

Review

# Modulation of native and recombinant GABA<sub>A</sub> receptors by endogenous and synthetic neuroactive steroids

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## Abstract

Upon administration, certain pregnane steroids produce clear behavioural effects including, anxiolysis, sedation, analgesia, anaesthesia and are anti-convulsant. This behavioural profile is characteristic of compounds that act to enhance the actions of GABA acting at the GABA<sub>A</sub> receptor. In agreement, numerous studies have now demonstrated these steroids to be potent, positive allosteric modulators of the GABA<sub>A</sub> receptor. The pregnane steroids are synthesized in the periphery by endocrine glands such as the adrenals and the ovaries, but are also made by neurons and glial cells in the central nervous system itself. Hence, these compounds could play both an endocrine and a paracrine role to influence neuronal excitability by promoting inhibition. Here we review evidence that the pregnane steroids are highly selective and extremely potent GABA<sub>A</sub> receptor modulators and that their effects at 'physiological' concentrations (low nanomolar) may be influenced by the subunit composition of the GABA<sub>A</sub> receptor. This feature may underlie recent findings demonstrating the effects of the neurosteroids on inhibitory synaptic transmission to be brain region dependent, although recent reports suggest that phosphorylation mechanisms may additionally influence neurosteroid sensitivity of the GABA<sub>A</sub> receptor. Numerous synthetic steroids have been synthesized in an attempt to therapeutically exploit the behavioural effects of the pregnane steroids and progress with this approach will be discussed. However, the demonstration that the steroids may be made within the central nervous system offers the alternative strategy of targeting the enzymes that synthesize/metabolise the neurosteroids to exploit this novel endocrine/paracrine interaction. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* GABA; GABA<sub>A</sub> receptor; Neuroactive steroids; Transmitter-gated ion channel

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## 1. Introduction

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The rapidity with which certain pregnane steroids induce sedation and anesthesia is incompatible with a genomic

locus of action [82]. The demonstration in the mid-1980s that these steroids potently and selectively enhanced the actions of GABA acting at the major inhibitory receptor in the central nervous system, the GABA<sub>A</sub> receptor, provided a more feasible and logical target [39]. The GABA<sub>A</sub> receptor is a member of the cysteine–cysteine loop transmitter-gated ion channel family that includes glycine, nicotinic and 5-HT<sub>3</sub> receptors. The binding of GABA to this receptor opens an associated chloride selective ion channel that increases neuronal membrane conductance, effectively shunting the influence of excitatory neurotransmitters such as glutamate [55]. The GABA<sub>A</sub> receptor is the target for a number of therapeutically important drugs including benzodiazepines, barbiturates and general anaesthetics such as propofol and etomidate. The pregnane steroids share many of behavioural actions of these compounds being anxiolytic, anticonvulsant, sedative, analgesic and at high doses anaesthetic [32,52]. The GABA<sub>A</sub> receptor is a heteropentamer drawn from a repertoire of  $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho_{1-3}$  subunits [5,6]. Importantly, these proteins have a distinct distribution within the central nervous system and subunit composition influences not only the physiological, but additionally the pharmacological properties of the receptor. Furthermore, recent studies utilizing transgenic mice suggest that some of the behaviours produced by the benzodiazepines are mediated by distinct receptor isoforms (e.g. their sedative and amnesic actions are  $\alpha_1$ -dependent, the anxiolytic effects being  $\alpha_2$ -dependent [77]). In view of this precedent, this review will discuss in detail the influence of GABA<sub>A</sub> receptor subunit composition upon positive allosteric regulation by pregnane steroids. Although subunit dependency, as assessed from studies performed on recombinant receptors, appears to be far more subtle than that documented for the benzodiazepines, it may nonetheless be relevant in the context of concentrations of the steroids that are the likely to occur in vivo under various physiological conditions. Moreover, there are strong indications that GABA-ergic synaptic transmission may be differentially regulated by pregnane steroids in different brain regions, an effect that might be attributable to variations in GABA<sub>A</sub> receptor subunit composition.

## 2. The interaction of pregnane steroids with transmitter-gated ion channels

The glycine-gated chloride channel is a genetic close relation of the GABA<sub>A</sub> receptor and is composed of five transmembrane crossing subunits selected from one  $\beta$  and four  $\alpha$  subunits [10]. In addition to enhancing the function of GABA<sub>A</sub> receptors certain general anaesthetics can additionally act as positive allosteric modulators of the glycine receptor [9,10]. By contrast, endogenous pregnane steroids such as 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (3 $\alpha$ ,5 $\alpha$ -TH PROG) and the synthetic anaesthetic alphaxalone (3 $\alpha$ -

hydroxy-5 $\alpha$ -pregnan-11,20-dione) are inactive at both native and recombinant glycine receptors [4,39,71,94] (see also Table 1). However, not all pregnane steroids are inert at the glycine receptor as the water soluble general anaesthetic minaxolone (2 $\beta$ -ethoxy-3 $\alpha$ -hydroxy-11 $\alpha$ -dimethylamino-5 $\alpha$ -pregnan-20-one) produces a large enhancement of glycine-evoked currents, albeit at concentrations of the anesthetic some 20-fold greater than those required to produce an equivalent effect at the GABA<sub>A</sub> receptor (Table 1).

Nicotinic receptors located in the central nervous system have been implicated in a wide range of behaviours including: analgesia, anxiety, memory acquisition, synaptic plasticity and neuronal excitotoxicity [1]. Neuronal nicotinic receptors are made from  $\alpha$  and  $\beta$  subunits ( $\alpha_{2-10}$ ,  $\beta_{2-4}$ ) which can combine in certain combinations to form both hetero-oligomeric and homo-oligomeric ( $\alpha_{7-9}$ ) receptors that have distinct physiological and pharmacological properties. Certain nicotinic receptor isoforms are extremely sensitive to some general anesthetic agents. However, whereas pregnane steroids such as alphaxalone and 3 $\alpha$ ,5 $\alpha$ -TH PROG enhance GABA<sub>A</sub> receptor function at nanomolar concentrations, micromolar concentrations of these steroids inhibit nicotinic receptor function

Table 1  
The selectivity of action of alphaxalone and minaxolone

Receptor	Alphaxalone ( $\mu$ M)	Minaxolone ( $\mu$ M)
GABA <sub>A</sub> ( $\alpha_1\beta_2\gamma_2$ :EC <sub>50</sub> )	2.2±0.3 (78±3%)	0.5±0.1 (93±5%)
Glycine ( $\alpha\beta$ :EC <sub>50</sub> )	60 No effect	11±1 (89±4%)
AMPA (IC <sub>50</sub> )	60 No effect	100 No effect
NMDA (IC <sub>50</sub> )	30 No effect	30 No effect
Nicotinic ( $\alpha_4\beta_2$ :IC <sub>50</sub> )	5±1	19±3
Nicotinic ( $\alpha_7$ :IC <sub>50</sub> )	13±2	11±1
5-HT <sub>3</sub> (h5-HT <sub>3A</sub> :IC <sub>50</sub> )	~50	8±1

All experiments were performed on oocytes voltage-clamped at -60 mV. The sources of receptor were GABA<sub>A</sub>, human  $\alpha_1\beta_2\gamma_2$ ; Glycine, human  $\alpha_1$  rat  $\beta$  for alphaxalone and rat spinal cord mRNA for minaxolone; Kainate and NMDA, rat cerebellar mRNA; Nicotinic, rat  $\alpha_4\beta_2$  and chick  $\alpha_7$ , 5-HT<sub>3</sub>, human 5-HT<sub>3A</sub>. All experiments upon GABA<sub>A</sub> and glycine receptors utilized the EC<sub>10</sub> concentration of the natural agonist. For the other receptors, the appropriate EC<sub>50</sub> was used. For GABA<sub>A</sub> and glycine receptors, the steroid EC<sub>50</sub> and the maximal potentiation produced (expressed as a percentage of the maximum response to GABA or glycine) are given in parenthesis. For kainate, NMDA, nicotinic and 5-HT<sub>3</sub> receptors, the IC<sub>50</sub> values are given where appropriate.

