



Published in final edited form as:

Psychopharmacology (Berl). 2014 September ; 231(17): 3479–3491. doi:10.1007/s00213-014-3568-4.

11-TRIFLUOROMETHYL-PHENYLDIAZIRINYL NEUROSTEROID ANALOGUES: POTENT GENERAL ANESTHETICS AND PHOTOLABELING REAGENTS FOR GABA_A RECEPTORS

Zi-Wei Chen^{1,5}, Cunde Wang³, Kathiresan Krishnan³, Brad D. Manion¹, Randy Hastings¹, John Bracamontes¹, Amanda Taylor³, Megan M. Eaton¹, Charles F. Zorumski^{3,4,5}, Joseph H. Steinbach^{1,4,5}, Gustav Akk^{1,5}, Steven Mennerick^{3,4,5}, Douglas F. Covey^{1,2,3,5}, and Alex S. Evers^{1,2,5}

¹Department of Anesthesiology, Washington University in St. Louis, St. Louis, Missouri 63110

²Department of Developmental Biology, Washington University in St. Louis, St. Louis, Missouri 63110

³Department of Psychiatry, Washington University in St. Louis, St. Louis, Missouri 63110

⁴Department of Anatomy and Neurobiology, Washington University in St. Louis, St. Louis, Missouri 63110

⁵Department of the Taylor Family Institute for Innovative Psychiatric Research, Washington University in St. Louis, St. Louis, Missouri 63110

Abstract

Rationale—While neurosteroids are well-described positive allosteric modulators of GABA_A receptors, the binding sites that mediate these actions have not been definitively identified.

Objectives—To synthesize neurosteroid analogue photolabeling reagents that closely mimic the biological effects of endogenous neurosteroids and have photochemical properties that will facilitate their use as tools for identifying the binding sites for neurosteroids on GABA_A receptors.

Results—Two neurosteroid analogues containing a trifluoromethyl-phenyldiazirine group linked to the steroid C11 position were synthesized. These reagents, CW12 and CW14, are analogues of allopregnanolone (5 α -reduced steroid) and pregnanolone (5 β -reduced steroid), respectively. Both reagents were shown to have favorable photochemical properties with efficient insertion into the C–H bonds of cyclohexane. They also effectively replicated the actions of allopregnanolone and pregnanolone on GABA_A receptor functions: they potentiated GABA-induced currents in *Xenopus laevis* oocytes transfected with $\alpha_1\beta_2\gamma_2L$ subunits, modulated [³⁵S]*t*-butylbicyclophosphorothionate binding in rat brain membranes and were effective anesthetics in *Xenopus* tadpoles. Studies using [³H]CW12 and [³H]CW14 showed that these reagents covalently label GABA_A receptors in both rat brain membranes and in TSA cells expressing either α_1 and β_2 subunits or β_3 subunits of the

GABA_A receptor. Photolabeling of rat brain GABA_A receptors was shown to be both concentration-dependent and stereospecific.

Conclusions—CW12 and CW14 have the appropriate photochemical and pharmacological properties for use as photolabeling reagents to identify specific neurosteroid binding sites on GABA_A receptors.

Keywords

Neurosteroids; photolabeling; GABA_A receptors

Introduction

Certain endogenous pregnane steroids and their synthetic analogues (broadly termed neurosteroids) are potent, rapidly acting intravenous anesthetics in vertebrates (Atkinson et al. 1965; Gyermek and Soyka 1975; Selye 1941). The sedative/anesthetic actions of neurosteroids have been shown to be mediated by GABA_A receptors (Harrison et al. 1987). At low concentrations, neurosteroids potentiate the effect of GABA on GABA_A receptors whereas at higher concentrations they can also directly activate the ion channel. Evidence from multiple sources indicates that neurosteroid actions are mediated by two (or more) distinct classes of binding sites on the GABA_A receptor: (1) Radioligand binding assays show that neurosteroids allosterically enhance [³⁵S]TBPS binding to GABA_A receptors at low concentrations (NS1 site) and inhibit it at higher concentrations (NS2 site) (Evers et al. 2010; Srinivasan et al. 1999). (2) Single channel electrophysiological studies demonstrate that prototypical neurosteroids such as allopregnanolone (3 α ,5 α P) produce several discrete kinetic effects on $\alpha_1\beta_2\gamma_2$ GABA_A receptors that appear to occur at distinct, non-overlapping steroid concentrations and that specific neurosteroid analogues (e.g. B285, etiocholanolone) produce some, but not all of these single channel effects (Akk et al. 2010; Akk et al. 2007). These data indicate that different neurosteroid binding sites mediate mechanistically distinct components of the potentiation of GABA responses and that neurosteroid analogues can be selective agonists for specific neurosteroid binding sites (Akk et al. 2004; Li et al. 2007). (3) Site-directed mutagenesis studies have shown that mutations in the first (TM1; Q241) and fourth (TM4; Y410 and N407) transmembrane domains (TMDs) of the α_1 subunit of the GABA_A receptor prevent both neurosteroid potentiation and activation, whereas mutations in the TM1 (T236) region of the α_1 subunit and the TM3 (Y284) region of the β_2 subunit selectively prevent direct activation (Hosie et al. 2006). A model based on these data proposes a potentiating site between TM1 and TM4 of the α_1 subunit, and an activating site in the cleft between TM1 of the α_1 subunit and TM3 of the β_2 subunit (Hosie et al. 2009b; Hosie et al. 2007). Electrophysiological experiments examining the effects of a variety of structurally modified neurosteroids on GABA_A receptors with various mutations in the putative binding sites do not fully support this model, leaving it unclear whether these mutations represent portions of neurosteroid binding pockets or are sites involved in transduction of neurosteroid binding signal at a different site (Li et al. 2009).

