Neurosteroids Mediate Habituation and Tonic Inhibition in the Auditory Midbrain

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Disney, Anita and Mike B. Calford. Neurosteroids mediate habituation and tonic inhibition in the auditory midbrain. *J Neurophysiol* 86: 1052–1056, 2001. Habituation of the behavioral response to a repetitive stimulus is a well-established observation in perceptual studies and is considered a basic form of nonassociative learning. There is also a long history of physiological studies suggesting that central nervous system habituation is mediated by inhibition. At higher levels of the sensory pathways, such inhibition is mainly contributed by GABAa receptor mechanisms. Concepts of modification of synaptic efficacy that apply to excitatory amino acid synaptic transmission do not have direct parallels with these inhibitory synapses: quantal release of GABA rapidly saturates available receptors at a synapse, placing an upper limit on responsiveness to increased transmitter release. However, pharmacological modulation of GABAa-receptor efficacy with exogenous agents (e.g., benzodiazepines and β-carbolines) is known to occur through allosteric mechanisms that modulate the effectiveness (positive and negative) of GABA at this receptor. The most potent endogenous modulators are 5α-reduced steroids. Production of these steroids was attenuated in adult rats with systemic injection of Finasteride, a competitive substrate for 5α-reductase. This treatment was sufficient to block habituation of the evoked midbrain response to repetitive presentation of an acoustic click. This result confirms that simple habituation is due to an increase in active inhibition, the increase being mediated by steroid modulation of the GABAa-receptor. Finasteride treatment also brought about a 23% increase in the evoked response to a click stimulus, suggesting that 5α-reduced steroids normally contribute to tonic inhibition in the rat inferior colliculus.

INTRODUCTION

For some time it has been known that the enzymatic machinery for the production of 5α-reduced progesterone-derived steroids, which modulate transmission at GABAa-synapses, is found throughout most of the mammalian brain. 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP; allopregnanolone) and 3α-tetrahydro-deoxycorticosterone (3α-THDOC) are both known to be potent positive modulators of inhibition mediated by the binding of GABA to its a-type receptor (Majewska et al. 1986). Both of these steroids can be synthesized de novo, or from intermediate substrates, in the brain (Hu et al. 1987; Jung-Testas et al. 1989). Brain levels of allopregnanolone have been shown to increase rapidly ($<$3 min) in response to anxiety producing stimuli and situations (e.g., Barbaccia et al. 1996; Purdy et al. 1991) and in response to ethanol administration (VanDoren et al. 2000). However, it has not been clear whether these steroids have a role in modulating GABAergic inhibition in neural processing in the absence of such priming.

The requirement for an endogenous modulator of GABAa-receptor efficacy is clear. GABA is the major inhibitory neurotransmitter at higher levels of the CNS. Acting at the ionotropic GABAa receptor, it is involved in many essential brain processes. Indeed the balance between inhibition and excitation defines the functioning of many neural systems, particularly those involved in sensory processing (Calford et al. 1998; Dykes et al. 1984; Park and Pollak 1993). However, the functional need for modulation of GABAa-receptor-mediated inhibition raises a paradox as minimal GABA release is considered sufficient for saturation. Quantal release of GABA rapidly saturates the GABA binding sites of GABAa-receptors at a synapse and opens a maximal number of Cl^- channels (Mody et al. 1994), limiting the effectiveness of increased transmitter release. One mechanism to control efficacy at such inhibitory synapses is to vary the number of receptors. With long-term changes in excitability, it is well established that GABAa-receptor numbers can increase or decrease (Jones 1993). However, even under optimal conditions, this mechanism is too slow (Nusser et al. 1998) to play a role in many of the situations in which inhibitory efficacy varies. Control by phosphorylation of receptors may provide one solution (Poisbeau et al. 1999). An alternative possibility is alteration of the kinetics of the ion channel. Pharmacologically applied GABAa-receptor modulators (benzodiazepines; β-carbolines) that prolong, or shorten, the time during which ions can pass across the cell membrane are well known. Allopregnanolone and THDOC are the most potent endogenous positive modulators of the GABAa-receptor (Majewska et al. 1986). The present study was designed to establish whether these steroids play a role in modulation of GABAergic inhibition in physiological circumstances that do not involve anxiolytic priming or potentially excitotoxic levels of activation.