[17,18,23,67]. Similarly, only relatively high concentrations of alphaxalone, or minaxalone cause inhibition of nicotine-induced currents mediated by neuronal  $\alpha_4\beta_2$  heteromeric, or  $\alpha_7$  homomeric, nicotinic receptors (Table 1). Finally, there is a close correspondence between the structure of those pregnane steroids that are behaviourally active and those active as GABA<sub>A</sub> receptor modulators (see Section 6). For example the orientation of the hydroxyl group at the 3 position of the steroid A ring is known to be critically important for both the behavioural and GABA<sub>A</sub> receptor effects of the anesthetic pregnane steroids (3 $\alpha$  active; 3 $\beta$  inert) (Table 1), whereas betaxalone (3 $\beta$ -hydroxy-5 $\alpha$ -pregnane-11,20-dione) the 3 $\beta$ -ol diastereomer of the anaesthetic alphaxalone, is behaviourally inactive, but equieffective with alphaxalone as an antagonist of neuronal nicotinic receptors [23].

The divergent structure-activity relationships for the behavioural and nicotinic receptor antagonist effects of the pregnane steroids, coupled with their relatively low potency at nicotinic receptors cf. GABA<sub>A</sub> receptors, suggests that nicotinic receptors do not mediate the behavioural effects of the pregnane steroids.

The 5-HT<sub>3</sub> receptors are closely related to nicotinic receptors, being cation conducting ion channels, composed of five subunits, but drawn from a limited repertoire of only two proteins (5-HT<sub>3A</sub> and 5-HT<sub>3B</sub>) which can form homomeric (5-HT<sub>3A</sub>), or heteromeric (5-HT<sub>3A</sub>/5-HT<sub>3B</sub>) receptors [26]. Indeed, the 5-HT<sub>3B</sub> subunit was only recently isolated [26] and therefore most pharmacological studies performed to date have utilized 5-HT<sub>3</sub> receptors endogenous to certain neurons and cell lines, or homooligomeric recombinant receptors composed of 5-HT<sub>3A</sub> receptor subunits. Importantly, the pharmacological properties of 5-HT<sub>3</sub> receptors are highly-species dependent, and this should be taken into account when considering these proteins as potential targets for mediating the actions of the neurosteroids [47]. However, we find human 5-HT<sub>3</sub> receptors (5-HT<sub>3A</sub> homooligomeric receptors) to be relatively insensitive to alphaxalone with inhibition of 5-HT-evoked currents being achieved only with high micromolar concentrations of this anaesthetic (Table 1) and betaxalone the behaviourally inert diastereomer being equieffective in this respect. Similarly, 3 $\alpha,5\alpha$ -TH PROG is only a relatively weak antagonist of this receptor [78]. Hence, in summary this receptor seems an unlikely target for mediating the behavioural effects of the pregnane steroids. However it should be noted that some steroids (e.g. 17 $\beta$ -estradiol and estrone), are reported to be extremely potent (nanomolar), agonist-dependent inhibitors of murine 5-HT<sub>3</sub> receptors [88].

Glutamate, acting through AMPA (DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isopropionic acid), kainate and NMDA (N-methyl-D-aspartate) receptors mediates much of the fast excitatory synaptic transmission in the mammalian central nervous system [27]. Glutamate receptors are multi-subunit proteins (there is debate as to whether the stoichiometry is

four or five) that have a distinct membrane topology from those of the cysteine loop receptors and hence constitute a separate family [27]. However, we find that even micromolar concentrations of the pregnane steroids alphaxalone and minaxalone (2 $\beta$ -ethoxy-3 $\alpha$ -hydroxy-11 $\alpha$ -dimethylamino-5 $\alpha$ -pregnan-20-one) have little or no effect on kainate-, or NMDA receptor-mediated currents recorded either from *Xenopus laevis* oocytes or from hippocampal neurons [55] (Table 1). Note that although these pregnane steroids have little or no effect at ionotropic glutamate receptors, pregnenolone sulphate acts to enhance NMDA-mediated responses with no effect on kainate or AMPA receptors [34].

In conclusion, both naturally occurring and synthetic pregnane steroids such as 3 $\alpha,5\alpha$ -TH PROG, alphaxalone and minaxalone are potent positive allosteric modulators of the GABA<sub>A</sub> receptor being effective from 1 nM to 1  $\mu$ M, whereas high micromolar concentrations of these steroids have little, or no effect on ionotropic glutamate, glycine, or 5-HT<sub>3</sub> receptors. Neuronal nicotinic receptors are antagonized by such high concentrations of these steroids. However, importantly this effect exhibits a distinct structure-activity relationship from that for the behavioural effects of these steroids. Hence, the pregnane steroids appear to be highly selective for the GABA<sub>A</sub> receptor.

### 3. Neurosteroid modulation of the GABA<sub>A</sub> receptor: isoform selectivity

The GABA<sub>A</sub> receptor is composed of five subunits drawn from a repertoire that includes:  $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho_{1-3}$  [5,6]. Subunit composition influences both the physiological and pharmacological properties of the receptor, and, importantly, these subunits have a distinct distribution throughout the central nervous system [70,92]. The behavioural profile of the pregnane steroids is in some respects similar to that of the benzodiazepines, e.g. having anxiolytic, anticonvulsant and sedative actions [32]. For benzodiazepines such as diazepam it is now apparent that these behaviours maybe mediated by distinct receptor isoforms (e.g. the sedative and anxiolytic actions being mediated by  $\alpha_1$ - and  $\alpha_2$ -containing receptors, respectively [77]). Hence, it is important to establish whether the pregnane steroids differentiate across the various GABA<sub>A</sub> receptor isoforms. Indirect evidence that this might be the case is provided by radioligand binding and chloride flux studies performed in distinct brain regions [33,66,72]. Furthermore, electrophysiological studies have clearly demonstrated that the pregnane steroids act differentially on synaptic GABA<sub>A</sub> receptors of different brain regions, although whether this is caused by receptor heterogeneity remains to be determined (see below). We have utilized the *Xenopus laevis* oocyte expression system to determine the influence of the GABA<sub>A</sub> receptor subunit composition on

the potency ( $EC_{50}$ ) and maximal ( $E_{MAX}$ ) GABA-enhancing actions of  $3\alpha,5\alpha$ -TH PROG.

