DNLL LESION animal (scale bar = 50 μm). Darkly labelled cells are positive for allopregnanolone/alloTHDOC. (D-F) In addition, the entire extent of the inferior colliculus (25X) in parasagittal sections, with the region shown in A-C indicated by 'X' (scale bar = 500 μm). The number of labelled cells in the ICC was significantly greater 30 minutes after the KA injection (B and E) into the contralateral DNLL than at baseline (A and D).
Finasteride prevented the effect observed in DNLL LESION animals at 30 minutes post-KA (C and F).

5.5 Neurosteroid Immunoreactivity in the SNR

The areal extent of the increase in 5α-reduced steroids in DNLL LESION animals was investigated by assaying another midbrain area, the substantia nigra pars reticulata (SNR). Cell bodies and large dendrites of neurons were labelled in the SNR, but not glia. The immunoreactive cell density of the SNR at baseline (11650 ± 1220 labelled cells/mm³; Fig. 14A and 14D) did not differ from that at 30 minutes post-KA in DNLL LESION (11210 ± 1200 cells/mm³; Fig. 14B and 14E) or FINASTERIDE + DNLL LESION (10880 ± 980 cells/mm³; Fig. 14C and 14F) rats (p > 0.05, single factor, treatment, ANOVA).

Figure 14. The Number of Neurosteroid Immunoreactive Cells in the SNR Was the Same Under All Treatment Conditions. (A-C) Corresponding regions of the SNR (400X) from one normal, one DNLL LESION and one FINASTERIDE + DNLL LESION animal (scale
Darkly labelled cells are positive for allopregnanolone/alloTHDOC. (D-F) In addition, the entire extent of the substantia nigra (25X) in parasagittal sections, with the region shown in A-C indicated by 'X' (scale bar = 500 μm). There was no difference between the number of labelled cells in the SNR of normal (A and D), DNLL LESION (B and E) and FINASTERIDE + DNLL LESION (C and F) animals.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>ICC cells/mm³</th>
<th>SNR cells/mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18500 ± 1620</td>
<td>11650 ± 1220</td>
</tr>
<tr>
<td>DNLL LESION</td>
<td>31020 ± 1730</td>
<td>11210 ± 1200</td>
</tr>
<tr>
<td>FINASTERIDE + DNLL LESION</td>
<td>14690 ± 1490</td>
<td>10880 ± 980</td>
</tr>
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Table 3. Summary of the ICC and SNR Immunoassays. Tabled values are the number of allopregnanolone/alloTHDOC immunoreactive cells per mm³ ± standard error.

5.6 Independent Confirmation of Local Regulation of Neurosteroids in the ICC

As a check against observer bias, the counting procedure was repeated independently by an experienced histologist (see section 2.6). Inter-rater reliability was 0.96 (Pearson's r) and the statistically significant difference (ICC: normal vs DNLL LESION group) was confirmed. It can be concluded that the assay with anti-allopregnanolone/alloTHDOC antisera showed an increased proportion of labelled neurons in the ICC about 20 minutes after the lesion of the contralateral DNLL (kainic acid generally took 10 minutes to deactivate the DNLL) and that this effect was blocked by the prior systemic administration of finasteride. Under all three treatment conditions,
the proportion of labelled neurons within the SNR (contained mainly within
the same sections as the ICC) did not change.
6 RESULTS

Neurosteroid Distribution Across Brain Areas: Immunohistochemistry

6.1 Neurosteroid Distribution Outline

Allopregnanolone/alloTHDOC immunoreactivity was widely distributed across the rat brain (Table 4). The cell body and thick dendrites of many neurons were immunolabelled (see section 7.12 for discussion of immunoreactive neuronal types). Although many of these cells were spinous, the spines themselves did not stain. For instance, pyramidal cells of the cerebral cortex and hippocampus, and the medium sized spiny neurons of the striatum. Glial-like labelling was not observed. To enable a simple comparison between brain regions, the regional density of labelled cells and processes was classified as very low, low, moderate, high or very high. Reference to brain areas accords with the atlas of Paxinos and Watson (1998), unless otherwise defined or further specified. Descriptions of the cellular distribution of allopregnanolone/alloTHDOC focus on brain areas from a systems perspective. Areas with either dense or sparse staining are highlighted.
6.2 Cerebral Cortex

Layers II-VI of isocortex and the transition zone between iso- and allocortex (allocortical areas are addressed in detail in sections below) exhibited a near uniform high density of labelled cell bodies and processes; there was very low density staining in layer I (Fig. 15). Pyramidal neurons constituted a large proportion of immunoreactive cells, with prominent labelling of pyramidal neurons continuing into allocortical regions (Fig. 16A). The labelling of the large apical dendrites of layer V pyramidal was visible into more superficial layers (Fig. 16B). The density of labelled somata in layer IV of sensory areas, which receives the majority of subcortical input, varied in different animals from low to high. Investigation of the determinant of the variability included the effect of swim-stress, different anaesthetic regimens, gender comparisons, manipulation of post-fixation protocols and tissue sectioning methods. No correlations were apparent.
(Previous page) **Figure 15. Layers II-VI of Sensory and Motor Areas Densely Stained.**

Cortical areas are marked out by arrowheads, and cortical layers are indicated by roman numerals (bregma -2.5 mm). M1, primary motor cortex; M2, secondary motor cortex; S1Tr, trunk representation in primary somatosensory cortex (S1); S1DZ, dysgranular zone; S1BF, barrel field.

**Figure 16. Cell Bodies and Apical Dendrites of Pyramidal Neurons Labelled in Isocortex, Allocortex and Transition Regions.** (A) Prominent pyramidal cell labelling in the agranular insular cortex continued in layers II and III of the allocortical piriform cortex (bregma -0.7 mm; layers according to Haberly and Price (1978)). Ins, insular cortex; Pir, piriform cortex. (B) Apical dendrites of pyramidal cells in layer V of primary somatosensory cortex stained along their extension into supragranular layers (scale bar = 50 μm).

**6.3 Thalamus**

All nuclei comprising the thalamus appeared moderately stained with the few exceptions highlighted below. In the anterior lobe, the anterodorsal thalamic nucleus showed a high density of stained cells, so too the
paraventricular nucleus from anterior through posterior, amongst the midline and intralaminar groups. The density of staining was between moderate and high in the reticular nucleus, in its full extent across the rostral and lateral thalamus, and in the lateral nuclei, suggested to be the rat equivalent of the primate pulvinar (Takahashi, 1985; Fig. 17A).