Photoaffinity labeling with neurosteroid analogues provides a way to directly identify neurosteroid binding sites, rather than inferring their existence from changes in functional effects in mutagenesis experiments. We have previously reported on the use of the

neurosteroid analogue photolabeling reagent 6-Azi-pregnanolone (6-AziP) to identify voltage-dependent ion channel-1 (VDAC) (Darbandi-Tonkabon et al. 2003; Darbandi-Tonkabon et al. 2004) and β -tubulin (Chen et al. 2012a) as neurosteroid binding proteins and to identify a neurosteroid binding site on the β_3 subunit of homopentameric GABA_A receptors (Chen et al. 2012b). The use of aliphatic diazirines, such as 6-AziP, as photolabeling reagents has some specific limitations: These reagents preferentially undergo intra- rather than intermolecular reactions limiting their efficiency as photolabeling reagents. Additionally, the mechanism through which aliphatic diazirines photolabel may be through generation of a diazo intermediate rather than through a carbene. Thus, aliphatic diazirines frequently only label nucleophilic amino acids. In contrast, reagents containing a trifluoromethyl-phenyldiazirine (TPD) group generate a carbene following ultraviolet irradiation; this enables them to insert into any C–H bond and to preferentially undergo intermolecular interactions. To address the limitation of 6-AziP, we have synthesized and tested two novel compounds, CW12 and CW14, as neurosteroid analogue photolabeling reagents. These reagents have a TPD group attached via an ether linkage at the C11 position on the steroid backbone. CW12 is a 5 α -reduced steroid (analogue of allopregnanolone), whereas CW14 is 5 β -reduced (analogue of pregnanolone (3 α ,5 β P)) to provide analogues of the two major endogenous neurosteroids, which have been shown to have similar but non-identical effects on GABA_A receptors (Evers et al. 2010).

An effective photolabeling reagent must meet several criteria: First it must have photochemical properties that allow it to efficiently label any amino acid on a protein. Second, the reagent must closely reproduce the physiological effects of the compound it is designed to mimic. In this study we describe the photochemical properties of CW12 and CW14 and their neurosteroid-like effects on GABA_A receptors and tadpoles. We also demonstrate that these reagents photolabel GABA_A receptors in both brain and in a heterologous expression system.

Methods

Synthesis of CW12 and CW14

The synthesis of CW12 (5 α -reduced; compound **9a**) and CW14 (5 β -reduced; compound **9b**) followed the reaction schemes shown in Supplemental Figures 1–2. Spectroscopic and elemental analysis data for CW12 and CW14 can also be found in the Supplemental Materials. The 3,20-dione precursors (compounds **10a** and **10b**) used for preparation of [³H]CW12 and [³H]CW14, were synthesized following the reaction scheme shown in Supplemental Figure 2. The radiosynthesis of [³H]CW12 and [³H]CW14 followed the reaction scheme shown in Supplemental Figure 3. The purity of CW12, CW14 and their tritiated analogues was determined using straight phase TLC. The non-radiolabeled compounds were visualized by charring and migrated as single bands. [³H]CW12 and [³H]CW14 were visualized by autoradiography as single bands that co-migrated with the non-radiolabeled standards (data not shown). These data indicate the purity of CW12 and CW14 and that the borohydride reductions produced the desired radiolabeled compounds and did not produce a diol (reduction of the 20-ketone).

