Sites of positive allosteric modulation by neurosteroids on ionotropic γ-aminobutyric acid receptor subunits

Susumu Ueno[a,*], Masato Tsutsui[a], Yumiko Toyohira[a], Kouichiro Minami[b], Nobuyuki Yanagihara[a]

[a]*Department of Pharmacology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishiku, Kitakyushu, Fukuoka 807-8555, Japan*
[b]*Department of Anesthesiology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishiku, Kitakyushu, Fukuoka 807-8555, Japan*

Received 7 April 2004; accepted 14 April 2004

Available online 27 April 2004

Edited by Jesus Avila

Abstract Neurosteroids are known as allosteric modulators of ionotropic γ-aminobutyric acid (GABA) receptors. Here, we investigated sites of positive allosteric modulation by allotetrahydrodeoxycorticosterone (5α-THDOC) at GABA receptors using the technique of chimeragenesis and the *Xenopus* oocyte expression system. Our findings have demonstrated that the region from transmembrane segment (TM) 4 to the C-terminus of the $GABA_A$ receptor α1 subunit is crucial for the action of 5α-THDOC, but insufficient for the action of another neurosteroid allopregnanolone, suggesting that a specific region critical for neurosteroid action at GABA receptors exists in the domain between TM4 and the C-terminus of GABA receptor subunits.

Keywords: $GABA_A$ receptor; $GABA_C$ receptor; Glycine receptor; Neurosteroid; Chimera; *Xenopus* oocyte

1. Introduction

In the mammalian central nervous system (CNS), fast inhibitory synaptic transmission is mediated mainly by the γ-aminobutyric $acid_A$ ($GABA_A$) receptor, a member of the ligand-gated ion channel superfamily, whose members include glycine receptors, nicotinic acetylcholine (nACh) receptors, and serotonin type 3 receptors [1]. To date, a number of clinically important drugs such as benzodiazepines, barbiturates, and volatile and intravenous anesthetics are known to act as positive allosteric modulators of $GABA_A$ receptors, and their sites of actions on $GABA_A$ receptor subunits have been investigated at the molecular level using site-directed mutagenesis [2].

Neurosteroids (neuroactive steroids) are synthesized de novo in the CNS and may alter neuronal excitability via the cell surface through an interaction with specific neurotransmitter receptors [3]. Many studies have demonstrated that neurosteroids positively and negatively modulate the function of members of the ligand-gated ion channel superfamily [4], and most of those studies have focused on the positive allosteric actions at $GABA_A$ receptors. It has been suggested that, as for other allosteric modulators, there may be a specific region for neurosteroid binding on the $GABA_A$ receptor molecule, a theory supported by previous reports that the interaction of neurosteroids with the $GABA_A$ receptor is dependent on subunit composition [5,6].

The $GABA_C$ receptor, another type of ionotropic GABA receptor, is composed of GABA ρ1 and/or ρ2 subunits as a pentamer. This receptor, which is mainly expressed in the retina, has channel function and drug sensitivity properties different from the $GABA_A$ receptor [7]. In particular, the $GABA_C$ receptor has been thought to be insensitive to most potentiators of $GABA_A$ receptors, including neurosteroids. In a recent study, however, allotetrahydrodeoxycorticosterone (5α-THDOC), a metabolite of corticosterone that produces anticonvulsant [8], anxiolytic [9–11], and sleep-modulating effects [12,13] in vivo due to a positive allosteric modulation of $GABA_A$ receptor function, has been demonstrated to potentiate the GABA-induced current in homomeric GABA ρ1 receptors at micromolar concentrations [14]. Although the concentration of neurosteroid used in that study was higher than that used in previous studies of $GABA_A$ receptors, this result suggests that the $GABA_C$ receptor is also modulated by neurosteroids, raising the possibility that there may be a common region of the $GABA_A$ and $GABA_C$ receptor subunits specific for the action of 5α-THDOC.