Figure 17. Sensorimotor Thalamic Areas Moderately Labelled, with Denser Staining in the Lateral and Reticular Nuclei. (A) LP, lateral posterior nucleus; LD, laterodorsal nucleus; Po, posterior nuclear group; Rt, reticular nucleus; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus (lateral 2.5 mm approximately; scale bar = 500 μm). (B) DLG, dorsal lateral geniculate; VLG, ventral lateral geniculate (bregma -3.8 mm; scale bar = 250 μm).

With the exceptions addressed, it was noted that immunolabelled cells were found in all of the sensory areas, including the ventroposterior nuclei, medial and lateral divisions, the posterior nucleus, gustatory/visceral nucleus, medial geniculate, all divisions, and lateral geniculate, dorsal and
ventral (Fig. 17A and 17B). The ventrolateral and ventromedial nuclei, relating to motor function, also stained.

6.4 Hypothalamus

The periventricular zone of the hypothalamus, wherein lies the majority of neurons containing hormone-releasing hormones (Markakis, 2002), generally stained more densely than the moderately immunoreactive medial and lateral zones, the magnocellular preoptic nucleus (possibly the rat homologue of the basal nucleus of Meynert) of the lateral zone excepted. In particular, the periventricular nucleus, supraoptic nucleus (considered a displaced portion of the periventricular nucleus), paraventricular nucleus and arcuate nucleus had numerous labelled cells (Fig. 18). The mammillary region of the medial and lateral zones appeared weaker stained than other regions.

Figure 18. Numerous Neuroendocrine Cells of the Periventricular Zone Stained.
3V, 3rd ventricle; AH, anterior hypothalamic area; Arc, arcuate nucleus; f, fornix; LH, lateral hypothalamic area; Pa, paraventricular nucleus; Pe, periventricular nucleus; TC, tuber cinereum; VMH, ventromedial hypothalamic nucleus (bregma -2.1 mm).
6.5 Hippocampal Formation

6.5.1 Entorhinal Cortex

Immunolabelling occurred in the superficial layers (I-III) that provide input to the dentate gyrus (also to the hippocampal fields and subiculum; Dolorfo and Amaral, 1998) and the deep layers (IV-VI) that are a source of output from the hippocampal formation to cortical and subcortical areas (Insausti et al., 1997). With reference to the lamination scheme applied by Insausti et al. (1997), the essentially cell-free layer I showed very low staining of processes extended from layer II neurons. A high density of stained cell bodies and dendrites was present in layers II and III, from which the perforant pathway mainly originates. There was high density labelling of processes that traversed the acellular layer IV, the lamina dissecans. Layers V and VI contained a high density of stained somata and dendrites.

6.5.2 Dentate Gyrus

Granule cells are the principal neurons of the dentate gyrus and their axons, the mossy fibres, terminate in the CA3 field (CA, cornu ammonis; Claiborne et al., 1986). Very high numbers of granule cell somata were labelled (Fig. 19A), and their processes were stained throughout the
molecular layer. Labelled cell bodies in the molecular layer were rare. A few mossy cells and their dendrites stained in the polymorphic layer.

6.5.3 CA3, CA2 and CA1 Fields

CA3 pyramidal neurons innervate the CA3, CA2 and CA1 fields (Ishizuka et al., 1990), and CA1 pyramidal cells project to the deep layers of the entorhinal cortex and subiculum (Swanson and Cowan, 1977). There was a very high proportion of pyramidal cells stained in all fields (Fig. 19A). Although basal dendrites represent a substantial fraction of the dendritic tree, apical processes were more densely stained. Labelling of the apical dendritic extension occurred from the cell body to the hippocampal fissure (Fig. 19B). A very low number of cell bodies were stained in the stratum oriens and stratum radiatum.

Figure 19. A Very High Proportion of Granule and Pyramidal Cells Stained in the Dentate Gyrus and Hippocampal Fields. (A) Somatic labelling was essentially
confined to the principal cell layers. DG, dentate gyrus; Gr, granular layer; HiF, hippocampal fissure; Mo, molecular layer; Or, stratum oriens; Po, polymorphic layer; Py, pyramidal cell layer; Ra, stratum radiatum (bregma -3.8 mm; scale bar = 500 μm). (B) Apical dendrites of pyramidal cells, shown here in CA1, labelled across their full extent (scale bar = 50 μm).

6.5.4 Subiculum

The principal cell layer of the subiculum, which provides a major output from the hippocampal formation (Witter et al., 1990), stained very densely. Staining was continuous with the labelled pyramidal neurons of the CA1 field through the widening of that layer into the subiculum. Apical dendrites of pyramidal cells were labelled across their extension through the molecular cell layer, which had a very low density of immunoreactive cell bodies.

6.6 Basal Forebrain

6.6.1 Basal Ganglia

A high number of cell bodies and thick processes were found to be labelled in the caudate-putamen (CPu; Fig. 20A). The dense staining of the nucleus accumbens was continuous with the CPu. In the olfactory tubercle, a very high proportion of the striatal-like medium sized cells in
the dense/principal cell layer labelled, with some stained processes extended into the superficial plexiform and deep layers (Fig. 20B).

![Image](image_url)

**Figure 20. The Dorsal and Ventral Striatum Densely Stained.** (A) Many cells labelled in the caudate-putamen, spaced by fibre bundles of the internal capsule (lateral 2.5 mm approximately; scale bar = 100 μm). (B) In the olfactory tubercle, staining concentrated in the principal cell layer. DL, deep layer; PCL, principal cell layer; PL, plexiform layer (lateral 2.5 mm approximately; scale bar = 50).