[³H]CW12 was prepared from the 5 α -reduced (3 α)-3-hydroxy-11 α -methyl-11 β -(4-trifluoromethylidiaziriny)benzyloxyprogesterone-3,20-dione as follows: 2 mg of this 3,20 dione was dissolved in ethanol with 25 mCi of [³H]-Na-borohydride (\approx 20 Ci/mmol) and reacted at room temperature for 3 hours in the dark. The reaction was terminated by acidification and the steroidal reaction products extracted into diethyl ether. The products (3 α - and 3 β -epimers) were separated by preparative straight-phase thin layer chromatography (TLC) (hexane:ethyl acetate:: 1:1) and visualized by autoradiography. CW12 was identified by co-migration with a non-radiolabeled standard which was visualized by charring the TLC plate following aerosol application of 5% sulfuric acid/95% ethanol. [³H]CW12 (the 3 α -epimer) was scraped from the plate, eluted from the silica with chloroform:methanol::2:1 and stored in ethanol at -20°C . [³H]-CW14 was prepared in an analogous manner starting with the 5 β -reduced (3 α)-3-hydroxy-11 α -methyl-11 β -(4-trifluoromethylidiaziriny)benzyloxyprogesterone-3,20-dione. The 3 α -epimer is the minor product in the preparation of [³H]CW12, whereas the 3 α -epimer is the major product in the preparation of [³H]CW14 (Supplemental Figure 3).

To characterize the photoreactivity of the photolabeling reagents, [³H]CW12 and [³H]CW14 were dissolved in either ethanol or cyclohexane and irradiated for 5 min using a 450-watt Hanovia medium pressure mercury lamp filtered through a 1.5 cm thick saturated Cu₂SO₄ solution to absorb all light <315 nm (Darbandi-Tonkabon et al. 2003). The samples were then applied to reverse phase silica chromatography plates (Fisher Scientific, Pittsburgh, PA) and developed with a mobile phase of acetonitrile:H₂O::95%:5%. Non-radioactive standards were visualized by charring and tritiated products by autoradiography.

Membrane preparation

Rat brain membranes were prepared from frozen rat brains (Pel Freez Biologicals) using previously described methods (Darbandi-Tonkabon et al. 2003). Briefly, rat brain cortex was homogenized in ice cold 0.32 M sucrose (10 ml/g of tissue) and centrifuged for 10 min at 1,500g. The supernatant was centrifuged for 30 min at 10,000g to obtain the P2 pellet. The P2 pellet was washed with 50 mM K-Phosphate/200 mM NaCl, pH 7.4. The pellet was re-suspended in 50 mM K-Phosphate/200 mM NaCl, pH 7.4 and recollected by centrifugation for 20 min at 10,000g. Aliquots of the membranes were stored at -80°C .

Preparation of α_1 FLAG β_2 and β_3 FLAG GABA_A receptors in TSA cell membranes

Transformed human embryonic kidney (TSA) cells were stably transfected with rat α_1 FLAG and β_2 GABA_A receptor subunits or with rat β_3 FLAG subunits using methods identical to those described for expression of GABA receptors in QT6 cells (Evers et al. 2010). Cells were grown in suspension culture in Dulbecco's modified Eagle's medium/F12 medium containing 50 mM HEPES and 10% fetal bovine serum. TSA cells were harvested and membranes were prepared by using the protocol previously described for preparation of QT6 cell membranes (Evers et al. 2010).

Photolabeling

Photolabeling of rat brain membranes and TSA cell membranes was performed as previously described (Darbandi-Tonkabon et al. 2003). Briefly, membranes (400 μg RBM or

TSA cell) were incubated with [³H]CW12 or [³H]CW14 on ice for 30 min. The samples were then irradiated with UV light for 5 min using the Hanovia photoreactor. The membranes were collected by centrifugation and solubilized with SDS sample buffer (312.5 mM Tris-HCl, 5% SDS, 0.5 M dithiothreitol, 50% glycerol, and 0.1% bromphenol blue) or BN-PAGE loading buffer.

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

BN-PAGE was carried out as previously described (Wittig et al. 2006). Samples were dissolved in 25 mM NaCl, 25 mM Imidazole/HCl, 0.5 mM EDTA, 7% digitonin, 10% glycerol, 1% Coomassie blue G-250 and 100 mM 6-aminohexanoic acid. After centrifugation at 12,000g for 15 min at 4°C, the solubilized proteins were loaded onto 5–13% gradient gels, which were cast using a gradient mixer at 4°C. The gel solutions contained 50 mM BisTris and 500 mM 6-Aminocaproic acid. The cathode buffer contained 50 mM Tricine, 15 mM BisTris, and 0.02% Coomassie blue G-250; and the anode buffer contained 50 mM BisTris, pH7.0. For SDS-PAGE, samples were solubilized in Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue).

