Neurosteroids Involved in Regulating Inhibition in the Inferior Colliculus

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Saalmann, Yuri B., Ian G. Morgan, and Mike B. Calford. Neurosteroids involved in regulating inhibition in the inferior colliculus. J Neurophysiol 96: 3064–3073, 2006. First published September 13, 2006; doi:10.1152/jn.00786.2006. Fast inhibitory neurotransmission in the brain is largely mediated by the γ-aminobutyric acid-type A (GABA_A) receptor. The 3α,5α-reduced neurosteroids (e.g., allopregnanolone) are the most potent endogenous modulators of the GABA_A receptor. Although it is known that 3α,5α-reduced neurosteroid levels change during stress or depression and over the estrus cycle, a basic physiological role consistent with their pharmacological action remains elusive. We used the unique architecture of the auditory midbrain to reveal a role for 3α,5α-reduced neurosteroids in regulating inhibitory efficacy. After blocking the massive GABAergic projection from the dorsal nucleus of the lateral lemniscus (DNLL) to the contralateral central nucleus of the inferior colliculus (ICC) in anesthetized rats, a reactive increase in the efficacy of other inhibitory circuits in the ICC (separable because of the dominant ear that drives each circuit) was demonstrated with physiological measures—single-neuron activity and a neural-population-evoked response. This effect was prevented by blocking 3α,5α-reduced neurosteroid synthesis with a 5α-reductase inhibitor: finasteride. Immunohistochemistry confirmed that the DNLL blockade induced an increase in 3α,5α-reduced neurosteroids in the contralateral ICC. This study shows that when GABAergic inhibition is reduced, the brain compensates within minutes by locally increasing synthesis of neurosteroids, thereby balancing excitatory and inhibitory inputs in complex neural circuits.

INTRODUCTION

Various neurosteroids are synthesized in the brain where they act on ligand-gated ion channels and G-protein-coupled receptors, including N-methyl-D-aspartate (NMDA), α-aminooxyacetic-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, GABA_A, glycine, 5-hydroxytryptamine 3, nicotinic acetylcholine, oxytocin, and sigma 1 receptors (Rupprecht and Holsboer 1999). The 3α,5α-reduced derivatives of progesterone (3α-hydroxy-5α-pregnan-20-one, also known as 3α,5α-THP or allopregnanolone) and deoxycorticosterone (3α,5α-THDOC) are positive allosteric modulators of the GABA_A receptor, enhancing neuronal inhibition (Lambert et al. 2001; Majewska et al. 1986). Although the 3α,5α-reduced neurosteroids can reach effective physiological levels within minutes (Purdy et al. 1991), there appear to be no readily releasable pools (Corpechot et al. 1993; Korneyev et al. 1993). Rather their synthesis (Fig. 1) is upregulated on demand (Reddy and Rogawski 2002). Altered brain levels of 3α,5α-reduced neurosteroids have been demonstrated during stress (Purdy et al. 1991) and depression (Uzunova et al. 1998), over the estrus cycle (Palumbo et al. 1995), and after 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 established.

Based on their pharmacological action, 3α,5α-reduced neurosteroids would appear to have the capacity to modulate inhibition, facilitating a regulated balance between excitatory and inhibitory influences. To test this hypothesis, we sought a brain area that has at least two separable GABAergic inputs with the aim of blocking one of these to allow for reactive plasticity to occur at the remaining GABAergic synapses. Unlike most brain areas, the central nucleus of the inferior colliculus (ICC) receives GABAergic input from a number of external sources as well as from local interneurons (Gonzalez-Hernandez et al. 1996; Roberts and Ribak 1987; Zhang et al. 1998). For this reason, we chose to adopt the rat ICC as our model (Fig. 2) and, in an acute physiological experiment, permanently deactivated its major GABAergic projection from the contralateral dorsal nucleus of the lateral lemniscus (DNLL). This intervention leaves viable sources of GABAergic 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. The synthetic enzymes, 5α-reductase and 3α-hydroxysteroid oxidoreductase, which produce the various 3α,5α-reduced neurosteroids found in the rat (Fig. 1), have been demonstrated in the ICC (Li et al. 1997).