In comparison with the striatum, the labelling density was less – classed as moderate – in the globus pallidus and ventral pallidum. The entopeduncular nucleus, also of the pallidal complex, contained only a few labelled cells. In addition, labelled cells were found across the substantia nigra-ventral tegmental area. The labelling of processes highlighted the anterior-posterior alignment of dendritic fields in the substantia nigra pars reticulata (Fig. 14 in chapter 5).
6.6.2 Amygdala-Extended Amygdala

Although there was some variation in the density of cell staining across the amygdala-extended amygdala, moderate density labelling predominated (Fig. 21). The medial amygdaloid nucleus tended to be more densely stained amongst the subdivisions of the extended amygdala, and the same can be said for the basolateral nucleus amongst the cortical-like nuclei of the amygdala. Of the latter, the nucleus of the lateral olfactory tract had a very high number of immunoreactive pyramidal-like cells in layer 2. Labelling of their pial-directed apical dendrites sometimes continued into layer 1.

Figure 21. Moderate Density Labelling Predominated in the Amygdala and Extended Amygdala. Extended amygdala: Ce, central amygdaloid nucleus; Me, medial amygdaloid nucleus. Cortical-like nuclei: BL, basolateral amygdaloid nucleus; Co, cortical amygdaloid nucleus; La, lateral amygdaloid nucleus. Surround: CPu, caudate-putamen; op, optic tract; st, stria terminalis (A: bregma -3.3 mm, scale bar = 0.5 mm; B: bregma -3.6 mm).
6.6.3 Septum

There was a moderate density of stained cells across the septum, appearing higher in the dorsal part of the lateral septal division and lower in the septofimbrial nucleus, if not the whole posterior septal division (Fig. 22). The somata and thick processes of septal cells were labelled.

![Figure 22. The Septum Moderately Stained](image)

ac, anterior commissure; LSD, lateral septal division, dorsal; LSI, lateral septal division, intermediate; MS, medial septal nucleus; VDB, nucleus of the diagonal band, vertical limb (bregma 0.2 mm; scale bar = 0.5 mm).

6.6.4 Olfactory Bulb

The main and accessory olfactory bulb (MOB and AOB respectively) stained densely. In the MOB, glomeruli were clearly outlined by labelled cells, and the glomerular interior contained highly stained neuropil. Further into the MOB, the external plexiform layer (EPL) showed many labelled processes but only a few labelled cells. The mitral cell layer at the base of the EPL had a very high number of stained somata, with the labelling of the apical dendrite of mitral cells highlighting the dendritic extension to
glomeruli (Fig. 23). Deeper still, the granule cell layer was densely stained. In relation to the AOB, the periglomerular cells, mitral/polymorphic cells and granule cells all stained.

![Figure 23. The Main Olfactory Bulb Was Densely Stained, Including the Principal Input and Output Layers.](image)
The ovoid glomeruli, where the axons of olfactory receptor neurons converge, contained highly immunoreactive neuropil surrounded by a shell of stained small neurons. Most somata were labelled in the thin layer of the mitral cells, which provide the chief output from the bulb (Shipley et al., 1995).

GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer (scale bar = 100 μm).

6.7 Midbrain

6.7.1 Tectum

Immunoreactivity in the superior colliculus was greatest in the moderately stained superficial grey and upper optic layers, in which the projection from the retina terminates (Harting et al., 1973 (tree shrew); Stein, 1981; in
the rat, direct input from the retina to the intermediate grey has also been reported (Beckstead and Frankfurter, 1983)). Cell bodies and thick dendrites in these layers were neurosteroid-positive. The labelling ventral to the optic layer included some large cells (Fig. 24).

Immunolabelled cells were found across the inferior colliculus (IC). However, the density of stained cells appeared to be higher in the dorsal and external cortex than the moderately stained central nucleus (Fig. 13 in chapter 5). Of note, principal/disc-shaped/flat cells were predominantly labelled in the central nucleus, and some of the large multipolar cells in layer 3 of the external cortex stained. The nuclei of the lateral lemniscus, which provide input to the IC, were moderately immunoreactive.

Figure 24. Labelling in the Superior Colliculus Concentrated in the Superficial Grey and Upper Optic Layers. In, intermediate layers; Op, optic layer; Su, superficial grey; Zo, zonal layer (lateral 1.5 mm approx.; caudal scale bar = 500 μm).
6.7.2 Periaqueductal and Ventral Midbrain

Moderate immunolabelling of cells occurred in the periaqueductal grey (PAG). The slight gradient in the density of immunostained cells across the PAG corresponded with the packing density of cells within the PAG, with the lowest density medially and the highest density peripherally, especially dorsally.

Large neurons and their dendrites were immunolabelled in the red nucleus. These immunoreactive cells were located caudally, where the rubrospinal tract predominantly arises (Gwyn, 1971; Brown, 1974). In the midbrain reticular formation, there was a low density of staining overall, with more labelling in the retrorubral field than the deep mesencephalic tegmentum.

6.8 Cerebellum

The cell body and thick branches of the dendritic tree of a very high proportion of Purkinje cells labelled (close to 100%; Fig. 25). Amongst the very densely stained network of processes in the molecular layer, labelled cell bodies were rare. The granular layer of the cerebellar cortex and the rostral and caudal groups of the cerebellar nuclei were moderately stained.
6.9 Pons-Medulla

The staining density across the pons and medulla ranged from low in the pontine and medullary reticular formation to moderate in the parabrachial nucleus, cochlear nuclei, oromotor nuclei, vestibular nuclei and the precerebellar pontine nuclei (Fig. 26). Very large neurons constituted a sizeable proportion of labelled cells in the vestibular nuclei, including the prominently stained multipolar giant cells in the lateral vestibular nucleus. Mainly large multipolar neurons stained in the motor trigeminal nucleus.
SpVe, spinal vestibular nucleus; SuVe, superior vestibular nucleus (lateral 2 mm approximately).

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**Table 4. 3α,5α-Reduced Steroid Immunoreactivity Summary Table.** The density (■ 1 unit; □ ½ unit) of immunoreactive cell bodies and processes was evaluated in areas across the brain. The staining of cell bodies ranged from rare (■) to dense (■■■■■) and the labelling of processes varied from little visible extension (■) to a dense network (■■■■■). Nuclei that have a similar quality and quantity of immunoreactivity and that are functionally related have been grouped. * The density of labelled cell bodies varied from ■■ to ■■■■ in layer IV of sensory areas of the cerebral cortex (see section 6.2). – Essentially acellular layer.
7 DISCUSSION

7.1 Inhibitory Gain Control in the Auditory Midbrain

Increased neuronal excitability within the ICC, resulting from the deactivation of the inhibitory input from the contralateral DNLL, induced a reduction in ICC excitability in animals that had not received finasteride (sections 3.3 and 4.2). This was evident, after a delay of around 20 minutes, as increased first-spike latency and reduced spike counts for single-neuron recordings, and increased latency and decreased amplitude of local EP recordings, in response to the presentation of sounds delivered to the contralateral ear. Since the DNLL manipulation affected only ipsilateral-ear-driven inhibition within the ICC, we interpret the subsequent reduction in ICC excitability as an increase in the efficacy of intrinsic inhibitory circuits within the ICC (which can be stimulated via the contralateral ear), in response to the initial increased ICC excitability. We then sought a mechanism for this effect.