Gel slicing

After electrophoresis, the gels were cut into vertical columns and sliced in 1 mm horizontal slices using a DE 113 manual gel slicer (Hoeffer Scientific Instruments, San Francisco, CA). Slices were digested with 4 ml of tissue solubilizer consisting of 3a20TM and TS-2 (ratio 9:1) for 48 hours, and the radioactivity in each slice was determined by scintillation spectrometry (Bureau and Olsen 1993).

Western blotting

Proteins from SDS-PAGE or BN-PAGE gels were transferred onto polyvinylidene fluoride membranes using a semi-dry transfer instrument. For BN-PAGE gels, the transfer solution contained 50 mM Tricine and 15 mM BisTris. For SDS-PAGE gels, the transfer solution contained 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol. The membranes were blocked with 5% dried milk and incubated with antibodies to the α_1 (Santa Cruz, 1:200), γ_2 (Chemicon, 1:200) or β_2/β_3 subunits (bd17; Chemicon, 1:200) of the GABA_A receptor, or to β -tubulin (Santa Cruz, 1:100), or voltage-dependent anion channel-1 (Abcam, 1:200) for 1 h followed by peroxidase-conjugated secondary antibody (Santa Cruz, 1:10,000). Immunoreactive bands were visualized using the ECL-plus western blotting detection system (Amersham Bioscience).

Autoradiography

SDS-PAGE gels were fixed for 30 minutes in isopropanol: water: acetic acid (25:65:10) at room temperature and then dried under vacuum. The dried gels were placed in cassettes and exposed to [³H]-sensitive ultra-film (Kodak Biomax light film) at -70 °C for periods ranging from five days to two weeks (Darbandi-Tonkabon et al. 2003; Darbandi-Tonkabon et al. 2004).

[³⁵S]t-butylbicyclophosphorothionate ([³⁵S]TBPS) binding

[³⁵S]TBPS binding assays were performed using previously described methods (Covey et al. 2000; Hawkinson et al. 1994) with modification. Briefly, aliquots of membrane suspension (0.5 mg/ml final protein concentration in assay) were incubated with 1–2 nM [³⁵S]TBPS (60–100Ci/mmol, Perkin Elmer Life Science, Boston, MA), 5 µl-aliquots of steroid in Me₂SO solution (final steroid concentrations ranged from 1 nM to 10 µM) with or without 5µM GABA, in a total volume of 1 ml of assay buffer. The assay buffer was 50 mM K-phosphate buffer, pH 7.4, 200 mM NaCl. Nonspecific binding was defined as binding observed in the presence of 200 µM picrotoxin. Assay tubes were incubated for 2 hours at room temperature. A Brandel (Gaithersburg, MD) cell harvester was used to collect the membranes on Whatman glass fiber (GF/C) filter paper which was washed with 4 ml of ice-cold buffer three times, dried and dissolved in 4 ml ScintiVerse II (Fisher Scientific, Pittsburgh, PA). Radioactivity bound to the filters was measured by liquid scintillation spectrometry. Each data point was done in triplicate and all experiments were performed three times. The average specific binding values of each triplicate were used for curve fitting and IC₅₀ and EC₅₀ values are presented as the parameters of the curve fitting to the pooled data from the repeated experiments ± SEM. The data from binding experiments performed in the presence of GABA were fit to the Hill equation and data from binding experiments performed in the absence of GABA were fit to a 2-component binding equation using Sigma Plot version 8 (SPSS; Chicago IL) and Prism (GraphPad Software Inc, San Diego, CA) as previously described (Evers et al. 2010).

Xenopus oocyte electrophysiology

Stage V–VI oocytes were harvested from sexually mature female *X. laevis* (Xenopus One, Northland, MI) and defolliculated by shaking for 20 min at 37°C in collagenase (2 mg/ml). Capped mRNA encoding rat GABA_A receptor α₁, α₁(Q241L) β₂ and γ_{2L} subunits were transcribed *in vitro* using the mMESSAGE mMachin Kit (Ambion, Austin, TX) from linearized pBluescript vectors containing receptor coding regions. The α₁(Q241L) mutation was made using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and mutated subunits were fully sequenced to confirm that only the desired mutation had been produced (Akk et al. 2008). Subunit transcripts were injected in equal parts (20–40 ng total RNA) 8–24 h following defolliculation. Oocytes were incubated up to 5 days at 18°C in ND96 medium containing (in mM) 96 NaCl, 1 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamycin (50 µg/ml). The cDNAs for the rat GABA_A-receptor subunits were originally provided by A. Tobin, University of California, Los Angeles (α₁); P. Malherbe, Hoffman-La Roche, Switzerland (β₂); and C. Fraser, National Institute on Alcohol Abuse and Alcoholism (γ_{2L}).