The isoform of the  $\alpha$  subunit (co-expressed with the  $\beta_1$  and  $\gamma_2$  subunits) did not greatly influence the maximal GABA-modulatory effect of  $3\alpha,5\alpha$ -TH PROG with this steroid causing an ~6 to 7-fold increase of the current induced by an  $EC_{10}$  concentration of GABA, irrespective of the isoform of the  $\alpha$  subunit. However, GABA<sub>A</sub> receptors containing the  $\alpha_6$  subunit appeared exceptional as the neurosteroid increased the response (~12-fold) to above the apparent GABA maximum (Table 2). A comparison of the steroid  $EC_{50}$  reveals only a 3–4 fold difference across the  $\alpha$  isoforms (74–317 nM) (see Table 2). This relative lack of selectivity contrasts with the situation for the benzodiazepines where the isoform of the  $\alpha$  subunit greatly influences the effects of compounds such as diazepam. Indeed, whereas the presence of an  $\alpha$  (1, 2, 3 or 5) subunit is a prerequisite for a robust effect of diazepam, the pregnane steroids are active at recombinant receptors composed of only  $\beta_1$  and  $\gamma_2$  subunits and at homomeric ‘receptors’ containing the  $\beta$  subunit alone [59,80].

Although the influence of the  $\alpha$  isoform on the steroid  $EC_{50}$  is relatively modest it may be of physiological significance. Plasma levels of  $3\alpha,5\alpha$ -TH PROG normally fluctuate between 3 and 10 nM, but increase to 30–60 nM following mild stress and may reach 100 nM prior to parturition [68]. Interestingly we find that these ‘physiological’ concentrations (3–100 nM) of the neurosteroid are significantly less effective at GABA<sub>A</sub> receptors incorporating  $\alpha_4$  or  $\alpha_5$  subunits cf. those containing  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , or  $\alpha_6$  subunits (Table 2). The relative steroid insensitivity of  $\alpha_4$

subunit containing GABA<sub>A</sub> receptors is particularly intriguing as expression of this subunit in the hippocampus and cerebellum is increased following progesterone (i.e.  $3\alpha,5\alpha$ -TH PROG) withdrawal [30,86,87]. The GABA<sub>A</sub> receptors recorded from hippocampal neurons isolated from such progesterone withdrawn animals exhibit a benzodiazepine pharmacology characteristic of  $\alpha_4$  subunit-containing receptors [90], but are additionally insensitive to low concentrations of  $3\alpha,5\alpha$ -TH PROG, a property consistent with the reduced effect of the neurosteroid reported here (Table 2).

It is now evident that the isoform of the  $\beta$  subunit can influence the pharmacological properties of the GABA<sub>A</sub> receptor, e.g. the general anaesthetic etomidate and the anticonvulsant loreclezole selectively enhance the actions of GABA acting at  $\beta_2$ - and  $\beta_3$ - cf.  $\beta_1$ -containing receptors [9]. However, the GABA enhancing actions of the pregnane steroids are not influenced by nature of the  $\beta$  subunit isoform [36,80] (Table 2).

The presence of a  $\gamma$  subunit within the heteromeric GABA<sub>A</sub> receptor complex is an essential prerequisite for the benzodiazepines to act, as recombinant receptors composed of  $\alpha\beta$ ,  $\alpha\beta\delta$  or  $\alpha\beta\epsilon$  subunits are benzodiazepine insensitive [25,58,91,97]. By contrast, the  $\gamma$  subunit is not required for the actions of the pregnane steroids [73,84]. Indeed the incorporation of the  $\gamma_2$  subunit reduces the maximal effect of the steroid [compare the steroid  $E_{MAX}$  for  $\alpha_1\beta_1$  cf.  $\alpha_1\beta_1\gamma_2$  GABA<sub>A</sub> receptors (Table 2)]. The isoform of the  $\gamma$  subunit ( $\gamma_{1-3}$ ) has little influence on the maximal potentiation of GABA produced by the  $3\alpha,5\alpha$ -TH PROG, although comparison of the steroid  $EC_{50}$  determined for  $\alpha_1\beta_1\gamma_x$  ( $x=1-3$ ) receptors revealed the  $\gamma_3$ - and  $\gamma_1$ -containing receptors to be ~3.3- and 6.3-fold less sensitive than the  $\gamma_2$ -containing receptor. These data suggest that neuronal GABA<sub>A</sub> receptors containing the  $\gamma_1$  subunit maybe less sensitive to ‘physiological’ concentrations of the neurosteroid.

Recently, the GABA modulatory actions of certain anabolic steroids have been described. These steroids are structurally distinct from the pregnane steroids and are therefore unlikely to mediate their effects through a common site. Furthermore, in contrast to the pregnane steroids, the functional consequences of the anabolic steroids binding to the GABA<sub>A</sub> receptor are highly dependent upon the isoform of the  $\gamma$  subunit with the steroid causing enhancement and inhibition of GABA-evoked responses at  $\alpha_2\beta_3\gamma_2$  and  $\alpha_2\beta_3\gamma_1$  receptors, respectively [49]. This selectivity is not restricted to recombinant receptors as in the hypothalamus anabolic steroids, such as nandrolone, enhance GABA-evoked responses in the ventromedial nucleus (predominantly expresses  $\alpha_2\beta_3\gamma_2$  subunits) and inhibits them in the medial preoptic area (predominantly expresses  $\alpha_2\beta_3\gamma_1$  subunits). By contrast,  $3\alpha,5\alpha$ -TH PROG at the relatively high concentration of 1  $\mu$ M potentiates GABA-evoked responses in both neuronal types. However, given the influence of the  $\gamma$  isoform on

Table 2

The influence of the subunit composition of the GABA<sub>A</sub> receptor on the GABA-modulatory effects of  $3\alpha,5\alpha$ -TH PROG

Human recombinant receptor combination	$EC_{50}$ (nM)	$E_{MAX}$ (%)
$\alpha_1\beta_1$	380±10	143±2
$\alpha_1\beta_1\gamma_1$	559±22	62±8
$\alpha_1\beta_1\gamma_{2L}$	89±6	69±4
$\alpha_1\beta_1\gamma_3$	294±36	74±5
$\alpha_1\beta_2\gamma_{2L}$	177±2	75±4
$\alpha_1\beta_3\gamma_{2L}$	195±36	72±4
$\alpha_2\beta_1\gamma_{2L}$	146±11	66±6
$\alpha_3\beta_1\gamma_{2L}$	74±1	67±7
$\alpha_4\beta_1\gamma_{2L}$	317±25	72±6
$\alpha_5\beta_1\gamma_{2L}$	302±38	81±2
$\alpha_6\beta_1\gamma_{2L}$	220±12	131±6
$\alpha_6\beta_2\gamma_{2L}$	350±29	108±5
$\alpha_6\beta_3\gamma_{2L}$	264±33	90±9
$\alpha_1\beta_1\epsilon$	N.D.	15±2

All parameters are calculated from steroid concentration-effect relationships obtained from oocytes expressing human recombinant GABA<sub>A</sub> receptors. The  $EC_{50}$  is defined as the concentration of steroid which produces an enhancement of the GABA( $EC_{10}$ )-evoked current to 50% of the maximum potentiation produced by that steroid. The  $E_{MAX}$  is the maximum potentiation of the GABA ( $EC_{10}$ )-evoked current produced by the steroid expressed as a percentage of the GABA maximum.

the EC<sub>50</sub> of 3 $\alpha$ ,5 $\alpha$ -TH PROG (Table 2), clearly it would now be of interest to determine the actions of lower, i.e. physiological concentrations of 3 $\alpha$ ,5 $\alpha$ -TH PROG on these neuronal subtypes.