The sites of neurosteroid action on $GABA_A$ receptor subunits remain to be clarified at the amino acid level. In an attempt to determine these sites, chimeric receptor proteins comprised of $GABA_A$ receptor β subunits and the glycine receptor α subunit were constructed previously [15], based on evidence that the homomeric glycine α receptor is insensitive to several positive allosteric modulators of $GABA_A$ receptors, including neurosteroids [16,17], whereas the homomeric $GABA_A$ β receptor can be modulated by a neurosteroid anesthetic, alphaxalone [18]. This approach, however, needs further refinement, because the homomeric $GABA_A$ β receptor has unusual channel properties that may complicate investigation of the action of neurosteroids using chimeric $GABA_A$-receptor-β/glycine-receptor-α subunit proteins.

In the present study, we first investigated the action of 5α-THDOC, applying the approach of expressing homomeric receptors composed of chimeric proteins containing the GABA receptor ρ and glycine receptor α subunits. If critical regions of

* Corresponding author. Fax: +81-93-601-6264.
E-mail address: susumu@med.uoeh-u.ac.jp (S. Ueno).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.030

the GABA receptor ρ subunit were identified, the corresponding regions of the $GABA_A$ receptor subunits could also be demonstrated to be important for the action of neurosteroids.

2. Materials and methods

2.1. Materials

Adult female *Xenopus laevis* were obtained from Seac Yoshitomi (Fukuoka, Japan); GABA, 5α-THDOC, allopregnanolone, collagenase type 1A, penicillin–streptomycin solution, and gentamicin solution were from Sigma Chemical Co. (St. Louis, MO); glycine was from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of reagent grade. Our study was reviewed and approved by the Ethics Committee on Animal Experiments and Care of the University of Occupational and Environmental Health.

2.2. Construction of chimeric cDNAs

Chimeras C1 and C2 were constructed by methods described previously [19]. The C1 chimera was composed of the human glycine α1 (Glyα1) receptor subunit sequence from the N-terminus to the junction site in transmembrane segment (TM) 3 and the human GABA receptor ρ1 subunit sequence from the junction site to the C-terminus. C2 was the converse chimera of C1, consisting of the GABA receptor ρ1 subunit sequence from the N-terminus to the junction site and the Glyα1 receptor subunit from the junction site to the C-terminus. To construct other chimeras, the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce a *Cla*I restriction site into the cDNA sequence encoding the conserved amino acid sequence "ID" close to the beginning of TM4 in the human GABA receptor ρ1, human Glyα1 receptor, and human $GABA_A$ receptor α1 subunit (see Fig. 3), respectively, as subcloned into a modified pBK-CMV vector [20]. These chimeras have the Glyα1 receptor subunit sequence from the N-terminus to the junction sites located just before TM4, and the GABA receptor ρ1 (C8) or the $GABA_A$ receptor α1 (C9) subunit sequence from the junction site to the C-terminus. All point mutations were verified by double-stranded DNA sequencing.

2.3. Expression of chimeric receptors in Xenopus oocytes and electrophysiological recordings

Stage V and VI oocytes from adult female *X. laevis* were isolated, injected with 1.5–2.0 ng cDNA of wild-type human GABA ρ1, human Glyα1, human $GABA_A$ α1β2γ2S or chimeric receptor subunit, and two-electrode voltage-clamp recordings were performed as described previously [21,22]. Oocytes were voltage-clamped at −70 mV using the OC-725C Oocyte Clamp Amplifier (Warner Instruments, Inc., Hamden, CT), and currents were digitally recorded with PowerLab/200 and Chart software (ADInstruments, Grand Junction, CO). Glycine or GABA was applied for 30–180 s to obtain the maximum (peak) current used as a measure of drug response. We tested the capacity of neurosteroids to enhance the effect of the glycine/GABA concentration that produced 10% of the maximal effect (EC_{10}) of glycine/GABA. This EC_{10} was determined individually for each oocyte. We used 1–3 mM glycine/GABA to produce a maximal current. Neurosteroids were first dissolved in dimethyl sulfoxide (DMSO), then diluted in modified Barth's saline before use at a final DMSO concentration not exceeding 0.05%, which had no effect on agonist-evoked responses (data not shown). Oocytes were perfused with neurosteroids for 2 min before coapplication of glycine/GABA to allow for complete equilibration of the oocytes with neurosteroids. In all cases, a 15–20-min washout period was allowed following application of the neurosteroid/agonist solutions.