METHODS

Electrophysiology

Two recording methods were used to monitor the effects of deactivation of the contralateral DNLL on 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 the Animal Care and Ethics Committee of the University of Newcastle and conformed to the Animal Welfare
enzymes are in italics. We used finasteride, an inhibitor of 5-
reduced neurosteroids. DHP, dihydroprogesterone; THP, tetrahydroprogesterone; DHDOC, dihydrodeoxycorticosterone; THDOC, tetrahydrodeoxycorticosterone.

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. Anesthesia was induced in adult (8–14 wk) male Dark Agouti 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 cases or with Motorola (Sydney, Australia) piezoelectric drivers for EP cases. The output of each transducer through a hollow ear-bar was first calibrated using a Bruel and Kjaer (Naerum, Denmark) 4165 microphone 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, Sequim, WA), filtered (AudioControl C-131 1/3 octave equalizer, MountlakeTerrace, WA), and processed neuronal activity was displayed on-line in a computer window and recorded (Kaiser Instruments: MALab running on a Macintosh G4). EPs represent the average of 23 individual responses with band-pass 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 postsynaptic wave generated within the ICC following the method of Szczepaniak and Moller (1996). The zero crossing of the waveform 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. To gauge spontaneous (maintained) activity, recordings were summed over 400-ms periods for 20 presentations of a sham stimulus (subthreshold, typically –20 dB SPL).

Baseline responses from the ICC were monitored for ~1 h after which kainic acid (KA; Sigma-Aldrich, Sydney, Australia) was infused over 1 min into the contralateral DNLL (14 mM, 0.5–1.5 μl). KA disrupts neurotransmission and later induces excitotoxic cell death. Confirmation of DNLL deactivation was provided by initial electrophysiological responses consistent with a DNLL locus on implantation of the microsyringe, suppression of tone-evoked responses within the DNLL following the KA injection, ICC neuronal responses showing a reduction in ipsilateral-ear-evoked inhibition post-KA, and histological identification of the DNLL injection site. KA was dissolved in an isotonic saline solution, to which pontamine sky blue was added 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 ~100 μm. This was used to confirm the efficacy of the KA injection, which typically took ~10 min to fully deactivate sound-induced activity in the DNLL. As the DNLL projects tonotopically to the contralateral ICC, best frequencies were matched between the nuclei to ensure that the DNLL-derived inhibitory input to the contralateral ICC neuron being studied had been deactivated (best frequency of profiled ICC neurons >4 kHz). Recordings from the ICC continued at regular intervals after 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) ~2 h before recordings commenced (an average of 3 h 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 h after finasteride delivery), to determine the effect of

**Treatments**

FIG. 1. 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. DHP, dihydroprogesterone; THP, tetrahydroprogesterone; DHDOC, dihydrodeoxycorticosterone; THDOC, tetrahydrodeoxycorticosterone.
finasteride on baseline electrophysiology. Finasteride is a specific inhibitor of 5α-reductase (Steckelbroek et al. 1999). The finasteride solution [17β-N-(2-methyl-2-propyl)carbamoyl-4-aza-5α-androst-1-en-3-one; Steraloids, Newport, RI] was 6 mg/ml of 30% wt/vol 2-hydroxypropyl-β-cyclodextrin (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 hydroxypropyl-β-cyclodextrin and isotonic saline did not alter baseline neuronal responses within the ICC.