7.2 Neurosteroid-Mediated Inhibitory Gain Control

The 5α-reduced neurosteroids reduce neuronal excitability by potentiating the action of GABA at GABA-A receptors (Majewska et al., 1986; review: Lambert et al., 2001). Finasteride was used to block 5α-reduced
neurosteroid synthesis (sections 3.5 and 4.3). This treatment prevented the reduction in ICC excitability observed in DNLL LESION animals following deactivation of the contralateral DNLL. Moreover, ICC neurons showed unusual excitation to sounds delivered to the ipsilateral ear (the source of the ipsilateral ear-evoked ICC excitation could include the ipsilateral superior olivary nuclei or the contralateral ICC (Irvine, 1986)), and elevated spontaneous activity following contralateral DNLL deactivation in conjunction with blockade of neurosteroid synthesis, at a time-period corresponding to the onset and greatest influence of neurosteroid action in DNLL LESION animals. Thus it can be inferred that, in response to loss of the input from the contralateral DNLL, the remaining GABA-ergic inhibition in the ICC was potentiated as a result of increased 5α-reduced neurosteroid synthesis, limiting the increases in excitability to sound stimulation (both contralateral and ipsilateral) and spontaneous activity. Consistent with this interpretation was a significant increase (relative to baseline and FINASTERIDE + DNLL LESION animals) in the number of neurosteroid-immunoreactive cells within the ICC after deactivation of the contralateral DNLL (section 5.4).

7.3 Local Regulation of Neurosteroid Concentration

5α-reduced steroid levels in the brain persist and can be augmented after removal of peripheral sources (Corpechot et al., 1993; Cheney et al., 1995), establishing that 5α-reduced steroids are synthesised in the brain. Following DNLL deactivation, the increased immunoreactivity in the ICC
but not the SNR suggests that regional regulation of the level of 5α-reduced steroids is also possible (sections 5.4 and 5.5). Our experiments, nonetheless, do not directly differentiate between a local or peripheral site of 5α-reduction, in as much as peripheral sources of 5α-reduced steroids were not removed. Peripheral sources were left intact in order to determine the effect of 5α-reduced steroids under normal conditions (see section 7.10). However, the interpretation that 5α-reduction occurred in the ICC is supported by the fact that the trigger for increased synthesis of 5α-reduced steroids was a local decrease in inhibition. There was no direct influence on the hypothalamic-pituitary-adrenal axis to increase peripheral production of 5α-reduced steroids. Local 5α-reduction does not rule out the supply of the immediate precursors of 5α-reduced steroids from the circulation. Furthermore, the demonstration that the increase in immunoreactivity for allopregnanolone/alloTHDOC was restricted to the affected pathway (ICC) and not general (comparison to SNR) suggests local brain synthesis of 5α-reduced steroids rather than systemic delivery.

7.4 Neurosteroid Action Through GABA-A Receptor Modulation

Pharmacological enhancement of GABA-A receptor-mediated inhibition has generally been shown to increase the latency and decrease the rate of sound-evoked action potentials of ICC neurons (Faingold et al., 1991; Vater et al., 1992). Similarly, evoked potentials (EPs) have increased latency and reduced amplitude under conditions of increased inhibition of the ICC (Bagri et al., 1989; Szczepaniak and Moller, 1996). It is consistent,
therefore, that 5α-reduced neurosteroids, as positive allosteric modulators of the GABA-A receptor, should produce corresponding electrophysiological changes within the ICC. Preliminary results from our laboratory suggest a reduction in auditory midbrain excitability with allopregnanolone administration. In addition, GABA-A receptor subunit combinations conferring neurosteroid sensitivity are known to be localised within the ICC (for example, α1β2γ2 subunits: Wisden et al., 1992).

7.5 Neurosteroid Effects on Different Cell Classes

We found a consistent pattern of response change with our two recording methods: single-neuron and EP. The local EP arises from the activity of neurons in the vicinity of the recording electrode. As only a subset of ICC neurons receives inhibitory input from the contralateral DNLL (review: Pollak et al., 2003), only these ICC EI neurons will be immediately affected by DNLL deactivation. Therefore the resultant ICC EP, reflecting the contribution of neurons whose excitability is raised as a direct result of deactivation of the contralateral DNLL, as well as that of neurons unaffected by the experimental manipulation, shows a less prominent increase in amplitude (and reduction in latency) than one might predict from the ICC single-neuron recordings which targeted the EI class of neuron (EPs shown in Figs. 9, 10 and 11 were recorded to contralateral ear stimulation, so any increase in ICC excitability reflects a reduction in tonic inhibition only). However, the marked reduction in ICC excitability observed during EP recordings, following the immediate effect of
deactivation of the contralateral DNLL, suggests that the action of neurosteroids may not be limited to formerly EI neurons.