2.4. Data analysis

Each data point represents the mean ± S.E.M. from 4 to 38 oocytes obtained from at least two different frogs.

3. Results and discussion

3.1. The action of 5α-THDOC on C1 and C2 chimeric receptors expressed in Xenopus oocytes

We first studied the effect of micromolar concentrations of 5α-THDOC on $GABA_C$ and glycine receptor function. For wild-type GABA ρ1 receptors expressed in *Xenopus* oocytes, 20 μM 5α-THDOC produced up to about 110% potentiation of the current induced by the EC_{10} of GABA (Figs. 1A and 2), which is consistent with the evidence demonstrated previously [14]. In contrast, at wild-type Glyα1 receptors 5α-THDOC did not show much potentiation (up to about 45%) of the current induced by the EC_{10} of glycine (Figs. 1A and 2). Since both GABA ρ1 and Glyα1 receptor subunits are members of the ligand-gated ion channel superfamily, share similar structure, and form functional homomeric receptors, construction of chimeric proteins between GABA ρ1 and Glyα1 subunits should be useful for the investigation of sites of 5α-THDOC action.

Therefore, to examine which regions are critical for the enhancing effect of 5α-THDOC, we used chimeras C1 and C2, which were constructed previously ([19]; Fig. 1B). Both are functional when expressed homomerically in oocytes and activated by glycine and GABA, respectively. At C1, 20 μM of 5α-THDOC produced about 180% potentiation of the EC_{10} glycine-induced current (Figs. 1C and 2). The converse chimera C2, however, produced only as much potentiation as observed with the wild-type Glyα1 receptor (Figs. 1C and 2).