**Immunocytochemistry**

Further DNLL lesion and finasteride + DNLL lesion experiments were conducted for the purpose of neurosteroid immunoassays. Procedures were as described in the preceding text, but at 30 min post-KA, animals were perfused transcardially with saline followed by 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M phosphate buffer (PB). For the purpose of establishing baseline staining, additional animals were anesthetized with ketamine (100 mg/kg im)/acepromazine (10 mg/kg im) and perfused when pedal and corneal reflexes were no longer present. A final group of animals was administered finasteride 4 h prior to perfusion to control for potential effects of finasteride on the basal state. Brains were postfixed for 48 h in 4% PFA/0.1 M PB to which 30% sucrose was added for cryoprotection. Brain tissue was sectioned parasagitually at 50 μm using a freezing microtome. The ICC generally spanned 1,500 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 (no detergents were used to avoid possible leeching of steroids). Free-floating sections were blocked using 1% donkey serum (DS; Sigma-Aldrich) in 0.1 M PB for 1 h. They were then incubated in 1:2,500 sheep anti-allopregnanolone primary antibody (purchased from Dr. R. Purdy, Department of Psychiatry, University of California San Diego) in 1% DS/0.1 M PB, for 48 h at 4°C on an orbital shaker (used throughout the immunoassay). After 3 × 10-min rinses in 0.1 M PB, sections were incubated overnight in 1:200 anti-sheep/goat immunoglobulin biotinylated secondary antibody (from donkey; Amsterdam Biosciences, Sydney, Australia) in 0.1 M PB at 4°C. After further rinses, sections were placed in 1:200 streptavidin biotinylated horseradish peroxidase complex (Amersham-Biosciences) in 0.1 M PB for 5–6 h 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 min and then transferred into a solution of 0.015% hydrogen peroxide/0.05% DAB/0.1 M PB for around 3 min. Finally, tissue was thionin counterstained. Sections were air-dried on gelatinized slides, mounted in DPX and coverslipped. According to radioimmunoassay, the primary antibody shows minimal cross-reactivity (<1%) with compounds present in rat (Bernardi et al. 1998). Our own competitive binding assays with brain slices have revealed that the majority of immunolabeling is prevented by solubilized allopregnanolone with some cross-reactivity of the primary antibody with 3α,5α-THDOC; this is not undesirable, as our interest lay in monitoring 3α,5α-reduced neurosteroids not necessarily allopregnanolone. The number of labeled cells was quantified using a three-dimensional counting method (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 ×100 oil-immersion objective (Leitz, Pl Apo Oel, NA 1.32) at a final magnification of ×1,000, under Köhler illumination. Labeled cells (defined by dark staining and clear cellular shape/features) were counted if they were either wholly within a rectangular prism (70 × 35 × 50 μm, idealized section thickness), marked out by an eyepiece reticle as the section was moved vertically or 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 forward, 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 well-defined midbrain nucleus, the substantia nigra pars reticulata (SNR), found in the same sagittal sections as the ICC. This served to delimit the region of altered neurosteroid synthesis and to control for bias in immunolabeling. As a check against observer bias, the counting procedure (performed by Saalmann) was repeated completely independently by an experienced histologist (Shannon Waldron) using a ×100 oil-immersion objective (Zeiss, Plano Neofluar, NA 1.30) fitted to an Olympus BX60 light microscope. Inter-rater reliability was 0.96 (Pearson’s r) and the statistically significant difference (ICC: control vs. DNLL lesion group) was confirmed. A positive correlation has been demonstrated between the level of neurosteroids measured by radioimmunoassay and the density of neurosteroid-immunoreactive cells (Frye and Vongsch 2001). The images in Fig. 7 were acquired using a Zeiss Axioplan 2: imaging and Axiocam and Axiovision software.

**Results**

Establishing the model

A test of the overall hypothesis first required a demonstration that loss of the major GABAergic 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 stimulated increased efficacy of the remaining, mostly interneuron-mediated and contralateral-ear-dominated, GABAergic inhibition. Finally we postulated that the increased inhibitory efficacy would not occur if the effect of 3α,5α-reduced neurosteroids at the GABA_A receptor were blocked. Given that no effective competitive antagonist for the 3α,5α-reduced neurosteroid binding site was identified until very recently (and is unsuitable for in vivo experiments) (Mennerick et al. 2004), we addressed this hypothesis with the use of the synthetic steroid finasteride, which blocks 5α-reduced neurosteroid production. We used two recording paradigms to determine changes in the ICC response to dichotic sounds: single-neuron recordings and EP recordings—locally recorded EP electrophysiology. We also determined relative changes in 3α,5α-reduced neurosteroid concentration with immunohistochemistry.