The influence of the  $\epsilon$  subunit on neurosteroid modulation is controversial as the co-expression of this subunit together with  $\alpha$  and  $\beta$  subunits has been reported to produce either steroid-insensitive [25] or -sensitive GABA<sub>A</sub> receptors [91]. We find that the co-expression of  $\alpha_1$ ,  $\beta_1$  and  $\epsilon$  subunits in oocytes results in the formation of GABA<sub>A</sub> receptors that are relatively insensitive to the GABA-modulatory actions of 3 $\alpha$ ,5 $\alpha$ -TH PROG, although this steroid does apparently appear to directly gate this receptor isoform (Table 2). However,  $\epsilon$ -containing receptors exhibit spontaneous channel openings in the absence of GABA, which greatly complicates the interpretation of how the neurosteroid interacts with this receptor isoform [65].

GABA<sub>A</sub> receptors that incorporate the  $\delta$  subunit have been reported to be insensitive to the GABA-modulatory effects of the pregnane steroids [97]. However, more recently a comparison of the steroid sensitivity of  $\alpha_4\beta_3\delta$  and  $\alpha_4\beta_3\gamma_2$  GABA<sub>A</sub> receptors (stably expressed in cell lines) found alphaxalone and 3 $\alpha$ ,5 $\alpha$ -TH PROG to produce a larger potentiation of the GABA-evoked current in cells expressing the  $\delta$  subunit [12]. This latter result would appear to be in agreement with a behavioural study utilizing  $\delta$  knock out mice which demonstrated this genetic manipulation to reduce the anesthetic effects of 3 $\alpha$ ,5 $\alpha$ -TH PROG and to abolish the anticonvulsant and anxiolytic effects of ganaxolone (3 $\alpha$ -hydroxy-3 $\beta$ -methyl-5 $\alpha$ -pregnane-20-one) [61].

GABA receptors assembled as a homopentamer of  $\rho$  subunits ( $\rho 1-3$ ) form chloride selective ion channels with a pharmacological profile that is distinct from that of the hetero-oligomeric assemblies discussed above. The latter has encouraged some authorities to employ the nomenclature 'GABA<sub>C</sub> receptor' when describing native or recombinant assemblies of  $\rho$  subunits (e.g. [95]). Homopentamers of the  $\rho 1$  subunit are susceptible to positive allosteric regulation by alphaxalone, 3 $\alpha$ ,5 $\alpha$ -TH PROG and 3 $\alpha$ ,5 $\alpha$ -TH DOC. Although this is qualitatively similar to the action of these compounds at GABA<sub>A</sub> receptor isoforms, potentiation of current responses mediated by the  $\rho 1$ -homooligomer occurs only at low concentrations of GABA and in response to high concentrations of the steroids. Moreover, in contrast to their positive allosteric effect at the GABA<sub>A</sub> receptor, the 5 $\beta$ -ol epimers of such compounds inhibit responses to GABA at the  $\rho 1$ -homooligomer [63]. This suggests that orientation of the A and B ring fusion of the steroids (i.e. *cis* or *trans* in the 5 $\beta$ - and 5 $\alpha$ -series, respectively) is an important determinant of the action of pregnane steroids at receptors formed from  $\rho$ -subunits [63]. This aspect of the structure-activity relationship is at variance with that found for the GABA<sub>A</sub> receptor, where the orientation of the hydrogen

atom at C5 of the steroid is relatively unimportant (see also Section 6).

#### 4. Neurosteroid modulation of GABA<sub>A</sub> receptors: molecular mechanism of action

Early experiments that investigated the influence of the anesthetic pregnane steroids on GABA-evoked membrane current noise and GABA-evoked single channels demonstrated that nanomolar concentrations of these compounds had no effect on the single channel conductance of the GABA<sub>A</sub> receptor, but acted primarily to prolong the mean channel open time [4,19,23,53]. Additionally, these studies revealed that at concentrations in excess of those required for enhancement of GABA-evoked responses, these compounds had a second action, to directly gate the GABA<sub>A</sub> receptor chloride channel complex [19,53]. In this latter respect the depressant steroids are similar to a number of general anaesthetics including pentobarbitone, propofol and etomidate [9]. However, the neurosteroids are distinguished from these general anaesthetics as the maximal GABA-mimetic effect of the steroids is extremely limited [44].

A more detailed single channel analysis of the GABA-modulatory actions of the pregnane steroids in mouse spinal neurons, revealed that these compounds potently promoted the probability of the GABA-gated ion channel entering naturally occurring intermediate, or long lived, kinetic states at the expense of brief channel openings [89]. This perturbation of single channel kinetics is similar to that reported for the depressant barbiturates, although the steroids additionally increase the frequency of single channel opening [89]. These studies were all performed using the prolonged application of low, non-saturating, concentrations of GABA. However, these experiments may be of physiological relevance as evidence is now emerging that the excitability of some neurons may be partly controlled by tonic GABA and the enhancement of such a background current by the neurosteroids would be expected to have a considerable influence on neuronal excitability [3,11].

By contrast, during the vesicular release of GABA, it appears that at least for some central synapses a small number of postsynaptic GABA<sub>A</sub> receptors will be briefly exposed to relatively high, if not saturating, concentrations of GABA. Hence, to better understand the molecular mechanisms that underlie the actions of neurosteroids on inhibitory synaptic transmission, studies utilizing the rapid and brief application of high concentrations GABA are more pertinent. Under these conditions, the application of GABA to nucleated membrane patches made from cerebellar granule cells induced a fast rising current which decayed biphasically [96]. The fast decay time constant is proposed to result from channels oscillating between bound-open and -closed conformations, with the slow time

constant being a consequence of receptors entering and exiting various desensitized states [48]. Under these conditions, the neuroactive steroid  $3\alpha,21$ -dihydroxy- $5\alpha$ -pregnan-20-one ( $3\alpha,5\alpha$ -TH DOC) prolongs the slow decay time constant, it is postulated, by slowing the recovery of  $GABA_A$  receptors from desensitization (as receptors exiting desensitization may reconstitute this would result in a prolongation of the GABA-evoked current, see [96]). Consistent with this molecular mechanism,  $3\alpha,5\alpha$ -TH DOC, in the presence of a saturating concentration of GABA, was found to increase the probability of the channel being in the open state by increasing the number of delayed channel openings [96]. Such a molecular mechanism may underlie the effects of the pregnane steroids on GABA-ergic synaptic currents (see below).