Habituation to repetitive stimulation is a near-ubiquitous nervous-system phenomenon. In contrast to adaptation, which occurs in peripheral structures such as photoreceptors, CNS habituation occurs at rates of stimulation that do not approach the biophysical limits of synapses or put metabolic demands on neurons. It is thus considered to involve an active inhibitory component and is the classical form of nonassociative learning

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(Groves and Thompson 1970). Electrophysiological recording has shown that the amplitudes of averaged evoked potentials (EPs) recorded in the central auditory pathway decrease over the course of repeated presentations of a response-eliciting stimulus (Webster 1971). It has also been shown that the degree and time course of these decreases in responsiveness can be manipulated through administering GABAa-receptor modulators (Jongsma et al. 1998; Webster and Aitkin 1971). In the present study, we determined whether there is any involvement of neurosteroids in the observed time course of such response decrements in the rat auditory midbrain nucleus—the inferior colliculus (IC).

The decreases in EP amplitude in the IC observed in previous experiments involving repetitive stimulation (Chakravarty and Faingold 1996; Webster 1971; Webster and Aitkin 1971) were maintained during continuous, rapid presentation of stimuli for between 20 and 30 min. This prolonged inhibition, already known to be GABAa-receptor mediated, may well be of the kind that requires positive modulation to be maintained over time. The synthesis of the 5α-reduced steroids can be blocked using Finasteride, a competitive inhibitor of 5α-reductase (Faller et al. 1993). We therefore investigated whether such blockade alters the course of the response amplitude decrements recorded during repetitive presentation of an acoustic click stimulus.

METHODS

Single-session, nonrecovery experiments were performed on 32 adult male rats (pigmented—Dark Agouti) aged between 8 and 15 wk and weighing 250–350 g. Anesthesia was induced by intramuscular injection of ketamine (100 mg/kg; Troy Laboratories) and acepromazine (10 mg/kg; Troy Laboratories); 10 min prior to anesthetic injections, an analgesic was administered subcutaneously (carprofen, 3 mg/kg; Zenocarp, Heriot Agvet). Throughout the experiment, pedal and eye-blink reflexes were monitored, and supplementary doses of ketamine were given as needed.

Locally derived EPs in response to acoustic click stimuli applied to the contralateral ear were recorded from the IC through a microelectrode. During the recording session, animals were held in a stereotaxic frame inside a sound-attenuating, electrically shielded room. Stimuli were generated using a 12-bit D/A with controlled output attenuators (Kaiser Instruments) driven by the MALab application running on an Apple Macintosh G3. Click stimuli were produced with 80 μs positive polarity square pulses, passed through a headphone amplifier (Tucker Davis), driving a piezoelectric transducer (Motorola) fitted to a hollow ear-bar. Fast-Fourier analysis revealed that the bulk of the energy of the click was $<$15 kHz. Locally manufactured tungsten-in-glass recording electrodes of impedance 1–1.5 MΩ, at 300 Hz, with 5–30 μm of exposed tungsten, were used in all experiments. Electrodes entered the pia at 1.7 mm rostral and 1.9 mm lateral of lambda and were advanced at an angle of 43° dorsorostral to ventrocaudal from vertical to a depth of 3,400 μm. Potentials were amplified differentially (A-M Systems 1800) with band-pass filtering 100 Hz to 10 kHz. The signal was digitized at 19.5 kHz (MALab event processor, Kaiser Instruments), viewed on-line (continuous averages), and stored for off-line analysis.

Preexperimental acoustic stimulation was kept to a minimum. Initially, using low presentation rates, the threshold sound level for a discernible EP was determined. Later recordings were obtained with the stimulus level 35 dB above threshold. After determining thresholds, no stimulus presentations were made for 30 min. No quantitative recordings were made within 1.5 h of induction of anesthesia to allow a return to normal neurosteroid levels following any handling-induced increase.