GABA currents were measured with a Warner OC725 two-electrode voltage-clamp amplifier 2–5 days following RNA injection in a bath of unsupplemented ND96 medium. Intracellular recording pipettes had a resistance of ~1 MΩ when filled with 3M KCl. Each oocyte was initially challenged with GABA alone and then with a single concentration of CW12 or CW14; this was done to provide a same-oocyte GABA comparator and to avoid the complication of slow neurosteroid washout. Control responses to another potentiator (a

non-photolabeling steroid or pentobarbital) were obtained from the same oocyte. Compounds were applied alone (direct activation experiments) or co-applied with GABA (potentiation experiments) using a gravity-flow perfusion system. Holding potential was -70 mV, and peak current during 20 s drug applications was used for quantification. Data were acquired and analyzed with pCLAMP software (Molecular Devices, CA). Statistical differences were determined using a two-tailed Student's t-test.

Tadpole anesthetic assay

Assays for neuroactive steroid-induced loss-of-righting reflex (LRR) and loss-of-swimming reflex (LSR) in *Xenopus laevis* tadpoles were performed and analyzed as previously described (Covey et al. 2000).

Animal experiments including tadpole anesthesia assays, *Xenopus* oocyte harvesting and use of rat brain tissue comply with ethical principles of laboratory animal care. The experimental protocols were approved by the Washington University Animal Studies committee and are in compliance with current United States law.

Results

Photochemistry of CW12 and CW14

To examine the ability of CW14 and CW12 to undergo bimolecular insertion reactions (essential for photolabeling), CW14, [^3H]CW14 and its 3β -epimer (3β -[^3H]CW14) were irradiated with UV light in either ethanol or cyclohexane and the photolysis products were analyzed on reverse-phase TLC. CW14, [^3H]CW14 and 3β -[^3H]CW14 all migrated as single bands in the absence of UV-irradiation, with CW14 ($R_f = 0.38$) running slightly slower (less polar) than its 3β -epimer ($R_f = 0.4$) (Figure 2). Following UV irradiation in ethanol, two major photolysis products were observed for CW14 (denoted by * and ** in Figure 2); both products were more polar than the parent compound. UV irradiation in cyclohexane also produced two major photolysis products, one slightly more polar (denoted by * in Figure 2) and one significantly less polar (denoted by # in Figure 2) than the parent compound. The non-polar product was uniquely observed in cyclohexane, indicating that CW14 can undergo carbene-mediated bimolecular insertion, forming an adduct with cyclohexane. Photolysis products of significantly higher polarity were also observed (top of the plate) following irradiation of [^3H]CW14 in either ethanol or cyclohexane. These products were not further identified, but are likely to result from intramolecular reactions since they were observed following photolysis in either ethanol or cyclohexane. These products were not observed following photolysis of non-radiolabeled compounds. 3β -[^3H]CW14 produced photolysis products in both ethanol and cyclohexane with migration patterns on reverse-phase TLC similar (but slightly more polar) to those observed with [^3H]CW14 (Figure 2). [^3H]CW12 showed a pattern of photolysis products in ethanol and cyclohexane similar to those observed with [^3H]CW14, with clear evidence of formation of a cyclohexane adduct (not shown).

The biological activity of CW12 and CW14

To determine whether CW12 and CW14 are neurosteroid-like modulators of GABA_A receptors, we employed three criteria: (1) their ability to modulate GABA-elicited current in oocytes transfected with GABA_A receptor subunits; (2) their ability to modulate [³⁵S]TBPS binding to native GABA_A receptors (rat brain membranes) with a pattern similar to that observed with endogenous neurosteroids and; (3) their ability to function as anesthetics in *Xenopus* tadpoles.