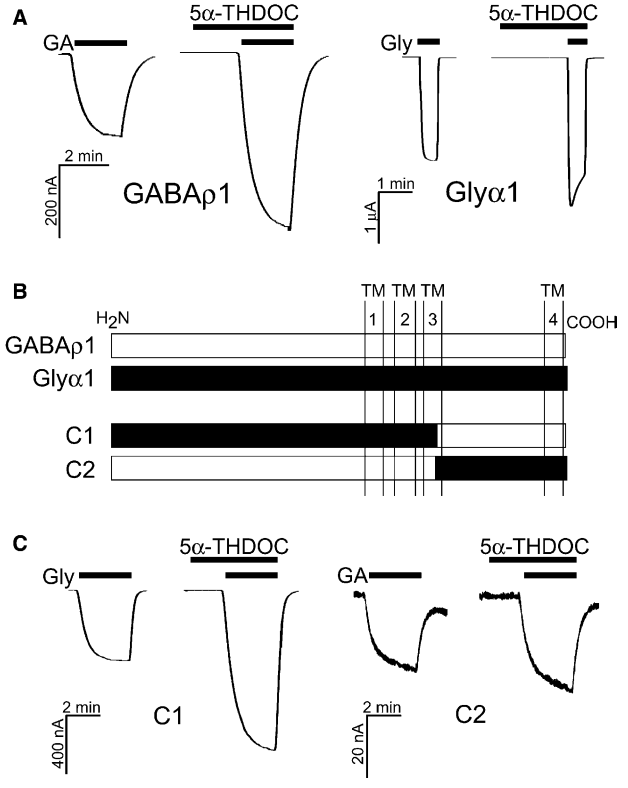

Fig. 1. (A) Sample tracings obtained from a single *Xenopus* oocyte expressing wild-type GABA ρ1 or glycine α1 (Glyα1) receptors homomerically demonstrate the effects of 5α-THDOC (20 μM) on currents induced by the EC_{10} of GABA (GA) or glycine (Gly). 5α-THDOC was preapplied for 2 min before co-application of agonists. (B) Schematic representations show the construction of the wild-type GABA receptor ρ1 (open bars) or the Glyα1 receptor subunit (closed bars), and the C1 or C2 chimeric subunit, respectively, which have been described previously [19]. (C) Sample tracings obtained from a single *Xenopus* oocyte expressing C1 or C2 chimeric receptors homomerically demonstrate the effects of 5α-THDOC (20 μM) on currents induced by the EC_{10} of GABA (GA) or glycine (Gly). 5α-THDOC was preapplied for 2 min before co-application of agonists.

We also tested lower concentrations (5 and 10 μM) of 5α-THDOC at wild-type and chimeric receptors. Significant potentiation was not observed at wild-type Glyα1 and C2 chimeric receptors, but much greater potentiation was produced at wild-type GABA ρ1 and C1 chimeric receptors (Fig. 2). These results suggest that the region from TM3 to the C-terminus of the GABA ρ1 subunit contains residues critical for the enhancing effect of 5α-THDOC.

3.2. Construction of new chimeric subunit cDNAs and their functional expression in Xenopus oocytes

We next constructed new chimeras, C8 and C9, to investigate in more detail the region critical for the action of 5α-THDOC (Fig. 3). We hypothesized that such action of 5α-THDOC, that is, a non-genomic effect of 5α-THDOC on GABA receptor function, would involve in the extracellular segments including TM4, rather than the intracellular segments of the receptor subunits. Therefore, we constructed those chimeras whose junction site is located close to the beginning of TM4 of the subunits. The cDNAs of those chimeras were individually injected into oocytes and tested with glycine to assay for functional expression. Although both chimeras have extracellular glycine-binding domains, no significant glycine-evoked response was obtained from oocytes expressing C8 chimeric receptors (Fig. 4). This could be because this chimeric receptor protein could not, if homomerically expressed, form a functional chloride channel or be inserted into the plasma membrane. Since the $GABA_A$ receptor is a main target for the actions of neurosteroids in vivo, the goal of the present study was to determine the sites of neurosteroid action on $GABA_A$ receptor subunits as well as GABA ρ1 receptors. Therefore, we studied the chimera C9, which had the same construct from the N-terminus to the junction site as C8 but had the $GABA_A$ receptor α1 subunit from the junction site to the C-terminus. C9 chimeric receptors expressed in oocytes produced an inward current with glycine, with a fast peak time, similar to the one for the wild-type Glyα1 receptor (Fig. 4A, inset), although the EC_{10} glycine concentration was higher (188 ± 20 μM), compared to the wild-type receptor (103 ± 6 μM).