7.6 Previous Manipulations of Inhibition in the Auditory Midbrain

The initial investigations that contributed to our understanding of how ICC neuronal responses are influenced by the contralateral DNLL involved single-ICC neuron recordings made during reversible DNLL deactivations (Li and Kelly, 1992; Faingold et al., 1993). These studies showed raised ICC excitability upon deactivation of the contralateral DNLL, corresponding to the initial effects post-KA in our experiments. However, no counteracting plasticity was reported. This can be accounted for by considering the time course of the reversible DNLL deactivations (see below). Our single-neuron recordings showed ICC neuronal excitability (after the initial increase) started to decrease by 20 to 50 minutes post-KA, reaching a minimum between 30 to 90 minutes post-KA. Faingold et al. (1993) effected reversible unilateral DNLL deactivation with lidocaine or 4,5,6,7-tetrahydroisoxazolo-(5,4-c)pyridin-3-ol, with recovery to baseline ICC responses occurring within 20 minutes. So ICC inhibition returned to its usual level in the Faingold et al. experiments, removing the trigger for increased neurosteroid synthesis, before neurosteroid action had become evident in our experiments. Li and Kelly (1992) used kynurenic acid in the contralateral DNLL to suppress ICC inhibition activated by the ipsilateral ear, with partial recovery after 30 minutes and full recovery to ICC baseline responses after 45 to 60 minutes. If neurosteroid levels were raised, any
neurosteroid action would overlap DNLL recovery and easily be interpreted as such. This is a possibility as there was complete cessation of tone-evoked DNLL activity for 45 to 60 minutes, yet ICC inhibition was on the rise by 30 minutes. Note that ICC EP recordings, that highlighted the plasticity counteracting the initial effect of permanent deactivation of the contralateral DNLL, have not been made during reversible DNLL deactivations.

7.7 Inhibitory Gain Control in Complex Neural Circuits

The experiments on the effects of contralateral DNLL lesions on the inferior colliculus demonstrated a form of inhibitory gain control which was blocked by finasteride treatment. In DNLL LESION animals, there was sufficient gain of contralateral ear-activated and tonic inhibition to return contralateral ear-evoked spike counts to around baseline and to elevate latency. The adjusted level of tonic inhibition suppressed the high spontaneous activity and strong ipsilateral ear-evoked excitation that otherwise manifested in FINASTERIDE + DNLL LESION animals. The ipsilateral ear-evoked excitation, which was not reported in the reversible DNLL deactivation studies (Li and Kelly, 1992; Faingold et al., 1993; see section 7.6), may have been ‘unmasked’ with the block of counteracting plasticity by finasteride after DNLL lesion. However, LTP has been demonstrated in the ICC under conditions of reduced inhibition (Hosomi et al., 1995; Zhang and Wu, 2000; Wu et al., 2002; see section 1.4.2), and such conditions would result from the combined effect of contralateral
DNLL lesion and finasteride. Regarding the studies of Hosomi et al. (1995), Zhang and Wu (2000) and Wu et al. (2002), LTP was apparent 10 minutes after inducing stimuli (tetanic stimulation of the LL, usually at 50 Hz for 20 seconds) and was maximal by 20 minutes. In our FINASTERIDE + DNLL LESION experiments, the high spontaneous activity, ipsilateral ear-evoked excitation and reduced firing latency which were recorded from the ICC by 30 minutes post-kainate (effectively 20 minutes after complete deactivation of the contralateral DNLL; Figs. 5F, 6B, 7B and 8) may represent the expression of LTP at brainstem synaptic inputs.

7.8 Neurosteroids in the Context of Known Forms of Inhibitory Plasticity

In this thesis, the data have been interpreted according to a model of inhibitory synaptic plasticity in which certain neurosteroids increase the effectiveness of GABA-ergic inhibition in response to increased excitability. An alternative interpretation of some of the results could be that GABA-ergic inhibition is enhanced by an increase in the number of GABA-A receptors. However, translocation of GABA-A receptors from a cytoplasmic to a post-synaptic membrane locus, or de novo synthesis, requires a time-course of more than one hour (Wan et al., 1997; Nusser et al., 1998). Until this occurs, up-regulation of GABA-A receptor-mediated inhibition would have to be provided by an increase in GABA-evoked chloride conductance, as pre-synaptic GABA release saturates post-synaptic GABA-A receptors (Edwards et al., 1990; De Koninck and Mody,
1994). As neurosteroid concentrations can be raised within minutes, and the effect of neurosteroids can be switched off by their metabolic transformation, 5α-reduced neurosteroids provide the rapid and flexible modulation of GABA-ergic inhibition required to explain our results.

7.9 Biochemical Events Leading to Increased Neurosteroid Synthesis

The sequence of biochemical events leading from reduced GABA-ergic inhibition to increased neurosteroid synthesis is not fully understood. However, elevated cAMP (adenosine 3',5'-cyclic monophosphate) levels (cortex: Barbaccia et al., 1992; retina: Guarneri et al., 1994) and calcium (retina: Guarneri et al., 1998; hippocampus: Kimoto et al., 2001) have been shown to increase neurosteroid synthesis. Increased ICC excitability could lead to increases in the intracellular levels of cAMP and calcium through reduced GABA-B receptor and increased ionotropic glutamate receptor activation, respectively. cAMP and calcium activate protein kinases which in turn directly activate steroid acute regulatory protein (StAR; Arakane et al., 1997) or act indirectly via phosphorylation of steroidogenic factor-1 (SF-1), which binds to the StAR promoter (Sugawara et al., 2000). StAR, in co-operation with diazepam binding inhibitor (DBI) and the mitochondrial benzodiazepine receptor, facilitates cholesterol transport to the mitochondrial cytochrome P450 side chain cleavage enzyme (West et al., 2001; Bose et al., 2002). Cholesterol is converted to pregnenolone by side chain cleavage (review: Warner and
Gustafsson, 1995), the putative rate-limiting step in neurosteroid synthesis. More neurosteroid precursors, whether from central or peripheral sources, would lead to increased production of 5α-reduced neurosteroids. As cycloheximide (protein synthesis inhibitor) has been shown to attenuate enhanced neurosteroid synthesis (Roscetti et al., 1994), increased synthesis of neurosteroid synthetic enzymes or associated regulatory factors (for example, StAR, SF-1, DBI) may be required for sustaining elevated neurosteroid levels.

7.10 Central and Peripheral Contributions to Steroid Levels in the Brain

A large number of cells throughout the brain, from the medulla through to the cerebral cortex, stained positively for 5α-reduced neurosteroids (chapter 6). However, there were regional differences in the density of staining, being high in the olfactory bulb, striatum and cerebral cortex and low in the brainstem reticular formation. We did not remove peripheral sources of 5α-reduced neurosteroids in our animals because we were interested in the normal level and distribution of these compounds in the brain. The peripheral contribution to brain allopregnanolone has been reported at approximately 30% (Cheney et al., 1995). Nevertheless, the heterogenous regional distribution of 5α-reduced neurosteroids in the brain is unlikely attributable to circulating steroids, as systemically administered 5α-reduced neurosteroids and their precursors distribute uniformly through the brain (Cheney et al., 1995). This suggests that the
observed staining pattern, that is the relative staining between areas, is essentially derived from 5α-reduced neurosteroids that were synthesised in the brain, although the absolute level is partly attributable to peripheral sources.