Responses in ICC were compared between animals in three treatment groups: DNLL lesion—KA-induced unilateral DNLL deactivation, control—a control for the deactivation procedure with KA delivered outside the DNLL, and finasteride + DNLL lesion—underwent DNLL deactivation but also had the synthesis of 5α-reduced neurosteroids blocked by pretreatment with finasteride (Fig. 1). 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 has previously been shown to prevent increases in neurosteroid synthesis in rat brain (Concas et al. 1998) and to be physiologically effective (Disney and Calford 2001).
Inhibitory plasticity: effect on the individual neuron

DNNL lesion group: ICC single-neuron recordings. Within 10–15 min 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. 3, A and B, and 4A), corresponding with the decline in activity within the contralateral DNNL. Furthermore, there was a small initial increase in the response of ICC neurons to stimulation through the contralateral ear alone (Figs. 3B and 4A), suggesting a reduction in tonic inhibitory input to the ICC with deactivation of the contralateral DNNL. This induced increase in responsiveness of ICC neurons was followed by a reduction in excitability within the ICC (Figs. 3C and 4A); spike counts started to decrease by 20–50 min post-KA, reaching a minimum between 30 and 90 min post-KA. Onset and sustained response components were similarly affected. The delayed reduction in ICC excitability was mirrored in mean first-spike latency determinations. Significant increases in latency were observed at all sound levels by 20 min post-KA (2-factor, time and sound level, repeated-measures ANOVA, followed by Bonferroni-corrected multiple comparisons with initial value, \( P < 0.025 \)), reaching a maximum between 30 and 90 min post-KA (Fig. 5).

Control group: ICC single-neuron recordings. There was no change in ICC responses (control group studies involved single-neuron recordings only; 4 animals) after KA injections placed outside the contralateral auditory nuclei (Figs. 3, G–I, and 4C). In one animal, a control KA injection was made, and 90 min later, KA was also injected into the DNNL. Although the control injection produced no change in ICC neuronal responses, the second injection brought about the plasticity response typically observed in the DNNL lesion group. This
The control case, spike counts remained stable throughout the experiment (C).

due to a nonspecific effect of KA injection.

excitability observed in DNLL lesion animals, and that it is not

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 nonspecific effect of KA injection.

In the absence of neurosteroid synthesis (finasteride +

DNLL lesion group), the effect of DNLL lesions was revealed
in three aspects of increased excitability in single-unit re-

sponses: anomalous ipsilateral-ear-stimulated excitation, in-
creased spontaneous activity, and increased response to con-

tralateral-ear stimulation. There was a fundamental difference
in the behavior 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 acti-

vated by the ipsilateral ear (Figs. 3, D and E, and 4B),

ipsilateral-ear stimulation elicited excitatory responses (Figs.
3F and 4B). This anomalous excitation appeared in ICC neu-

rons (EI classification prior to the lesion) by 15–35 min
post-KA, with maximum ipsilateral-ear drive between 30 and
80 min 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 com-
ponents (4 of 7 animals). Ipsilateral-ear drive manifested as an
offset response in one animal. These responses were strong,
and in four of seven animals, the maximum ICC spike count
achieved after KA administration was produced in response to
ipsilateral stimulation alone. An unmasking of anomalous
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).

In addition to the unmasking of ipsilateral-ear-evoked excitation, a large elevation in spontaneous activity occurred within the ICC of finasteride-treated animals after deactivation of the contralateral DNLL (Figs. 3F and 4B). Spontaneous activity within the ICC, prior to deactivation of the contralateral DNLL, was very low in all experimental groups (usually ≤5 spikes, summed over 20 periods of 400 ms). However, after DNLL deactivation in finasteride-treated animals, ICC spontaneous activity increased from 15 to 40 min post-KA (7 of 8 animals), reaching a maximum 30 to 70 min post-KA (20–350
DNLLE LESION GROUP: ICC EP RECORDINGS. Local 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 (Fig. 6, A and C). Consistent with the fact that the DNLLE to ICC input provides inhibition dominated by the ipsilateral-ear pathway, there was little immediate effect of DNLLE deactivation on the EP recorded in response to contralateral-ear stimulation. However, after ~10 min, 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 min post-KA [for the group, F(8,40) = 14.5, P < 0.001, significant effect of time, 1-way repeated-measures ANOVA]. Latency increased, reaching a maximum 25–60 min post-KA. At 30 min post-KA, the group average amplitude as a proportion of the pre-KA level was 0.95 (compared with 0.75 for the DNLLE lesion group; P < 0.05, t-test independent). At the same time, latency was only altered by 0.125 ms (compared with 0.47 ms for the DNLLE lesion group; P < 0.05, t-test independent).