The basis of the experimental design was to compare the degree of habituation of the acoustic click EP in the IC of rats with Finasteride blockade of 5α-reductase with that of controls. Figure 2, which presents a data summary, also presents a summary of the experimental design. Four groups of animals were used. Two experimental groups received 60 mg/kg Finasteride [1,(5α)-andrsotene-4-aza-3-one-*N*-tert-butyl-17β-carboxaminde, Sigma, 6 mg/ml in 30% wt/vol 2-hydroxypropyl-β-cyclodextrin, Sigma, in 0.9% saline] by intraperitoneal injection; two control groups received only the vehicle solution (2.5–3 ml; 30% wt/vol 2-hydroxypropyl-β-cyclodextrin, Sigma, in 0.9% saline). The concentration of Finasteride was based on previous reports. Lephart et al. (1996) have reported a 60–80% reduction in enzyme activity and behavioral effects in pregnant rats with 50 mg/kg Finasteride given subcutaneously. Ongoing experiments in this laboratory with a different paradigm (Saalmann and Calford 1999) have shown the delivery method and concentration to be effective in reducing induced inhibition in the IC. A 90-min period with no acoustic stimulation followed Finasteride or vehicle injections; this should be sufficient to allow any stress-induced elevation in neurosteroids to return to normal (Barbaccia et al. 1996). In one group of experimental animals and one control group, a baseline measure of habituation was obtained prior to Finasteride or vehicle injection. All comparisons of EP amplitude were based on averages of 20 presentations at specified time periods. For graphical presentation, these were referenced to the amplitude at either the start of the stimulus presentation period under consideration or to the initial value. For statistical comparisons, raw EP amplitudes were used in a mixed-model analysis-of-covariance design with the initial EP amplitude or that at the start of a run as the covariant using the SPSS package.

On completion of recording, the characteristic frequency (CF) and the threshold to a pure tone stimulus were noted before a lethal dose of pentobarbital sodium was administered. The fact that the EP to pure-tone pulses showed frequency tuning consistent (CF near 8 kHz) with the location of the electrode tip (and which varied with depth) confirmed that the EP under study was locally generated. After perfusion/fixation brains were sectioned and stained. Electrode tracks were located by track damage and reconstructed to confirm the recording site as the central nucleus of the inferior colliculus.

Experimental procedures were approved by the Animal Experimentation and Ethics Committee of the Australian National University and conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

RESULTS

The form of the EP obtained in the IC in response to contralateral click stimuli (Fig. 1*A*) closely resembles that obtained by Semple and Aitkin (1980) to electrical stimulation of the cochlear nucleus. In that study, manipulation of the rate of electrical stimulation allowed interpretation of the early wave as presynaptic and the later, and larger, wave as postsynaptic. Consideration of previous reports (Webster and Bock 1971) and preliminary experiments led to adoption of a 5-Hz presentation rate as sufficient to induce mild habituation of the postsynaptic EP-component and an insignificant decrement of the presynaptic component. Thus within 2.5 min, the postsynaptic EP showed a rapid and sustained reduction to ~90% of the initial value that was maintained throughout 25 min of stimulation (Fig. 1*B*). Apart from a small initial decrement within the first minute of presentation, this habituation was completely blocked in a repeat period of presentation of the click stimulus started 90 min after Finasteride injection (Fig. 1*C*). Control animals given only the vehicle solution showed the normal pattern of habituation as established in the baseline determination.