Electrophysiological studies

To study the effects of CW12 and CW14 on GABA_A receptor currents, we first tested their modulation of GABA-elicited chloride currents in *Xenopus laevis* oocytes expressing $\alpha_1\beta_2\gamma_2$ subunits of GABA_A receptors. Figure 3A shows representative traces of currents activated by 2 μ M GABA in the absence and presence of neurosteroids. 2 μ M GABA is well below the EC₅₀ concentration of GABA, providing sufficient dynamic range to measure neurosteroid potentiation. GABA-elicited currents were enhanced by the endogenous neurosteroids, pregnanolone (3 α 5 β P) and allopregnanolone (3 α 5 α P). The average current produced by 0.5 μ M 3 α 5 α P + 2 μ M GABA varied between 3.15 \pm 0.36 times (n = 4; same oocyte comparator for CW14) and 2.16 \pm 0.36 times greater than the current induced by 2 μ M GABA alone (n = 7; same oocyte comparator for CW12). 0.5 μ M CW14 (Fig. 3A1) and CW12 (Fig. 3A2) produced substantially greater potentiation of the GABA-elicited currents. The average current produced by 0.5 μ M CW14 + 2 μ M GABA (n=4) was 9.75 \pm 1.94 times greater than the current induced by 2 μ M GABA alone and the current elicited by 0.5 μ M CW12 + 2 μ M GABA (n = 7) was 7.12 \pm 2.65 times greater. The potentiating effects of CW12 and CW14 on GABA-elicited currents were maximal at 0.5 μ M (not shown), whereas 3 α 5 α P and 3 α 5 β P have EC₅₀ values of 2.3 \pm 0.03 μ M (Wittmer et al. 1996) and 1.2 \pm 0.40 μ M (Covey et al. 2000) respectively. In contrast to 3 α 5 α P and 3 α 5 β P, the potentiating effects of CW14 and CW12 were not completely eliminated after washing with ND96 solution for 1 min. The higher hydrophobicity of CW14 and CW12 is likely the reason for the slow washout.

There are numerous positive allosteric modulators of GABA-elicited currents that act at distinct sites on GABA_A receptor. The α_1 (Q241L) mutation has been shown to selectively reduce or eliminate potentiation by numerous neuroactive steroids (Akk et al. 2008; Hosie et al. 2006), and can be thus employed as a specific indicator of the involvement of the classic neurosteroid site(s). To confirm that the steroid analogues CW14 and CW12 act via the classic neurosteroid potentiation site(s), we tested steroid-modulation in oocytes expressing α_1 (Q241L) $\beta_2\gamma_2$ receptors. As shown in Figure 3B1, coapplication of 0.5 μ M CW14 with 0.2 μ M GABA potentiated the peak response to 1.24 \pm 0.07 (mean \pm SEM; 5 cells) times that of GABA alone. While this potentiating effect was statistically significant (P<0.05; t-test vs. no effect), the magnitude of CW14 potentiation in the mutated receptors was markedly reduced in comparison to wild-type (compare Figure 3B1 vs 3A1). Application of 0.5 μ M CW12 produced no significant potentiation (1.10 \pm 0.07 times control, 4 cells) on the peak response elicited by 0.2 μ M GABA in the α_1 (Q241L) containing receptors (Figure 3B2). Control experiments were conducted to demonstrate lack of effect of the α_1 (Q241L) mutation on potentiation by another allosteric potentiator, pentobarbital (PB), whose binding site (Chiara

et al. 2013) does not overlap with the neurosteroid site. Coapplication of 100 μM PB with 0.2 μM GABA enhanced the maximal response to 8.78 ± 1.45 times control (pooled data from 5 cells used in experiments with CW14 and 4 cells used with CW12; Figure 3B1 and 3B2).

We also tested the ability of CW12 and CW14 to directly activate GABA_A chloride currents in the absence of GABA (Figure 3C). 10 μM CW12 activated a current 0.32 ± 0.18 times the current elicited by 2 μM GABA (n=4); this is similar to the current directly elicited by 10 μM 3 α 5 α P (0.37 ± 0.07 ; n=8). Markedly smaller current were directly elicited by 0.1 – 1.0 μM CW12 (n = 4 at each concentration). 10 μM CW14 directly activated a small GABA_A chloride current (0.05 ± 0.01 times control; n = 4), similar to the current directly elicited by 10 μM 3 α 5 β P (0.06 ± 0.03 ; n = 10).