### 5. Neurosteroids and inhibitory synaptic transmission

The first studies which addressed this issue used hippocampal neurones in culture and demonstrated that pregnane steroids such as alphaxalone and  $3\alpha,5\alpha$ -TH PROG prolonged the decay of evoked inhibitory postsynaptic currents (IPSCs), but had little effect upon the amplitude, or the rise time of these synaptic currents [40] (Fig. 1). More recently, the pregnane steroids have been shown to produce a similar, selective prolongation of the decay of evoked IPSCs, miniature inhibitory postsynaptic currents (mIPSCs — recorded in the presence of tetrodotoxin) and spontaneous inhibitory postsynaptic currents (sIPSCs — not evoked, but recorded in the absence of tetrodotoxin) recorded from neurones in the hippocampus (CA1 pyramidal and dentate granule cell neurones), the cerebellum (Purkinje neurones) and the hypothalamus (supraoptic nucleus) [13–15,22,28,37,38]. Additionally,  $3\alpha,5\alpha$ -TH PROG is reported to increase the amplitude of sIPSCs recorded from neurones located in the ventromedial nucleus and the medial preoptic area of the hypothalamus [49].

Synaptic  $GABA_A$  receptors from different brain regions are differentially influenced by neurosteroids such as  $3\alpha,5\alpha$ -TH PROG and  $3\alpha,5\beta$ -TH PROG and  $5\alpha$ -TH DOC as nanomolar concentrations prolong mIPSCs in rat hippocampal CA1 and cerebellar Purkinje neurones, whereas micromolar concentrations are required to similarly influence mIPSCs in the hypothalamus [13,15,22,28,37,38]. These differences are unlikely to result from a differential access of the neurosteroid to these synapses, as within the same hippocampal slice (made from a 20-day-old rat), nanomolar concentrations of  $3\alpha,5\beta$ -TH PROG prolong mIPSCs recorded from CA1 neurones, but micromolar concentrations of the steroid are necessary to produce a similar effect in dentate granule neurones [37,38]. Interestingly, mIPSCs recorded from dentate granule neurones of younger animals (10-day-old) are influenced by nanomolar concentrations of the pregnane steroids, suggesting that the

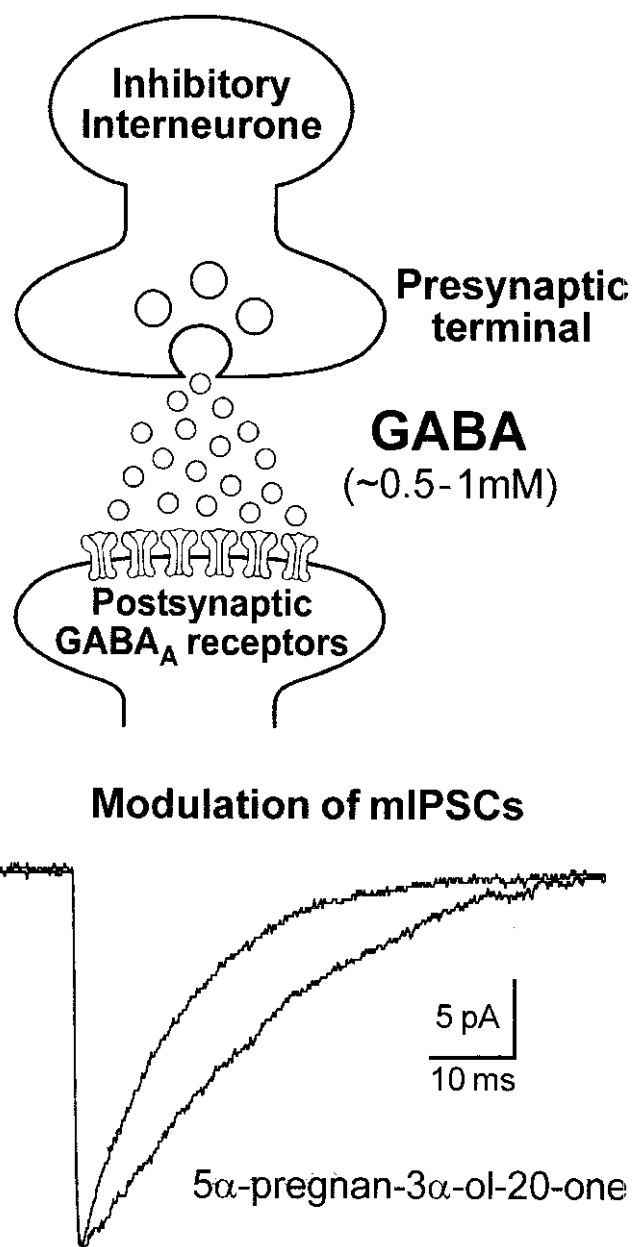


Fig. 1. Diagrammatic representation of a miniature inhibitory post-synaptic current (mIPSC) at a GABA-ergic synapse and its modulation by the neurosteroid  $5\alpha$ -pregnan- $3\alpha$ -ol-20-one ( $3\alpha,5\alpha$ -TH PROG). Note the steroid-induced prolongation of the synaptic current decay which results in enhancement of inhibition.

neurosteroid sensitivity of synaptic  $GABA_A$  receptors may be developmentally regulated [22].

A dramatic example of  $GABA_A$  receptor/neurosteroid plasticity occurs in magnocellular oxytocin neurones located in the supraoptic nucleus of the hypothalamus. In rats 1 day prior to parturition,  $3\alpha,5\alpha$ -TH PROG levels are relatively high and sIPSCs recorded from such neurones are prolonged by this steroid. However, upon parturition the circulating levels of  $3\alpha,5\alpha$ -TH PROG fall and in unison, the sIPSCs become steroid-insensitive. Physiologi-

cally this is important as it produces a reduction of neuronal inhibition and, as a consequence, permits the timed release of the oxytocin that is necessary for parturition and lactation [13,14].

It is conceivable that neurosteroid synaptic plasticity could result from changes in the subunit composition of the synaptic GABA<sub>A</sub> receptors. In the dentate gyrus, GABA<sub>A</sub> receptor subunit composition is known to undergo considerable developmental changes during the time (10–20 days) that these inhibitory synapses become less sensitive to the neurosteroid [31,46,50]. Furthermore, the change in steroid sensitivity which occurs in the magnocellular neurones post parturition is accompanied by an increase in the ratio of the  $\alpha_2$ - $\alpha_1$  GABA<sub>A</sub> subunit mRNA in these neurones [15]. Whether this  $\alpha$  subunit switch alone is sufficient to produce neurosteroid insensitive receptors is not clear, as studies on recombinant receptors demonstrate little difference in the  $3\alpha,5\alpha$ -TH PROG sensitivity of  $\alpha_1$ - and  $\alpha_2$ -containing receptors (e.g. Table 2), although a reduced metabolite of this steroid ( $5\alpha$ -pregnane- $3\alpha,20\alpha$ -diol) is less potent at the latter [8]. Alternatively, these neurones may express additional subunits that could contribute to their changed pharmacology (e.g. the  $\epsilon$  subunit). Furthermore, it is conceivable that the properties of synaptically clustered  $\alpha_2$ -containing receptors could differ from those of recombinant receptors expressed in a non-neuronal host cell. Such comparisons may be further compromised as the studies on recombinant receptors utilized relatively slow methods of agonist application, compared to the rapid and brief application of saturating concentrations of GABA proposed to occur at the synapse.