7.11 Basal Level of Neurosteroids

We anaesthetised rats with ketamine (100 mg/kg im) and acepromazine (10 mg/kg im) prior to paraformaldehyde perfusion and removal of the brain for immunohistochemistry. Ketamine was selected to induce anaesthesia as it has less effect on the brain content of steroids than pentobarbital, clonazepam, ethanol, chloral hydrate and urethane (Korneyev et al., 1993a). Korneyev et al. (1993a) reported that ketamine (160 mg/kg ip), at a higher dose than we employed, does not alter pregnenolone levels but reduces progesterone concentration to a small degree. This influence on a precursor of 5α-reduced steroids might, if anything, lead to a reduction of allopregnanolone and alloTHDOC in the brain. Furthermore, activation of NMDA receptors has been shown to increase neurosteroid synthesis (Kimoto et al., 2001). As ketamine is an NMDA receptor antagonist, it is unlikely that our anaesthetic regimen elevated neurosteroid levels. There have been no reports of acepromazine affecting brain steroids.

In addition, a number of measures were taken to minimise the possibility of stress-induced steroidogenesis prior to euthanasia. Firstly, in order to
habituate animals to the type of handling involved with administering the anaesthetic agents, regular handling occurred prior to the day of experiments. Secondly, the potentially better tolerated intraperitoneal route of anaesthetic administration was used in a series of experiments instead of intramuscular injections. However, there was little/no change in neurosteroid distribution. Finally, maintaining anaesthesia for an extended period of time prior to perfusion (greater than two hours), for the purpose of allowing steroid levels to return to baseline, produced similar immunohistochemical staining to that seen in our standard protocol (section 2.5). This suggests that the level of neurosteroids that we determined immunohistochemically represents basal conditions.

7.12 Neurosteroids Are Preferentially Located in Particular Cell Types

The majority of allopregnanolone/alloTHDOC-positive cells had the position, morphology and size of putative excitatory neurons. For example, pyramidal-like cells of the cerebral cortex and hippocampus, thalamic sensory relay neurons (see section 7.18 for discussion of the ventroposterior thalamic nuclei) and disc-shaped/flat cells of the ICC. In contrast, putative local circuit GABA-ergic neurons did not appear to be labelled in most brain areas. This suggests that mainly excitatory neurons use neurosteroids to regulate inhibition. Nevertheless, GABA-ergic neurons providing long-range projections were immunoreactive, such as cerebellar Purkinje cells, reticular thalamic neurons, striatal cells and SNR.
neurons. In principle, this allows for control over long-range modulatory influences without affecting local inhibitory networks. Putative glia, identified by nuclear confinement of Nissl stain and typical glial morphological features, were not found to be immunolabelled.

7.13 Neurons as a Site of Neurosteroid Synthesis

Whereas it was once assumed that steroidogenesis in the CNS was effected by glial cells, there is increasing evidence that neurons play a large role. Neuronal synthesis of allopregnanolone/alloTHDOC from cholesterol or nearer precursor is suggested by knowledge that allopregnanolone/alloTHDOC is synthesised in the brain (see section 7.10) and that only neurons were allopregnanolone/alloTHDOC-immunoreactive. The enzymes required for the de novo synthesis of 5α-reduced neurosteroids have been demonstrated in neurons (cerebellum: Tsutsui et al., 2003; somatosensory/pain pathway: Patte-Mensah et al., 2003). Although allopregnanolone/alloTHDOC-immunoreactive glia were not observed, it is possible that precursors of allopregnanolone/alloTHDOC could be synthesised in glia, which contain the relevant enzymatic machinery (Jung-Testas et al., 1989; Patte-Mensah et al., 2003), serving as a source of substrates for neurons. However, double labelling experiments for 3α,5α-reduced steroids and glial fibrillary acidic protein are needed to further clarify the role of glia in neurosteroidogenesis. The neuronal allopregnanolone/alloTHDOC content can also derive from uptake of circulating allopregnanolone/alloTHDOC or precursor. This may
be facilitated by the lipophilicity of the steroids and, more specifically, mediated by organic anion transport proteins (Kullak-Ublick et al., 1998; review: Hagenbuch and Meier, 2003).

7.14 Intracellular Localisation of Neurosteroids

The immunostaining of cell bodies and thick dendrites places allopregnanolone/alloTHDOC in membranes and within aqueous intracellular space. The latter was unexpected considering the lipophilic nature of the steroids. Insight into the staining pattern is provided by the intracellular locations of the different neurosteroid synthetic steps. Cholesterol is metabolised to pregnenolone in mitochondria (review: Strauss et al., 2003). The lack of labelling of dendritic spines may reflect the rarity of mitochondria therein (Fiala et al., 2002). Progesterone and deoxycorticosterone production (from pregnenolone) and their 5α-reduction occurs in the smooth endoplasmic reticulum (SER; De Larminat and Blaquier, 1978; Li et al., 1997; Ishimura and Fujita, 1997). The additional presence of 3α-hydroxysteroid dehydrogenase (3αHSD), the enzyme that interconverts the dihydro and tetrahydro derivatives of progesterone/deoxycorticosterone, associates allopregnanolone/alloTHDOC and/or their precursors with the SER, which is extensive throughout the cytoplasm (Spacek and Harris, 1997). However, there is also a cytosolic fraction of 3αHSD. Cytosolic 3αHSD seems to preferentially operate in the reductive direction in vivo, while particulate 3αHSD tends to work oxidatively (Li et al., 1997). A predominantly
cytosolic site for the formation of allopregnanolone/alloTHDOC requires their solubilisation. Increased water solubility of steroids is achievable through chemical transformation, for example sulfation. With effective compartmentalisation of the intracellular space, diffusion of water-soluble steroids may be restricted. If the commercial primary antibody that we used for the immunoassays detected allopregnanolone/alloTHDOC and a water-soluble derivative(s), this would be consistent with the observed staining pattern, with the absence of immunoreactivity in thin dendrites and spines. Another steroid solubilisation method involves binding proteins. Examples of intracellular proteins that bind neurosteroids include unnamed 57 (Bukusoglu and Krieger, 1994), 60 (Darbandi-Tonkabon et al., 2003) and 64 (Bukusoglu and Krieger, 1994) kD proteins, microtubule-associated protein 2 (Murakami et al., 2000), voltage dependent anion channel proteins (McEnery et al., 1993; Darbandi-Tonkabon et al., 2003), progesterone receptor (Labombarda et al., 2003), 25-Dx (Labombarda et al., 2003), and GABA-A receptor subunits.