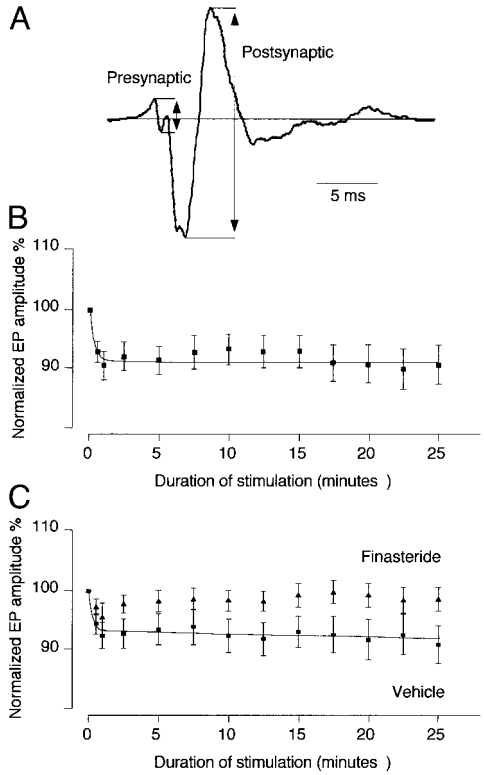

FIG. 1. *A*: example of an averaged (20 presentations) evoked potential (EP) recorded in response to a contralateral click stimulus 35 dB above threshold in the inferior colliculus (IC) of an individual animal. Pre- and postsynaptic components are identified. *B*: habituation of the postsynaptic EP over 25 min of continuous presentation at 5 Hz; means and SEs from 14 animals as percentages referenced to the initial value. Curve shows a fitted exponential decay (time constant, 1.31 min; plateau, 91.95%). *C*: lack of habituation of the click-EP over 25 min of continuous stimulus presentation when begun 90 min after treatment with Finasteride (8 animals). Finasteride attenuates the production of 5α-reduced steroids. Animals (6) receiving the vehicle solution showed normal habituation (time constant, 1.02 min; plateau, 89.94%).

Another striking result of the Finasteride treatment was an increase of ~25% in the EP-amplitude in response to the click stimulus (Fig. 2*A*). Thus the determination of a lack of habituation (Fig. 1*C*) was made with respect to this increased value. At a later period (145 min after Finasteride injection; Fig. 2*A*), presumably when Finasteride inhibition of 5α-reductase became less effective due to synthesis of new enzyme, a third period of repetitive stimulation revealed a significant decrement in the EP-amplitude. Interpretation of this later period is confounded by the dual effects of diminution of the Finasteride induced effects on habituation and induced enhancement of the EP. Irrespective, the final level reached was still well above (+23%) the equivalent level reached at the same time point with the vehicle-injected animals. Overall, the Finasteride-induced increase in the click EP suggests that 5α-reduced steroids have an ongoing role as positive modulators of GABAergic inhibition in the IC. A three-way (treatment $\times$ run $\times$ time) mixed-model ANOVA was performed on the amplitude of the presynaptic component, and none of the main effects was significant; specifically the initial 25-min period of repetitive stimulation resulted in a nonsignificant change to 96% of baseline and 90 min after Finasteride injection the presynaptic EP was at 101% of baseline. A difficult to interpret two-way interaction [run $\times$ time: $F(1,10) = 6.349$, $P = 0.023$] was significant. However, the differences in the presynaptic component change profiles cannot explain the effects observed in the animals given Finasteride: the presynpatic component was at only 101% of baseline 90 min after Finasteride was administered, while the postsynaptic component had increased to 120% of baseline by this time. This suggests that there was neither clear repetitive stimulation nor Finasteride-induced alterations in the amplitude of the input to the IC and that the observed changes in the postsynaptic EP probably originated within the IC.

The Finasteride-induced increase in the click EP was an unexpected outcome and provided a potential complication to the interpretation of the original design, which essentially planned for a within animal comparison of habituation before and after attenuation of 5α-reduced steroid synthesis. First, it was necessary to establish whether the 90-min period after Finasteride injection was sufficient for the induced increase to reach a steady state. This was tested in three animals by sampling the click EP with 4-s presentations of the repetitive acoustic-click stimulus at 15-min intervals after Finasteride injection. In each case, a plateau was reached prior to 90 min (Fig. 2*C*). Hence it is considered unlikely that the lack of change in the EP with repetitive stimulation in the Finasteride-injected animals resulted from a continual increase in the base click EP acting against an habituation. Rather the most parsimonious explanation is that the attenuated levels of 5α-reduced steroids were responsible for blocking habituation. Second, the presence of an initial baseline habituation run in the original experiment complicates the interpretation; despite the fact that EPs had returned to normal levels after the 90 min break in the vehicle-injected animals, it may be that in an environment of raised steroid synthesis (after the initial 25-min presentation) the subsequent injection of Finasteride had a complex effect. Consequently, a second set of animals was studied in which both experimental and control groups received only a 4-s presentation of the repetitive acoustic-click stimulus prior to Finasteride or vehicle injection (Fig. 2*B*). This was sufficient to obtain an EP averaged over 20 presentations to establish the base level in each animal. Even with minimal preinjection acoustic stimulation, the effect of attenuating the potential synthesis of 5α-reduced steroids resulted in a marked increase in the click EP. This allows the conclusion that tonic synthesis of these steroids normally enhances inhibitory components of the response to acoustic clicks in the IC. In all respects, for both Finasteride and vehicle treatments, the time course of the changes in EPs were very similar for the two presentation paradigms (compare Fig. 2, *A* and *B* from the time of injections).