The actions of certain anabolic steroids (nandrolone, stanozolol and  $17\alpha$ -methyltestosterone) on synaptic inhibition are dependent upon neuronal type and in this case the selectivity does seem to be dependent on the isoform of the GABA<sub>A</sub> receptor. Hence, these steroids enhanced the amplitude and prolonged the duration of sIPSCs in one part of the hypothalamus (the ventromedial nucleus), but inhibited their amplitude in another region (medial preoptic area). By contrast,  $3\alpha,5\alpha$ -TH PROG did not discriminate, being facilitatory in both neuronal types [49]. The major GABA<sub>A</sub> receptor isoform in the ventromedial nucleus is thought to contain  $\alpha_2$ ,  $\beta_3$  and  $\gamma_2$  subunits, whereas in the medial preoptic area  $\alpha_2$ ,  $\beta_3$  and  $\gamma_1$  subunits predominate [29]. Similarly,  $17\alpha$ -methyltestosterone enhanced GABA-evoked currents recorded from HEK293 cells expressing recombinant  $\alpha_2$ ,  $\beta_3$  and  $\gamma_2$  subunits, whereas it inhibited such currents recorded from cells expressing  $\alpha_2$ ,  $\beta_3$  and  $\gamma_1$  subunits [49].

Evidence is now emerging that the steroid sensitivity of synaptic GABA<sub>A</sub> receptors may be influenced by additional factors, including local steroid metabolism and phosphorylation. Hence, whereas the dentate granule neurones of 20-day-old rats are relatively insensitive to  $3\alpha,5\beta$ -TH PROG, or  $3\alpha,5\alpha$ -TH PROG [7,37,38], mIPSCs are prolonged by low nanomolar concentrations of ganaxolone [7]

the metabolically stable analogue of  $3\alpha,5\alpha$ -TH PROG [20]. Given their close structural similarity, it is highly unlikely that these two steroids bind to distinct sites on the GABA<sub>A</sub> receptor. Hence, a more parsimonious explanation for these data would be that the naturally occurring  $3\alpha,5\alpha$ -TH PROG is subjected to local metabolism in the dentate gyrus, whereas the synthetic steroid ganaxolone is not [7].

Phosphorylation/dephosphorylation of GABA<sub>A</sub> receptor subunits and/or their associated proteins is now established as an important regulatory mechanism whereby the internal biochemistry of the neurone can influence inhibitory receptor function, turnover and assembly [64,85]. Phosphorylation has additionally been implicated in neurosteroid modulation of the GABA<sub>A</sub> receptor [35,57], including synaptic GABA<sub>A</sub> receptors [16,28,37]. Inhibitors of protein kinase C have been reported to abolish, or reduce, the effects of pregnane steroids on inhibitory synaptic currents recorded from hypothalamic magnocellular neurones and hippocampal CA1 neurones, respectively [28,37]. Inhibition of protein kinase A also reduced the effects of the steroid in CA1 neurones, but such inhibitors were without effect in the hypothalamus [28,37]. Whether the kinases influence the effects of the neurosteroid by phosphorylating the synaptic GABA<sub>A</sub> receptors per se, or indirectly by phosphorylating proteins associated with the receptor is not yet established [85].

These data suggest that potentially, cell surface G-protein coupled receptors that mediate their effects through kinases could influence the steroid/GABA<sub>A</sub> receptor interaction. Whilst such signalling cross talk remains to be demonstrated, the complementary situation, i.e. the effects of a G-protein coupled receptor being indirectly influenced by the steroid/GABA<sub>A</sub> receptor interaction has recently been described [16]. Oxytocin, acting on hypothalamic magnocellular neurons causes a reduction of the amplitude of GABA-mediated sIPSCs, an effect that can be mimicked by activating protein kinase C with phorbol esters. However, this action of the hormone and indeed that of stimulating the kinase directly with phorbol esters on synaptic GABA<sub>A</sub> receptors is completely prevented by pre-applying  $3\alpha,5\alpha$ -TH PROG, provided that the synaptic GABA<sub>A</sub> receptors are neurosteroid sensitive (i.e. juvenile or pregnant rats but not postpartum animals see above). Hence, allosteric regulation of the GABA<sub>A</sub> receptor would appear to be a prerequisite for the neurosteroid to block the protein kinase C-mediated modulation of the synaptic GABA<sub>A</sub> receptors. It will be of interest to determine whether such signalling cross talk is restricted to the hypothalamus, or is representative of a more general mechanism within the brain.

## 6. Structure activity relationships for steroids at the GABA<sub>A</sub> receptor

Studies conducted in the mid- and late-1980s exploring the structural requirements for steroid modulation of the

GABA<sub>A</sub> receptor found optimal activity to be associated with structures containing a 5 $\alpha$ - or 5 $\beta$ -reduced pregnane (or androstane) skeleton, a hydroxyl substituent at C3 of the steroid A ring in the  $\alpha$  orientation and a keto group at either C20 of the pregnane steroid side chain, or C17 of an androstane ring system [33,41,69] (Fig. 2). The naturally occurring steroids 3 $\alpha$ ,5 $\alpha$ -TH PROG and 3 $\alpha$ ,5 $\beta$ -TH PROG, 3 $\alpha$ ,5 $\alpha$ -TH DOC and androsterone (3 $\alpha$ -hydroxy-5 $\alpha$ -androstane-17-one) possess such elements. Subsequent development of the structure-activity relationship utilizing a wide range of synthetic steroids has revealed that a saturated, or even closed, steroid ring system is not an absolute requirement for activity as evidenced by 4-pregnen-3 $\alpha$ -ol-20-one [43] and certain benz[e]indene compounds [76], respectively. In addition, 20-keto-reduced analogues of 3 $\alpha$ ,5 $\alpha$ -TH PROG and 3 $\alpha$ ,5 $\beta$ -TH PROG (i.e. pregnanediols) act as partial agonists at the steroid site, with potencies and efficacies that are determined by the *cis* or *trans* fusion of the A and B rings and the orientation ( $\alpha$  or  $\beta$ ), of the 20-hydroxyl moiety [8,60]. Such refinements, along with the effects of chemical substitutions, and in some instances epimerisation, at the C2, C3, C5, C10, C11 and C17 positions of the steroid ring system and C21 of the acetyl side chain have been reviewed in detail elsewhere [54]. The present discussion will focus upon developments with particular practical, or theoretical, significance.