7.15 Distribution of Neurosteroids Across the Brain

Using gas chromatography-mass fragmentography (GC-MF) or radioimmunoassay (RIA), the level of allopregnanolone in rat brain is generally reported as highest in the olfactory bulb, then striatum, followed by the cerebral cortex, hippocampus and cerebellum (Cheney et al., 1995; includes brainstem: Uzunov et al., 1996; includes hypothalamus: Bernardi et al., 1998; includes amygdala: Uzunova et al., 2003); human data has
also been published (Bixo et al., 1997; Weill-Engerer et al., 2002). Our evaluation of the density of allopregnanolone/alloTHDOC-immunostained cells and processes in the areas above is consistent with the GC-MF and RIA results, however we observed that allopregnanolone/alloTHDOC is concentrated within the hippocampus (Fig. 19) and cerebellum (Fig. 25) in the pyramidal and Purkinje cell layers respectively.

Neurosteroid immunoreactivity presented at various stages of processing in each of the sensory pathways. Using the auditory system to illustrate, allopregnanolone/alloTHDOC-positive cells and processes were contained in the cochlear nuclei, superior olivary complex, nuclei of the lateral lemniscus, inferior colliculus, medial geniculate body and the auditory cortex. Immunolabelling was also found in the motor and motor-related systems, including the brainstem oromotor nuclei, pretectal nuclei, red nucleus, cerebellum, ventrolateral and ventromedial thalamic nuclei and the motor cortex. There was immunostaining in numerous limbic structures, such as the archicortex, mediodorsal thalamic nuclei and various interconnected divisions of the septum, amygdala and hypothalamus. In addition, the periventricular zone of the hypothalamus, parabrachial nucleus, periaqueductal grey, paraventricular thalamic nucleus and the insular and perirhinal cortex, that is, multiple levels of central autonomic control, showed immunoreactivity. Modulation of GABA-ergic inhibition in a number of these areas by endogenous 5α-reduced neurosteroids has already been demonstrated (cerebral cortex: Grobin
and Morrow, 2001; septum: Van Doren et al., 2000; colliculi: Disney and Calford, 2001; see chapters 3 and 4; spinal cord: Keller et al., 2004).

7.16 Correlations Between Distributions of Neurosteroids and Their Synthetic Enzymes

StAR protein facilitates the transport of cholesterol to P450scc, which converts cholesterol to pregnenolone (Bose et al., 2002; see section 1.2.3; Fig. 2). The cellular distribution of allopregnanolone/alloTHDOC is strongly correlated with that of StAR. StAR mRNA and protein are present in the olfactory bulb, medium spiny neurons of the striatum, cerebral cortex, pyramidal cells of the hippocampus, Purkinje cells of the cerebellar cortex and the periventricular zone of the hypothalamus (mRNA: Furukawa et al., 1998; protein: King et al., 2002). This pattern mirrors the neuronal populations that contain the highest concentrations of neurosteroid immunoreactivity. In relation to P450scc, early studies located the enzyme mainly in glial cells (Le Goascogne et al., 1987; Iwahashi et al., 1990). More recently however, Furukawa et al. (1998) reported that P450scc mRNA is present in hippocampal pyramidal cells and cerebellar Purkinje cells; but weak hybridisation signals prevented transcript detection elsewhere. P450scc protein has since been demonstrated in neurons of the cerebral cortex, cerebellum and hippocampus (Kimoto et al., 2001; King et al., 2002). Progesterone is synthesised from pregnenolone by 3β-hydroxysteroid dehydrogenase, which is found in neurons of the olfactory bulb, cerebral cortex, cerebellum, hippocampus and hypothalamus.
(Furukawa et al., 1998; Zwain and Yen, 1999). Co-localisation of StAR, P450scc and 3β-hydroxysteroid dehydrogenase has been shown in the same cerebellar Purkinje cells (Furukawa et al., 1998), and essentially all Purkinje cells were allopregnanolone/alloTHDOC-positive in our study (Fig. 25).

5α-reduction appears to occur principally in neurons (Melcangi et al., 1993), consistent with the labelling of neurons by the anti-allopregnanolone/alloTHDOC antisera. However, the distribution of neurosteroids contrasts that of the regional activities of 5α-reductase. For instance, the level of 5α-reduction is highest in the pons-medulla and lowest in the cerebral cortex (Celotti et al., 1992; Li et al., 1997), whereas the density of neurosteroid immunoreactivity was lowest in the brainstem and highest in the forebrain. On the other hand, 3α-hydroxysteroid oxidoreductase (also called 3α-hydroxysteroid dehydrogenase) activity across the rat brain parallels neurosteroid labelling, with the exception of the caudate nucleus (Krieger and Scott, 1984; Li et al., 1997). Yet 3α-reduction has been reported to take place primarily in glial cells (Krieger and Scott, 1989; Melcangi et al., 1993), at odds with the seeming absence of allopregnanolone/alloTHDOC immunoreactive glia but widespread neuronal labelling. Reviewing the study of Krieger and Scott (1989), 3α-reduction in the olfactory tubercle was undiminished following the destruction of neurons by kainic acid. The interpretation that neuronal 3α-reduction is minimal relies upon the death of all neurons, but the vulnerability of neurons to kainic acid is variable (Coyle and Schwarcz,
In relation to Melcangi et al. (1993), steroid synthesis was evaluated in a neuronal culture from foetal or neonatal brain, and this subset of neuronal types may lack 3α-hydroxysteroid oxidoreductase-containing neurons. Finally, DBI and allopregnanolone/alloTHDOC labelling presented in pyramidal cells of the cerebral cortex and hippocampus (Alho et al., 1985). Although the low level of DBI in the striatum and substantia nigra (Alho et al., 1985) did not correlate with the neurosteroid concentration.