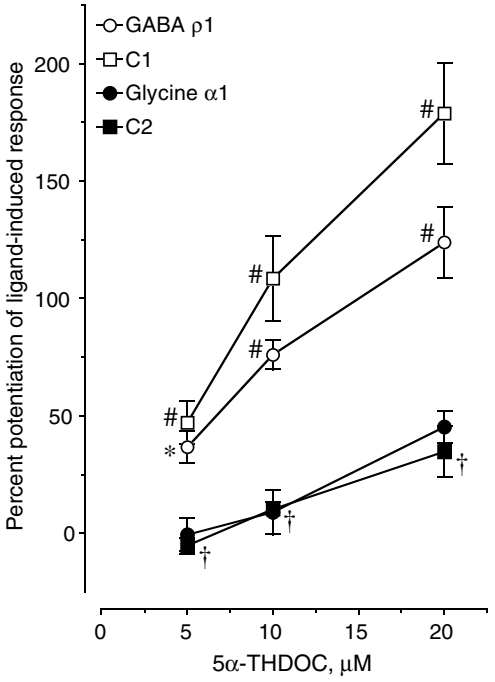

Fig. 2. The effects of various concentrations of 5α-THDOC on GABA-evoked responses at wild-type GABA ρ1 (open circles) and C2 chimeric receptors (closed squares), and glycine-evoked responses at wild-type Glyα1 (closed circles) and C1 chimeric receptors (open squares) expressed in *Xenopus* oocytes. The values of the EC_{10} concentrations used in this study were as follows: 0.34 ± 0.02 μM of GABA for wild-type GABA ρ1 receptor, 28 ± 4 μM of glycine for wild-type Glyα1 receptor, 103 ± 6 μM of glycine for C1, and 0.40 ± 0.01 μM of GABA for C2. The EC_{10} of GABA for C2 is significantly different ($P < 0.01$, using unpaired, two-tailed *t*-tests) from that for wild-type GABA ρ1 receptor, and the EC_{10} of glycine for C1 is also significantly different ($P < 0.001$, using unpaired, two-tailed *t*-tests) from that for wild-type Glyα1 receptor. All values are presented as means ± S.E.M. from 5 to 11 oocytes. $^{*}P < 0.01$ and $^{\#}P < 0.001$, compared to wild-type Glyα1 receptor, and $^{\dagger}P < 0.001$ compared to wild-type GABA ρ1 receptors using unpaired, two-tailed *t*-tests.

3.3. The action of 5α-THDOC and allopregnanolone on C9 chimeric receptors

Using an EC_{10} of glycine, we obtained about 140% potentiation of the glycine-evoked response at C9 chimeric receptors (Fig. 4A). These results suggest that the region from TM4 to the C-terminus of the $GABA_A$ receptor α1 subunit is critical for the action of 5α-THDOC. The corresponding region of the GABA ρ1 receptor subunit could also be involved in the action of 5α-THDOC, although we did not obtain the results from the chimeric C8 receptor. To our knowledge, this is the first evidence that the region from TM4 to the C-terminus of the GABA receptor subunit is important for neurosteroid action.

We then tested the ability of allopregnanolone, a progesterone metabolite and a potent positive modulator of $GABA_A$ receptors (Fig. 4B, inset), to potentiate the glycine response of the C9 chimera. 20 μM of allopregnanolone, however, produced only $33 \pm 12\%$ potentiation of the glycine-evoked currents at this chimera, which was similar to that obtained from the wild-type Glyα1 receptor (Fig. 4B), suggesting that the region from TM4 to the C-terminus of the $GABA_A$ receptor α subunit may not be sufficient for the action of allopregnanolone. This is consistent with the evidence that alphaxalone, a neurosteroid anesthetic, may act on $GABA_A$ receptors via the N-terminal side of TM2 of the α subunit [15] or a residue located in TM1 of the β subunit [23], since alphaxalone is a synthetic analog of allopregnanolone and has similar efficacy at $GABA_A$ receptors [24]. Moreover, as demonstrated in the present study (Fig. 4B) as well as previous work [14], allopregnanolone and alphaxalone do not enhance GABA ρ1 receptor function as much as 5α-THDOC, suggesting that allopregnanolone and alphaxalone may also act on $GABA_A$ and GABA ρ1 receptors via a mechanism different from 5α-THDOC. Further investigation is required to determine the region critical for the actions of allopregnanolone and alphaxalone at $GABA_A$ and $GABA_C$ receptors.