### 6.1. Steroids with increased oral bioavailability and resistance to metabolism

A serious impediment to the use of natural pregnane steroids as, for example, anticonvulsant, anxiolytic, or hypnotic drugs is their low oral bioavailability and short half-life [32]. Rapid hepatic metabolism, via conjugation, or oxidation of the 3 $\alpha$ -hydroxyl group that is crucial for steroid action at the GABA<sub>A</sub> receptor, underlies these pharmacokinetic features [32]. Moreover, oxidation of the 3 $\alpha$ -hydroxyl group to the ketone may introduce undesirable progestational activity by the formation of pregnanediones that bind to steroid hormone receptors [79].

Steroids protected from rapid metabolism may be synthesised by the incorporation of alkane and alkyl halide moieties at the 3 $\beta$ -position. Ganaxolone provides an example of such a compound. This 3 $\beta$ -methyl substituted analogue of 3 $\alpha$ ,5 $\alpha$ -TH PROG largely retains the potency and efficacy of the parent compound at the GABA<sub>A</sub> receptor [20]. Orally administered ganaxolone, unlike 3 $\alpha$ ,5 $\alpha$ -TH PROG has anticonvulsant activity against acute, chemically-induced, seizures in rats and the compound is also effective in rodent kindling models [20]. In a chronic dosing regimen, the efficacy of ganaxolone against chemically-induced seizures does not diminish with time, indicating that significant tolerance to the drug itself does not develop [74]. However, cross-tolerance between ganaxolone and diazepam does occur [74]. Interestingly,

the anticonvulsant activity of ganaxolone is increased in a rat model of catamenial epilepsy, wherein a sudden withdrawal of neurosteroids (derived from elevated progesterone levels in response to exogenous gonadotrophins) is associated with enhanced susceptibility to chemically-induced seizures. By contrast, the anticonvulsant potency of diazepam is reduced in this paradigm [75].

Studies in man suggest that ganaxolone may be of therapeutic value. In small-scale clinical trials, ganaxolone has been shown, as add-on therapy, to reduce the incidence of refractory seizures in children [62]. Similarly, ganaxolone appears efficacious, as add-on therapy, in the treatment of intractable infantile spasms [51]. In addition to the latter open-label trials, a recent randomised, double blind, design presented preliminary evidence that ganaxolone, as monotherapy during presurgical evaluations, does possess antiepileptic activity [56].

A degree of metabolic protection is also provided by the introduction of a 3 $\beta$ -trifluoromethyl group into 3 $\alpha$ ,5 $\alpha$ -TH PROG [32]. In 5 $\alpha$ -pregnanes (e.g. 3 $\alpha$ -hydroxy-3 $\beta$ -trifluoromethyl-5 $\alpha$ -pregnan-20-one), this modification appears to be associated with partial agonism, because efficacy is reduced in comparison to 3 $\alpha$ ,5 $\alpha$ -TH PROG and positive allosteric regulation by the latter is antagonised by the 3 $\beta$ -trifluoromethyl derivative [42]. Steroids with limited efficacy, including the pregnanediols, could, in principle, offer advantages over full-agonists in certain clinical settings. In 5 $\beta$ -pregnanes, 3 $\beta$ -substitution does not cause a reduction in efficacy [45].

### 6.2. Water-soluble steroids and intravenous anaesthesia

Attempts to develop water-soluble pregnane steroids suitable for intravenous administration have met with varying degrees of success [21,81]. The advantages of steroidal compounds over other chemical classes of intravenous anaesthetic are well illustrated by alphaxalone, a water-insoluble 11-ketone derivative of 3 $\alpha$ ,5 $\alpha$ -TH PROG, that enjoyed considerable success (as a 3:1 w/w mixture with alphadolone, i.e. Althesin) in the clinic [21]. Unfortunately, the fat solvent (Chremophor EL) chosen to administer alphaxalone caused rare anaphalactoid reactions and ultimately resulted in the cessation of the use of Althesin in human anaesthesia. Solubility in water, yet retention of anaesthetic potency, can be conferred by several chemical modifications of the steroid nucleus that include: esterification at the C21 position (e.g. hydroxydione, the sodium succinate derivative of pregnanedione), the incorporation of an 11 $\alpha$ -dimethyl amino group (i.e. minaxolone), or the introduction of a 2 $\beta$ -morpholinyl group [e.g. 21-chloro-3 $\alpha$ -hydroxy-2 $\beta$ -(4-morpholinyl)-5 $\alpha$ -pregnan-20-one methanesulphonate (ORG 20599) and 3 $\alpha$ -hydroxy-2 $\beta$ -(2,2-dimethyl-morpholin-4-yl)-5 $\alpha$ -pregnane-11,20-dione (ORG 21465)]. For those compounds that have been tested (i.e. minaxolone, ORG 20599 and ORG 21465) the chemical modification used to introduce