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 organization 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 (EI neurons: 47.8% of 69 cells) and normal (EI neurons: 45.8% of 118 cells) animals. The response latency of single neurons (median or component latency at similar ICC depths across groups. The median latency 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. For EP recordings, there was little variation in waveform shape or component latency at similar ICC depths across groups. 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 (Godfrey et al. 2000; Kotak et al. 1998). As 3α,5α-reduced neurosteroids have not been spikes, with 4 animals recording between 275 and 350 spikes). This contrasts with the DNLLE lesion group, in which only one animal showed an increase in spontaneous activity (from 30 min post-KA, reaching a maximum of 225 spikes 85 min post-KA). The response to contralateral-ear stimulation also showed an overall increase in excitability with the manifestation varying from cell to cell in a manner appropriate to the response profile (Figs. 3F and 4B). Thus onset, sustained and offset response components were differentially affected, and in half the cases, differences were apparent at high versus low sound levels. Furthermore, with contralateral-ear-evoked excitation, ICC neurons showed no change or reduced latency after the deactivation of the contralateral DNLLE in the presence of finasteride, in contrast with the DNLLE lesion group (Fig. 5).

Inhibitory plasticity: effect on the neuronal ensemble

FIG. 6. The effect of neurosteroids was evident on ensembles of neurons. A: evoked potential (EP) waveforms showing that an induced reduction in GABAergic inhibition within the ICC was quickly followed by a countering increase in remaining inhibition. After deactivation of the contralateral DNLLE, EP amplitude fell, reaching a minimum at 30 min post-KA. B: with neurosteroid synthesis blocked, there was no reduction in ICC EP amplitude after deactivation of the contralateral DNLLE. C: 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 DNLLE—treatments at a particular time were compared using Tukey’s HSD (after 2-factor, treatment and time, ANOVA, with repeated measures on time): **, P < 0.05; *, 0.1 > P > 0.05. D: for the 30 min post-KA vs. baseline comparison, the percentage change in peak-to-peak EP amplitude plotted against the change in EP latency, for each DNLLE lesion animal and finasteride + DNLLE lesion animal. There was a significant difference (P < 0.01, Hotelling’s T² test) between the amplitude and latency at 30 min post-KA vs. the pre-KA level.
shown to modulate glycnergic 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.

**Immunodetection of neurosteroids**

The manipulation of the physiological consequences of DNLL deactivation by finasteride points to an involvement of 5α-reduced neurosteroids. Immunohistochemical staining for cells containing 3α,5α-reduced neurosteroids was used to confirm such involvement. In the ICC, the anti-allopregnanolone antiserum labeled cell bodies and large dendrites of neurons that had a distinct disk-shaped appearance. This appearance is consistent with the excitatory “principal” cells described in the ICC of many species (Irving 1986). Gial-like cells (identified by nuclear confinement of Nissl stain, appropriate location and typical glial morphological features) were not labeled. Using an unbiased tissue sampling and counting procedure (see methods), we established the baseline labeling in the ICC as 18,500 ± 1,620 labeled cells/mm³ [mean ± SE (rounded to the nearest 10) of counts from 5 animals; Fig. 7A]. This increased significantly (P < 0.0001, t-test after single factor, treatment, ANOVA) to 31,020 ± 1,730 labeled cells/mm³ in DNLL lesion rats at 30 min post-KA (n = 5; Fig. 7B). At the same experimental time point, the labeled cell density of 14,690 ± 1,490 cells/mm³ in finasteride + DNLL lesion rats (n = 5) was not significantly different from baseline (expected-wise error rate of 0.05; Fig. 7C). Note that finasteride on its own (16,440 ± 1,080 labeled cells/mm³; n = 5) did not significantly affect the basal state within the experimental time frame.