DISCUSSION

The effect of interference of 5α-reduced neurosteroid synthesis in the present study adds a new perspective to the understanding of the roles attributed to neurosteroids (Rupprecht and Holsboer 1999). It is clear that, in addition to the previously reported roles in the short-term response to anxiety-related stimulation and in the response to alcohol, 5α-reduced neurosteroids are involved in modulating inhibition relevant to the processing of sensory stimuli. Two aspects of this modulation were uncovered in the present study in which neurosteroid synthesis was attenuated with Finasteride.

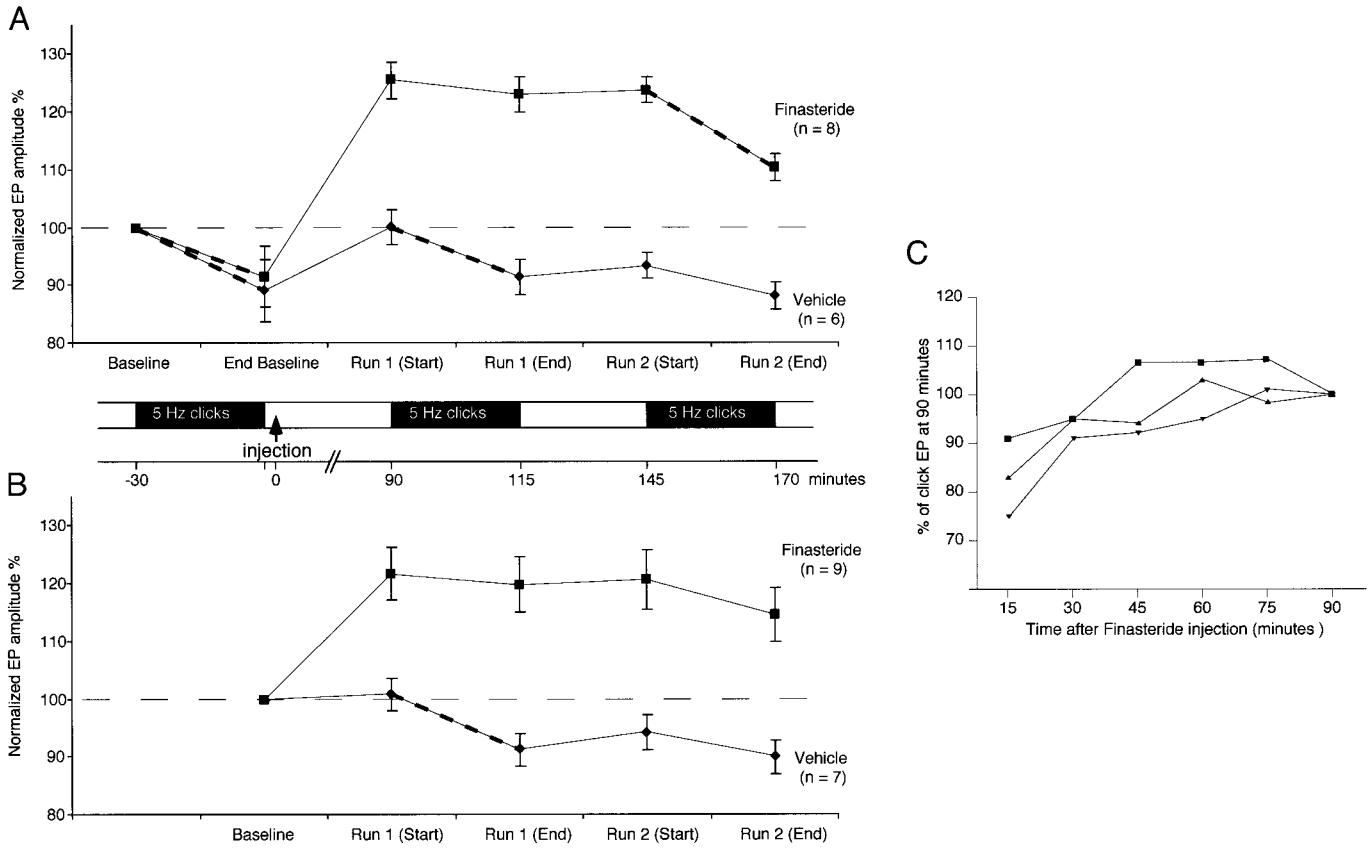

FIG. 2. *A*: summary of the effects of Finasteride injection on click EPs and habituation. For each period of repetitive stimulation, only the starting and final values (mean and SE) are illustrated; - - -, where there is a significant difference between these values ($P < 0.01$). There is a significant main effect between the Finasteride- and vehicle-injected groups ($F = 14.32$, $P < 0.0002$), and, as illustrated in Fig. 1*C*, during the *run 1* period (90 min after injection), there was no habituation in the Finasteride-injected animals. With the short period between *runs 1* and *2* (30 min), there is only partial recovery from habituation in the vehicle-injected group. *B*: results for a variant of the paradigm in which only a 4-s presentation was made to obtain the base EP prior to injection. Both Finasteride- and vehicle-injected groups show the same effects as illustrated in *A*, confirming that an effect of Finasteride is to reduce the effectiveness of a tonic inhibition of the IC response to acoustic-click stimuli. *C*: EP amplitudes for 3 animals sampled by 4-s presentations at 15-min intervals after Finasteride injection, illustrating that the induced growth had ceased by 90 min.

Firstly, 5α-reduced neurosteroid synthesis appears necessary for habituation of the response to repetitive stimulation. In animals treated with Finasteride, there was an initial decrease in EP amplitude over the first 2 min of repetitive stimulation. Thereafter, the EP amplitude during the 25 min of stimulation matched that obtained to a brief presentation. The reported time to increased neurosteroid synthesis is ~3 min in anxiety-priming paradigms (Purdy et al. 1991), and thus the initial decrease in EP amplitude is expected to be controlled by other mechanisms. Elucidation of the details of the amplitude and timing of this initial effect would require different methods than those used in this study, where the summing across animals may be problematic due to small differences in the timing of the effect. Second, it was found that the amplitude of the EP to click stimulation is, in