Modulation of [³⁵S]TBPS binding in rat brain membranes

Neurosteroids have a characteristic pattern by which they allosterically modulate binding of the convulsant [³⁵S]TBPS to GABA_A receptors (Evers et al. 2010; Srinivasan et al. 1999). To determine if CW12 and CW14 also produce this pattern, [³⁵S]TBPS binding to rat brain membranes was measured in the presence of various concentrations of CW12 and CW14 in the presence and absence of 5 μM GABA (Figure 4A). In the presence of GABA, CW12 and CW14 produced single component inhibition of [³⁵S]TBPS binding with IC₅₀ values of 12.3 ± 3.9 nM and 15.9 ± 5.0 nM respectively. CW12 and CW14 appeared more potent than 3 α 5 α P and 3 α 5 β P which have IC₅₀ values of 71 ± 18 nM and 74 ± 7 nM respectively (Covey et al. 2000). In the absence of GABA (Figures 4A1 and 4A2, control) CW12 and CW14 showed the characteristic neurosteroid pattern of allosteric modulation with enhancement of [³⁵S]TBPS binding at low steroid concentrations and inhibition at higher concentrations (Evers et al. 2010). CW12 enhanced [³⁵S]TBPS binding with an EC₅₀ = 57.3 ± 26.5 nM and inhibited binding with an IC₅₀ = 204.9 ± 54.5 nM. CW14 had an EC₅₀ = 18.4 ± 31.4 nM and an IC₅₀ = 1.3 ± 0.5 μM .

Tadpole anesthesia

To test the anesthetic activity of CW12 and CW14, loss of righting reflex (LRR) and loss of swimming reflex (LSR) was measured in prelimbud stage *Xenopus* tadpoles (Fig. 4B). The EC₅₀ values for LRR and LSR were 112 ± 14 nM and 747 ± 364 nM for CW14; and 110 ± 21 nM and 655 ± 43 nM for CW12 (n = 20 tadpoles at each concentration). These values are similar to those observed with 3 α 5 α P (LRR = 390 ± 40 nM; LSR = 5.50 ± 0.48 μM) and 3 α 5 β P (LRR = 63 ± 3 nM; LSR = 300 ± 0.05 nM).

CW12 and CW14 photolabeling of rat brain membranes

To determine if CW12 and CW14 are effective photolabeling reagents for neurosteroid binding proteins, rat brain membranes (400 μg) were photolabeled with [³H]CW12 or [³H]CW14, analyzed by SDS-PAGE and radiolabeled bands were identified by autoradiography. 1 μM [³H]CW12 photolabeled multiple proteins with the most prominent band at ≈ 52 -kDa (Figure 5A). Photolabeling with [³H]CW14 was concentration-dependent with prominent bands at ≈ 35 -kDa, ≈ 52 -kDa, ≈ 66 -kDa and ≈ 100 -kDa (Figure 5B). While the bands at ≈ 35 -kDa and ≈ 52 -kDa are likely to be VDAC (Darbandi-Tonkabon et al.

2003) and a GABA_A receptor subunit respectively, our previous work has shown that the ≈ 52 -kDa band may represent other neurosteroid binding proteins, such as β -tubulin (Chen et al. 2012a).

To clarify the identity of the photolabeled proteins, membranes were photolabeled with 10 μ M [³H]CW14 and analyzed by blue-native electrophoresis, so that GABA_A receptors would retain their pentameric structure (MW ≈ 250 -kDa) and be identifiable by immunoblotting against multiple GABA_A receptor subunits. As shown in Figure 6A, autoradiographic analysis of the blue native gel showed radiolabeled bands at ≈ 140 , ≈ 240 , ≈ 480 and ≈ 1200 -kDa. Immunoblots to the α_1 , $\beta_{2/3}$ and γ_2 subunits identified the band at ≈ 240 -kDa as the GABA_A receptor, confirming that CW14 effectively photolabels this protein. The photolabeled band at ≈ 480 -kDa was also stained by the GABA_A receptor antibodies suggesting that it is a dimer of pentameric GABA_A receptors. The photolabeled band at ≈ 140 -kDa was identified as VDAC by immunoblot against VDAC-1. The photolabeled band at ≈ 1200 -kDa was identified as β -tubulin by immunoblotting. The photolabeled bands between 480 and 1048-kDa were not identified, but may represent multimers of the GABA_A receptor based on weak immunostaining with anti-GABA_A subunit antibodies. One lane of the blue native gel was sliced and counted, indicating that the most prominently photolabeled protein was VDAC (≈ 140 -kDa) followed by the GABA_A receptor (≈ 240 and ≈ 480 -kDa). Additionally, photolabeling of rat brain membranes with [³H]CW14 and its 3 β -epimer ([³H]3 β -CW14) were compared by BN-PAGE (Fig. 6B). The photolabeling of VDAC, GABA_A receptors, and tubulin with [³H]CW14 demonstrated a concentration-dependent pattern (Fig. 6B left). However, minimal photolabeling was observed with 0.3 or 1 μ M [³H]3 β -CW14 (Fig. 6B right), indicating that photolabeling of both VDAC and the GABA_A receptors was stereospecific.