3.4. A possible interaction of the region from TM4 to the C-terminus of GABA receptor subunits with neurosteroids

The region from TM4 to the C-terminus of the subunit of the ligand-gated ion channel superfamily has been demonstrated to be involved in gating and allosteric modulation of channel function. For example, TM4 may be involved in affinity for acetylcholine and channel gating in mouse nicotinic and *Torpedo* acetylcholine receptors [25,26]. Moreover,

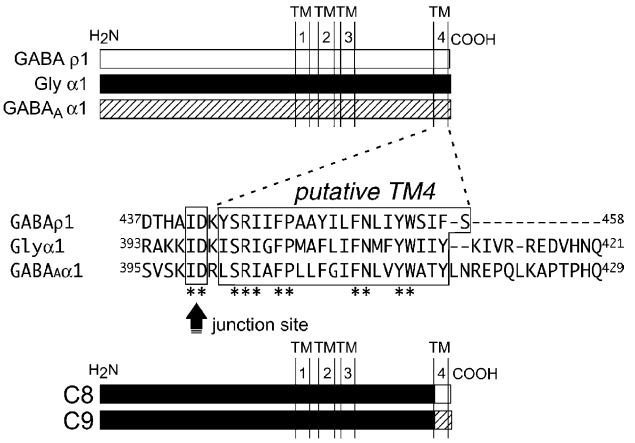

Fig. 3. Schematic representations demonstrate the construction of chimeras C8 and C9. The construction of the wild-type human GABA receptor ρ1 (open bars), human Glyα1 receptor (closed bars), and human $GABA_A$ receptor α1 (hatched bars) subunit (upper panel), and amino acid sequences around the TM4 region (including the C-terminus) of those subunits are shown, respectively (middle panel). Asterisks indicate the conserved amino acids among those subunits. To construct C8 and C9 chimeras, a unique restriction enzyme site was introduced into the cDNA sequence encoding the conserved amino acid sequence "ID" close to the beginning of TM4. The constructions of the resultant chimeric subunits are also shown schematically (lower panel).

Jenkins et al. [27] have recently suggested that the residues at the extracellular end of TM4 of the $GABA_A$ receptor α subunit may be important for the positive modulatory effects of inhaled anesthetics. Interestingly, it has been suggested that the C-terminal tail of the neuronal nACh receptor α4 subunit is required for potentiation by estradiol, acting as a steroid-binding site [28,29]. This may strongly support our results, suggesting that the corresponding regions of the $GABA_A$ and $GABA_C$ receptor subunits may also act as steroid-binding sites. Estradiol, however, has not been reported to be an allosteric modulator of $GABA_A$ and $GABA_C$ receptors, and not many amino acid residues are conserved in the TM4-to-carboxyl terminus region between the $GABA_A$/GABA ρ1 receptor and neuronal nACh receptor subunits. Therefore, further studies are required to investigate if, in the ligand-gated ion channel superfamily, the TM4-to-carboxyl terminus regions of the receptor subunits are involved in the potency and efficacy of different neurosteroids in the allosteric modulation of receptor function.

In conclusion, we demonstrate that there are regions specific for the action of neurosteroids at $GABA_A$ and $GABA_C$ receptor subunits and that one candidate may be located from TM4 to the C-terminus in the $GABA_A$ receptor α subunit. Our observations should help provide a better understanding of a structure–function relationship between neurosteroids and receptor subunits of members of the ligand-gated ion channel superfamily.