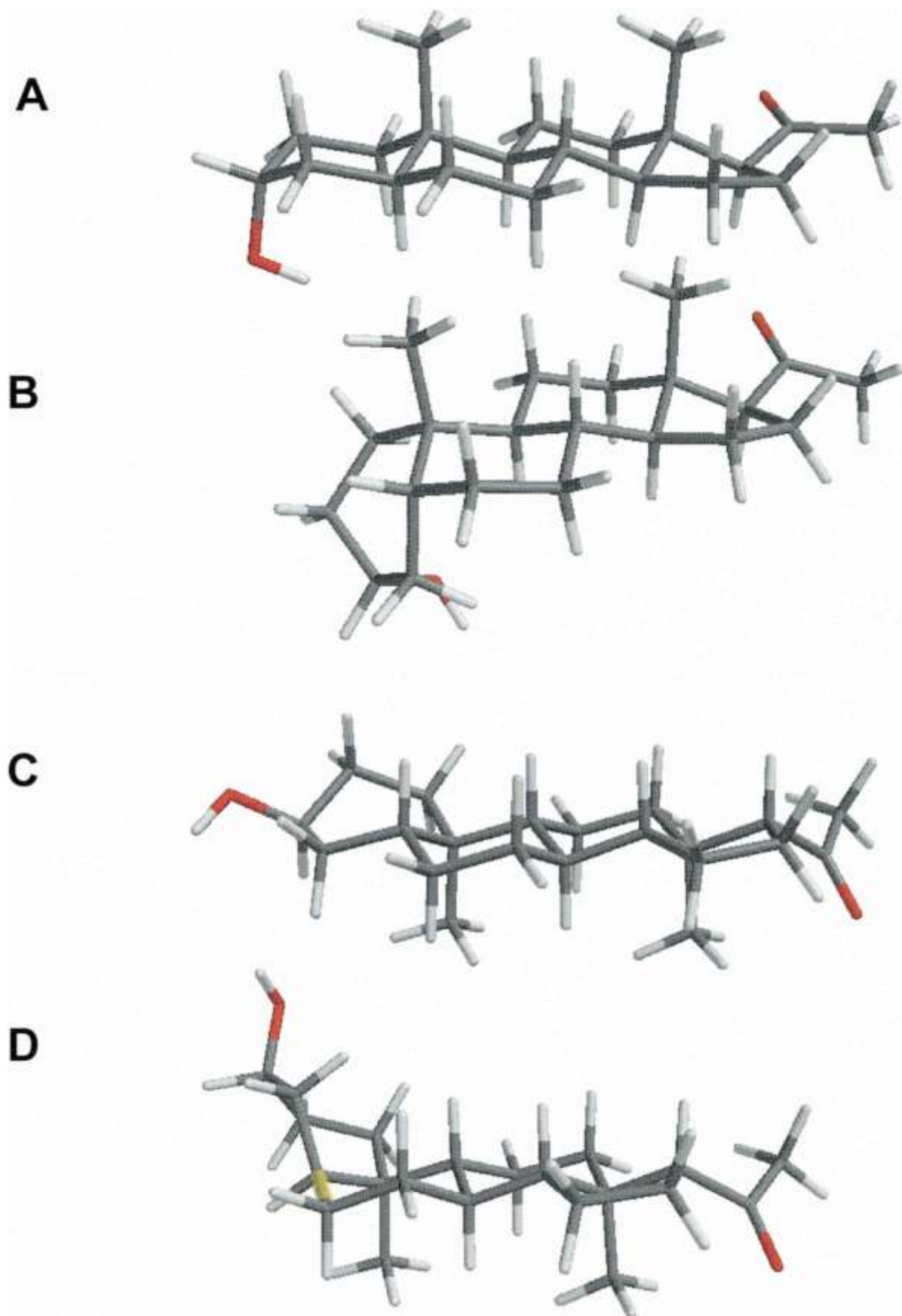


Fig. 2. Chemical structures of  $3\alpha,5\alpha$ -TH PROG (A),  $3\alpha,5\beta$ -TH PROG (B) and their respective enantiomers *ent*- $3\alpha,5\alpha$ -TH PROG (C) and *ent*- $3\alpha,5\beta$ -TH PROG (D).

solubility in water has little effect upon activity at the GABA<sub>A</sub> receptor [2,44,83]. It is clear that the steroid binding site of the GABA<sub>A</sub> receptor can tolerate rather bulky substituents at the 2 $\beta$ -position, because even a 2,6-dibutyl morpholinyl derivative of ORG 21465 is accommodated without loss of potency [2]. Unfortunately, although water-soluble steroidal anaesthetics demonstrated promise in animal models, clinical testing has revealed either pharmacokinetic, or side-effect, profiles that are unacceptable in humans, halting, at least at present, clinical development of this class of agent as general anaesthetic compounds.

## 7. Multiple steroid binding sites upon the GABA<sub>A</sub> receptor

The existence of multiple GABA<sub>A</sub> receptor isoforms may provide for binding sites at which steroids display differing affinities and/or efficacies. A recent study examining the effect of synthetic enantiomers of 3 $\alpha$ ,5 $\alpha$ -TH PROG and 3 $\alpha$ ,5 $\beta$ -TH PROG provides evidence for such heterogeneity in steroid action [24]. Across in vivo and in vitro assays that included loss of the righting reflex in tadpoles and mice, displacement of [<sup>35</sup>S]TBPS binding from rat brain membranes and potentiation of GABA-evoked currents recorded from rat hippocampal neurones, a robust and qualitatively consistent enantioselectivity of action was observed for the naturally occurring 3 $\alpha$ ,5 $\alpha$ -TH PROG versus the synthetic *ent*-3 $\alpha$ ,5 $\alpha$ -TH PROG [24,93] (Fig. 2). In addition, the enantiomers of the benz[e]indene BI-1 [98] and 17 $\beta$ -carbonitrile-substituted 5 $\alpha$ -androstanes [i.e. 3 $\alpha$ 5 $\alpha$ ACN [(3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile] and *ent*-3 $\alpha$ 5 $\alpha$ ACN [(3 $\beta$ ,5 $\beta$ ,8 $\alpha$ ,9 $\beta$ ,10 $\alpha$ ,13 $\beta$ ,14 $\alpha$ ,17 $\alpha$ )-3-hydroxyandrostane carbonitrile] [24,93], exhibit a similar correlation between GABA-modulatory activity and anaesthetic potency. These observations provide the most convincing evidence to date that certain pregnane steroids associate directly with the GABA<sub>A</sub> receptor, because enantiomers act dissimilarly only in a chiral (e.g. protein) environment. Hence, it is significant that the degree of enantioselectivity observed for 3 $\alpha$ ,5 $\beta$ -TH PROG versus *ent*-3 $\alpha$ ,5 $\beta$ -TH PROG and 3 $\alpha$ ,5 $\beta$ ACN versus *ent*-3 $\alpha$ ,5 $\beta$ ACN is vastly reduced across the same battery of assays. As expected from numerous studies (reviewed in [54]), little diastereoselectivity was observed between the epimers 3 $\alpha$ ,5 $\alpha$ -TH PROG and 3 $\alpha$ ,5 $\beta$ -TH PROG [24]. At least two interpretations of these data are possible: (i) the binding pocket on the GABA<sub>A</sub> receptor cannot accommodate *ent*-3 $\alpha$ ,5 $\alpha$ -TH PROG or (ii) that the pair 3 $\alpha$ ,5 $\beta$ -TH PROG/*ent*-3 $\alpha$ ,5 $\beta$ -TH PROG bind to a site distinct from that recognising 3 $\alpha$ ,5 $\alpha$ -TH PROG and *ent*-3 $\alpha$ ,5 $\alpha$ -TH PROG.

Most recently, it has been reported that the anabolic steroids 17 $\alpha$ -methyltestosterone, stanozolol and nandrolone also exert a rapid, non-genomic, modulation of

GABA<sub>A</sub> receptor activity [49]. In view of the selectivity of these anabolic steroids for particular isoforms of the GABA<sub>A</sub> receptor, coupled with their structures not possessing the crucial 3 $\alpha$ -hydroxyl group, it would appear most unlikely that they share a common site of action with the pregnane compounds discussed above and hence an additional steroid binding site on the GABA<sub>A</sub> receptor may be invoked.

## 8. Concluding remarks

Neurosteroids such as 3 $\alpha$ ,5 $\alpha$  TH PROG are clearly established as the most potent endogenous modulators of GABA<sub>A</sub> receptor-mediated inhibitory neurotransmission. The demonstration that de novo neurosteroid synthesis both in glial and neuronal cells results in levels sufficient to modulate GABA<sub>A</sub> receptor function argues in favour of a physiological/pathophysiological role for these locally released modulators. Although the pharmacological selectivity of neurosteroids versus different GABA<sub>A</sub> receptor isoforms is not as stringent as that displayed by benzodiazepines, 'physiological' concentrations of 3 $\alpha$ ,5 $\alpha$  TH PROG (i.e. 3–100 nM) appear to discriminate amongst different recombinant and native GABA<sub>A</sub> receptor subtypes. Furthermore, it is becoming apparent that additional factors (i.e. phosphorylation and metabolism) contribute to the degree of selectivity of neurosteroid action. Future investigations will determine whether these novel regulatory mechanisms might provide targets of therapeutic intervention.

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