As an internal control for bias in immunolabeling, and to establish that the increase in 5α-reduced steroids in DNLL lesion animals was specific to the ICC, other brain areas were examined. There was no general increase in the DNLL lesion animals. A midbrain area that appears in the same sections as the ICC, the substantia nigra pars reticulata (SNR), was examined in detail. Cell bodies and large dendrites of neurons were labeled in the SNR but not putative glia. The immunoreactive cell density of the SNR at baseline (11,650 ± 1,220 labeled cells/mm³; n = 5; Fig. 7D) did not differ from that at 30 min post-KA in DNLL lesion (11,210 ± 1,200 cells/mm³; n = 5; Fig. 7E), finasteride + DNLL lesion (10,880 ± 980 cells/mm³; n = 5; Fig. 7F), or finasteride only (11,320 ± 1,090 cells/mm³; n = 5) rats (P > 0.05, single factor, treatment, ANOVA).

**DISCUSSION**

Increased neuronal excitability within the ICC, directly resulting from the deactivation of the inhibitory input from the contralateral DNLL, was observed to be followed by a reduction in ICC excitability in animals that had not received finasteride. This was evident, after a delay of around 20 min, as increased first-spike latency and reduced spike counts in single-neuron recordings, and increased latency and decreased amplitude in local EP recordings, in response to the presentation of sounds delivered to the contralateral ear. Because 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). We then sought a mechanism for this effect.

The 3α,5α-reduced neurosteroids reduce neuronal excitability by potentiating the action of GABA at GABAA receptors (Lambert et al. 2001; Majewska et al. 1986). Finasteride was used to block 5α-reduced steroid synthesis. This treatment prevented the reduction in ICC excitability observed in DNLL lesion animals after deactivation of the contralateral DNLL. Moreover, with this treatment, ICC neurons developed unusual excitation to sounds delivered to the ipsilateral ear and elevated spontaneous activity after contralateral DNLL deactivation at a time corresponding to the onset and greatest influence of steroid action in DNLL lesion animals. Thus it can be inferred that in response to loss of the input from the contralateral DNLL, the remaining GABAergic inhibition in the ICC was potentiated as a result of increased 5α-reduced steroid synthesis, limiting the increases in excitability to sound stimulation (both contral- 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 steroid-immunoreactive cells within the ICC after deactivation of the contralateral DNLL.

The unusual ipsilateral-ear-evoked excitation (finasteride + DNLL lesion animals) may have simply been “unmasked” with the block of counteracting inhibitory plasticity by finasteride after DNLL lesion. Alternatively, the combined effect of DNLL deactivation and finasteride may have contributed conditions appropriate for the potentiation of excitatory inputs, manifesting as ipsilateral-ear-evoked excitation, high spontaneous activity, and reduced firing latency. Long-term potentiation (LTP) has been demonstrated in the ICC under conditions of reduced inhibition (Hosomi et al. 1995; Zhang and Wu 2000).

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, 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 3α,5α-reduced neurosteroids, as positive allosteric modulators of the GABA_A receptor, should produce corresponding electrophysiological changes within the ICC. Preliminary feasibility studies in 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 localized within the ICC (for example, α 1β 2γ 2 or δ subunits) (Pirker et al. 2000).

It has been established that 5α-reduced steroids are synthesized de novo in the brain as levels in the brain persist and can be augmented after removal of peripheral sources (Corpechot et al. 1993; Cheney et al. 1995). Our physiological 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 to determine the effect of 5α-reduced steroids under normal conditions. However, our demonstration of increased immunoreactivity in the ICC but not the SNR, after DNLL deactivation, indicates that regional regulation of the level of 5α-reduced steroids occurs. Taking into account that systemically administered 5α-reduced steroids do not preferentially localize in ICC (Cheney et al. 1995; Wang et al. 1995), the demonstration that the increase in immunoreactivity for allopregnanolone/3α,5α-THDOC was restricted to the affected pathway (ICC compared with SNR) suggests local brain synthesis of 5α-reduced steroids rather than systemic delivery. Our physiological findings are consistent with the interpretation that the trigger for increased synthesis of 5α-reduced steroids is a local decrease in inhibition that, without such synthesis, results in anomalous excitability. Local 5α-reduction does not rule out the supply of the immediate precursors of 5α-reduced steroids from the circulation.