the circumstances of the present paradigm, normally mildly suppressed by the tonic synthesis of neurosteroids. Both findings suggest that 5α-reduced neurosteroids have a more subtle role in neuronal functioning than has previously been thought. The finding of the tonic effect in particular raises the probability of a role in short- to medium-term gain control of neural circuits. This is a widespread phenomenon in motor and sensory systems (e.g., Katz et al. 1999; Lisberger et al. 1983). It has previously been reasoned that in some circumstances changes in response strength and receptive field size are explicable only with a mechanism for short-term inhibitory synaptic plasticity that responds to increased excitability (Clarey et al. 1996). Such a mechanism appears to require (see INTRODUCTION) modulation of GABA-synaptic efficacy of the form provided by the 5α-reduced neurosteroids.

The concept of habituation as a simple nonassociative learning process (Groves and Thompson 1970) is consistent with mediation by neurosteroid modulation of GABAa-receptors. It is well established that, at most levels, the response of the central auditory pathway to monaural stimulation involves excitatory and inhibitory components (Clarey et al. 1992). The present work suggests that synthesis of neurosteroids by glial cells is induced, or increased, by repetitive stimulation. The increased neurosteroid concentration produces a local positive modulation of the effect of GABA on a-type receptors. In contrast to associative-learning effects, as with long-term potentiation and depression, this effect would be a nonselective, spatially limited increase in inhibitory-synapse efficacy. There is insufficient knowledge at this time to understand the trigger mechanism for increased synthesis. Where increased synthesis has resulted from potentially damaging levels of excitation (Barbaccia et al. 1996; Purdy et al. 1991), it appears that increased free-radical concentration can induce activation

(Brown et al. 2000). However, this is unlikely to be a factor with the 5-Hz stimulation employed here. It is known that steroidogenesis is under the control of peripheral-type benzodiazepine receptor (PBR)-induced transport of cholesterol, and intermediate steroids, to the inner mitochondrial membrane (Do-Rego et al. 1998; Papadopoulos et al. 1997). Endogenous ligands of PBR are known (diazepam-binding-inhibitor derived peptides), but their mode of control and release is not (Do-Rego et al. 1998; Lacor et al. 1999). It is possible that excess GABA may be a stimulating factor because in the retina, GABAa-receptor agonists have been shown to stimulate steroidogenesis (Guarneri et al. 1995).

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