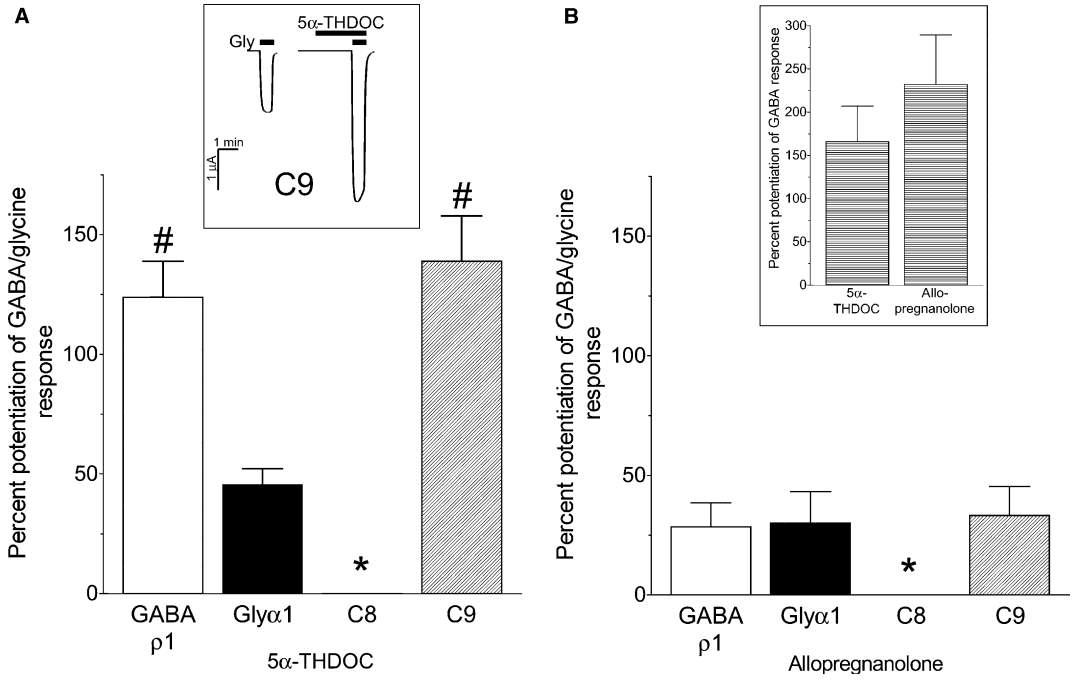

Fig. 4. The effects of 20 μM of 5α-THDOC (A) and allopregnanolone (B) on GABA or glycine-evoked responses in wild-type GABA ρ1 (open bar), Glyα1 (closed bar), and C9 chimeric receptors (hatched bar) expressed in *Xenopus* oocytes. Note that no glycine-evoked current was obtained from oocytes expressing C8 chimeric receptors (asterisks). The EC_{10} concentration of glycine for C9 is 188 ± 20 μM, which is significantly different ($P < 0.001$, using unpaired, two-tailed *t*-tests) from that for wild-type Glyα1 receptor. All values are presented as means ± S.E.M. from 4 to 12 oocytes. The data in (A) for the GABA ρ1 and Glyα1 receptors are from Fig. 2. $^{\#}P < 0.001$, compared to wild-type Glyα1 receptor using unpaired, two-tailed *t*-tests. Inset in (A), sample tracings obtained from a single oocyte expressing C9 chimeric receptors homomerically demonstrate the effects of 5α-THDOC on currents induced by the EC_{10} of glycine (Gly). 5α-THDOC (20 μM) was preapplied for 2 min before co-application of glycine. Inset in (B), the effects of 5α-THDOC and allopregnanolone on GABA-induced responses in wild-type $GABA_A$ α1β2γ2S receptors. Note that the concentration of both neurosteroids used is 1 μM. All values are presented as means ± S.E.M. from five oocytes.

Acknowledgements: The authors are grateful to Drs. George R. Uhl, Peter R. Schofield, Paul J. Whiting, and Neil L. Harrison for providing cDNAs used in this study, and Dr. R. Adron Harris for helpful suggestions. This work was supported by a Grant-in-Aid for Encouragement of Young Scientists from JSPS (12770052) and a University of Occupational and Environmental Health (UOEH) Research Grant for Promotion of Occupational Health to S.U.