7.17 Correlations Between Neurosteroid Distribution and GABA-ergic Innervation

Essentially all brain areas receive GABA-ergic input, and correlations are evident between the densities of GABA-ergic innervation and allopregnanolone/alloTHDOC-immunostaining. For instance, there was very high neurosteroid immunoreactivity in the olfactory bulb, where most of the granule and juxtaglomerular cells are GABA-ergic (Ribak et al., 1977), providing extensive intrabulbar inhibition (review: Shipley et al., 1995). For the most part, the pontine and medullary reticular fields have a low density of GABA-ergic cell bodies and axon terminals (the atlas of Mugnaini and Oertel, 1985), and this corresponds with the low numbers of neurosteroid-positive cells. GABA-ergic receptors and neurons in the superior colliculus are most dense in the superficial grey layer (Mize, 1992) as was allopregnanolone/alloTHDOC-labelling. The fact that allopregnanolone and alloTHDOC potentiate the action of GABA at GABA-
A receptors prompts the question of whether a relationship exists between the distributions of neurosteroids (chapter 6) and particular GABA-A receptor subunits. With reference to the immunohistochemical distributions of subunits reported by Fritschy and Mohler (1995) and Pirker et al. (2000), it appears that no individual subunit density spread is entirely predictive of the brain-wide neurosteroid labelling pattern (see Table 5). Nevertheless, as α1, β2, β3 and γ2 subunits are found throughout the rat brain – but with variation in the respective subunit distributions (Fritschy and Mohler, 1995; Pirker et al., 2000) – there is overlap with the similarly widespread neurosteroids. Taking into account that different subsets of GABA-A receptor subunits are present in different brain regions, neurosteroids may influence GABA-A receptors of varied subunit constitutions.

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Steroid</th>
<th>α1</th>
<th>β2</th>
<th>β3</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory Bulb</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Striatum</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Cerebral Cortex</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Cerebellar Cortex</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Ret. Thalamic N.</td>
<td>Mod.-High</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Cerebellar Nuclei</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Thalamus</td>
<td>Moderate</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Tectum</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Pontine Ret. F.</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>ND</td>
<td>High</td>
</tr>
<tr>
<td>Medullary Ret. F.</td>
<td>Low</td>
<td>Moderate</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

**Table 5. The Distribution of Neurosteroids and the Most Prevalent GABA-A Receptor Subunits in Selected Areas of the Rat Brain.** There is variation between the regional densities of allopregnanolone/alloTHDOC labelling ('steroid' column; condensed from chapter 6) and α1, β2, β3 and γ2 subunit immunoreactivity (data summarised from...
7.18 Neurosteroids Are Positioned to Affect Different Forms of Inhibition

Local inhibition in the ICC was modulated by neurosteroids following deactivation of the projection from the contralateral DNLL (chapters 3 and 4). As GABA-ergic local circuit neurons are distributed throughout the brain (see Mugnaini and Oertel, 1985), with levels of neurosteroids in many areas equal to or greater than that in the ICC (see Table 4), regulation of inhibitory interneurons by neurosteroids is unlikely to be exceptional. Nonetheless, GABA-ergic interneurons are rare/absent in a few areas, such as the ventroposterior (VP) and mediodorsal (MD) nuclei of the rat thalamus (Ottersen and Storm-Mathisen, 1984; Harris and Hendrickson, 1987; Kuroda and Price, 1991). The VP and MD were moderately allopregnanolone/alloTHDOC-immunoreactive, with both nuclei receiving GABA-ergic input from the reticular thalamic nucleus (Rt; dense input to VP: Pinault et al., 1995; also pallidal inputs to MD: Mogenson et al., 1987; Kuroda and Price, 1991). So it seems that neurosteroids are well placed to influence inhibition provided by long-range projections, at their termination in addition to their origin. As discussed in section 7.12, it appears that only long-range inhibitory neurons were neurosteroid-positive. Therefore, neurosteroid modulation of disinhibitory connections may be possible, but probably limited to those
synapses on long-range inhibitory neurons. Possible candidates include the cerebellar Purkinje cells and Rt neurons, which are essentially all GABA-ergic (Ottersen and Storm-Mathisen, 1984) and receive local and/or forebrain GABA-ergic input (Asanuma and Porter, 1990).

Neurosteroids are in an immediate position to affect inhibitory input to the cell body and thick dendrites, which strongly influence action potential firing. Such input to pyramidal cells in the isocortex is provided by basket cells, amongst others (Somogyi et al., 1998; Gupta et al., 2000). In contrast, GABA-ergic double bouquet cells, for example, target dendritic spines and thin dendrites (Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997). These contacts are suitably placed to regulate local post-synaptic responses, and 5α-reduced neurosteroids are poorly positioned to have an effect in this instance. A similar scenario exists in the CA1 region of the hippocampus with inputs to pyramidal cells from basket and Schaffer collateral-associated cells (Cope et al., 2002). There also appears to be a difference in the neurosteroid sensitivity of GABA-ergic synapses on apical versus basal dendrites of CA1-CA3 pyramidal cells. Orchinik et al. (2001) showed greater inhibition of binding of the chloride channel antagonist t-butylbicyclophosphorothionate by applied alloTHDOC in the stratum radiatum compared with the stratum oriens. Interestingly, we found apical dendrites to be densely allopregnanolone/alloTHDOC-immunoreactive.
Neurosteroid synthetic enzymes and neurosteroid-sensitive receptors have already been identified in a number of brain regions (review: Stoffel-Wagner, 2001), leading us to propose that our demonstration of neurosteroid-mediated gain control may have a broader applicability across the brain. Since physiologically relevant levels of 5α-reduced neurosteroids have been demonstrated in the cerebral cortex, hippocampus, striatum and cerebellum (Cheney et al., 1995), neurosteroids may be involved in the dynamic regulation of synaptic transmission and the activity of neuronal circuits in response to normal variations in processing demands. In more pathological situations, given that reduced inhibition can lead to a reactive increase in neurosteroid synthesis, endogenous GABA-agonistic neurosteroids may play a role in preventing progression to epileptiform activity and excitotoxicity (Reddy and Rogawski, 2002) in many circumstances.
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