Immunohistochemical assays for 3α,5α-reduced steroids in the ICC predominantly labeled cells with the appearance of excitatory principal cells. These constitute ≥60% of ICC neurons (Irvine 1986). This pattern of labeling is consistent with that in other areas of the brain stem, midbrain, thalamus, and cerebral cortex where cells with the profile of inhibitory interneurons and glia were not labeled (Saalmann et al. 2003). Thus the labeled neurons match with the expected site of action of these 5α-reduced steroids; that is, 5α-reduced steroids acting at GABA_A receptors on excitatory neurons, thereby reducing ICC excitability. The absence of immunoreactive glia was unexpected as glia are thought to be involved in the synthesis of 3α,5α-reduced steroids (Jung-Testas et al. 1989). However, recent work has reported that some neurons (spinal, sensory pathway) appear able to synthesize steroids de novo (Patte-Mensah et al. 2003, 2004).

Finasteride on its own had little effect on the basal state during the 4 h after injection as determined by the single-neuron and EP electrophysiological parameters of this study and immunohistochemical assays of 3α,5α-reduced pregnane steroids. However finasteride was effective in blocking the increased syntheses of 5α-reduced steroids after DNLL lesions. Whereas it would not change the interpretation that 5α-reduced steroids mediate the increased inhibition in ICC induced by loss of the DNLL input and that these effects are blocked by finasteride, it may be the case that our measures were insensitive to some baseline effects of finasteride administration. Previously we have reported that at 90 min after finasteride injection, the ICC EP to acoustic click stimuli had increased by ~20% and plateaued (Disney and Calford 2001), suggesting some form of basal influence by 5α-reduced steroids. This determination required following changes in the click EP within individual animals from the time of finasteride injection, which was not undertaken in the present study (here recordings started ~2 h after finasteride delivery). Our assay for cellular 5α-reduced steroids was based on counting immunohistochemically positive cells. This proved useful in confirming that finasteride blocked increased 5α-reduced steroid synthesis and showed no effect on baseline staining. It is possible that the concentration of 5α-reduced steroids could have been reduced by finasteride even if the number of immunolabeled cells was unaltered.

In this paper, we have interpreted the data according to a model of inhibitory synaptic plasticity in which neurosteroids increase the effectiveness of GABAergic inhibition in response to increased excitability. An alternative interpretation of some of the results could be that GABAergic inhibition is enhanced by an increase in the number of GABA_A receptors. However, translocation of GABA_A receptors from a cytoplasmic to a postsynaptic membrane locus, or de novo synthesis, requires a time course approaching ≥1 h (Kittler and Moss 2003; Nusser et al. 1998). Until this occurs, upregulation of GABA_A receptor-mediated inhibition would have to be provided by an increase in GABA-evoked chloride conductance. Increased release of GABA is not a viable control mechanism as minimal quantum presynaptic GABA release saturates postsynaptic GABA_A receptors (Edwards et al. 1990). Because neurosteroid concentrations can be raised within minutes, and the effect of neurosteroids can be switched off by their metabolic transformation, 3α,5α-reduced neurosteroids provide the rapid and flexible modulation of GABAergic inhibition required to explain our results.

The sequence of biochemical events leading from reduced GABAergic inhibition to increased neurosteroid synthesis is not fully understood. However, elevated adenosine 3′,5′-cyclic monophosphate (cAMP) 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_A 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 cooperation with diazepam binding inhibitor (DBI) and the mitochondrial benzodiazepine receptor, facilitates cholesterol transport to the mitochondrial cytochrome P450 side chain cleavage enzyme (Bose et al. 2002; West et al. 2001). Cholesterol is converted to pregnenolone by side chain cleavage (Stoffel-Wagner 2001), the putative rate-limiting step in neurosteroid synthesis. More neurosteroid precursors would lead to increased production of 3α,5α-reduced neurosteroids. As cycloheximide has been shown to attenuate enhanced neurosteroid synthesis (Rossetti et al. 1994), increased synthesis of neurosteroid synthetic enzymes or associated regulatory factors
neurosteroid-mediated gain receptors have already been identified in a number of brain regions (Pirker et al. 2000; Stoffel-Wagner 2001), leading us to propose that our demonstration of neurosteroid-mediated gain control may have a broader applicability across the brain. Because physiologically relevant levels of 3α,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 a variety of circumstances.

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GRANTS

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REFERENCES