References

[1] Ortells, M.O. and Lunt, G.G. (1995) Trends Neurosci. 18, 121–127.
[2] Olsen, R.W. (1998) Toxicol. Lett. 100–101, 193–201.
[3] Paul, S.M. and Purdy, R.H. (1992) FASEB J. 6, 2311–2322.
[4] Rupprecht, R. and Holsboer, F. (1999) Trends Neurosci. 22, 410–416.
[5] Hill-Venning, C., Belelli, D., Peters, J.A. and Lambert, J.J. (1997) Br. J. Pharmacol. 120, 749–756.
[6] Belelli, D., Casula, A., Ling, A. and Lambert, J.J. (2002) Neuropharmacology 4, 651–661.
[7] Zhang, D., Pan, Z.H., Awobuluyi, M. and Lipton, S.A. (2001) Trends Pharmacol. Sci. 22, 121–132.
[8] Kokate, T.G., Cohen, A.L., Karp, E. and Rogawski, M.A. (1996) Neuropharmacology 35, 1049–1056.
[9] Crawley, J.N., Glowa, J.R., Majewska, M.D. and Paul, S.M. (1986) Brain Res. 398, 382–385.
[10] Wieland, S., Lan, N.C., Mirasedeghi, S. and Gee, K.W. (1991) Brain Res. 565, 263–268.
[11] Rodgers, R.J. and Johnson, N.J. (1998) Pharmacol. Biochem. Behav. 59, 221–232.
[12] Mendelson, W.B., Martin, J.V., Perlis, M., Wagner, R., Majewska, M.D. and Paul, S.M. (1987) Psychopharmacology (Berlin) 93, 226–229.
[13] Müller-Preuss, P., Rupprecht, R. and Lancel, M. (2002) Neuroreport 13, 487–490.
[14] Morris, K.D., Moorefield, C.N. and Amin, J. (1999) Mol. Pharmacol. 56, 752–759.
[15] Rick, C.E., Ye, Q., Finn, S.E. and Harrison, N.L. (1998) Neuroreport 9, 379–383.
[16] Pistis, M., Belelli, D., Peters, J.A. and Lambert, J.J. (1997) Br. J. Pharmacol. 122, 1707–1719.
[17] Belelli, D., Pistis, M., Peters, J.A. and Lambert, J.J. (1999) Neurochem. Int. 34, 447–452.
[18] Sanna, E., Garau, F. and Harris, R.A. (1995) Mol. Pharmacol. 47, 213–217.
[19] Mihic, S.J., Ye, Q., Wick, M.J., Koltchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R.A. and Harrison, N.L. (1997) Nature 389, 385–389.
[20] Wick, M.J., Mihic, S.J., Ueno, S., Mascia, M.P., Trudell, J.R., Brozowski, S.J., Ye, Q., Harrison, N.L. and Harris, R.A. (1998) Proc. Natl. Acad. Sci. USA 95, 6504–6509.
[21] Ueno, S., Lin, A., Nikolaeva, N., Trudell, J.R., Mihic, S.J., Harris, R.A. and Harrison, N.L. (2000) Br. J. Pharmacol. 131, 296–302.
[22] Beckstead, M.J., Weiner, J.L., Eger 2nd, E.I., Gong, D.H. and Mihic, S.J. (2000) Mol. Pharmacol. 57, 1199–1205.
[23] Carlson, B.X., Engblom, A.C., Kristiansen, U., Schousboe, A. and Olsen, R.W. (2000) Mol. Pharmacol. 57, 474–484.
[24] Maitra, R. and Reynolds, J.N. (1999) Brain Res. 819, 75–82.
[25] Lee, Y.H., Li, L., Lasalde, J., Rojas, L., McNamee, M., Ortiz-Miranda, S.I. and Pappone, P. (1994) Biophys. J. 66, 646–653.
[26] Tamamizu, S., Lee, Y., Hung, B., McNamee, M.G. and Lasalde-Dominicci, J.A. (1999) J. Membr. Biol. 170, 157–164.
[27] Jenkins, A., Andreasen, A., Trudell, J.R. and Harrison, N.L. (2002) Neuropharmacology 43, 669–678.
[28] Paradiso, K., Zhang, J. and Steinbach, J.H. (2001) J. Neurosci. 21, 6561–6568.
[29] Curtis, L., Buisson, B., Bertrand, S. and Bertrand, D. (2002) Mol. Pharmacol. 61, 127–135.