CW14 photolabel recombinant GABA_A receptors expressed in TSA cells

To further confirm that CW14 labels GABA_A receptors, membranes (400 μ g) from native TSA cells or from TSA cells expressing recombinant $\alpha_1\beta_2$ -heteromeric or β_3 -homomeric GABA_A receptors were photolabeled with 10 μ M [³H]CW14. The photolabeled proteins were separated by SDS-PAGE followed by gel slicing and scintillation counting. Two broad peaks of radioactivity, centered at ≈ 52 -kDa and ≈ 100 -kDa were observed in the $\alpha_1\beta_2$ and β_3 -expressing membranes but not in the non-transfected TSA membranes (Figure 7). These peaks correspond to the molecular weights of monomers and dimers of GABA_A receptor subunits. These results indicate that [³H]CW14 labels β_3 and either β_2 or α_1 subunits of GABA_A receptors.

Discussion

This study was aimed at developing neurosteroid analogue photolabeling reagents for use in identifying pharmacologically relevant neurosteroid binding sites on GABA_A receptors. This requires that the neurosteroid photoaffinity analogues bind in the same location on the GABA_A receptor as do endogenous neurosteroids. Further, the photolabeling reagents must have photochemical properties enabling them to preferentially participate in intermolecular

crosslinking rather than intramolecular reactions and to covalently insert at any proximate amino acid in an unbiased manner.

In a previous study, we used the neurosteroid analogue photolabeling reagent 6-AziP to identify a novel neurosteroid binding site (F301) in the second transmembrane domain of β -homomeric GABA_A receptors (Chen et al. 2012b). This study did not identify the residues predicted to be involved in neurosteroid binding by site-directed mutagenesis (Hosie et al. 2009a; Hosie et al. 2006), indicating either that the residues identified by mutagenesis are relevant to transduction rather than binding of neurosteroids or that the predicted binding sites were simply not detected. One reason that 6-AziP may not label all neurosteroid binding sites is that the photolabeling moiety is an aliphatic diazirine. Diazirines are commonly used for protein photolabeling because they have an absorbance band centered at 350 nm, which is well away from the principal absorbance band of proteins (280 nm). Irradiation of a diazirine can, via elimination of molecular nitrogen, generate a carbene, which then participates in either an internal reaction (intramolecular) or a bimolecular insertion reaction. Carbenes can insert into any C–H bond and are thus not selective for specific amino acids. However, if an intramolecular reaction occurs, the carbene that is generated will be unavailable for bimolecular insertion. Irradiation can also convert a diazirine to a diazo intermediate that can then form a carbonium ion; the carbonium ion acts as an electrophile and can selectively label nucleophilic amino acid side chains (Brunner 1993). Aliphatic diazirines, such as 6-AziP, often undergo intramolecular reactions following carbene formation and may preferentially photolabel proteins through a diazo intermediate (Brunner 1993). This makes these reagents inefficient photolabeling reagents with a bias toward labeling nucleophilic amino acids (Pratt et al. 2000; Ziebell et al. 2004).

To overcome the photochemical limitations of 6-AziP, we synthesized neurosteroid analogues containing a 4-(3-trifluoromethyl-3*H*-diazirin-3-yl)-phenoxy (TPD) group. Following UV-irradiation, the TPD moiety preferentially generates a carbene (65%) rather than a diazoisomer (35%) and cannot undergo intramolecular rearrangement (Brunner et al. 1980). The TPD groups were placed at C-11 on the neurosteroid C-ring because previous studies had shown that neurosteroids with large hydrophobic substitutions at this position retain their ability to modulate GABA_A receptors (Shu et al. 2007). Two photolabeling reagents with 11-TPD groups were synthesized: CW12, an analogue of the 5 α -neurosteroid allopregnanolone and CW14, an analogue of the 5 β -neurosteroid pregnanolone (Figure 1).

Photochemistry

To test whether these reagents generate a carbene that preferentially undergoes intermolecular reactions we examined the ability of CW12 and CW14 to form unique adducts with cyclohexane (a solvent in which the only bond available for attack is a C–H) following UV-irradiation. CW14 and its 3 β -epimer both formed adducts with cyclohexane; these adducts migrated more slowly than the non-irradiated compounds on reverse-phase TLC indicating that they are less polar (denoted by # in Figure 2). Irradiation of CW14 in ethanol produced a product that was more polar than the non-irradiated compound (denoted by ** in Figure 2) indicating the probable formation of an ethanol-steroid adduct. Irradiation in both ethanol and cyclohexane produced a common product (denoted by * in figure 2), that

migrated as a slightly more polar band than the non-irradiated CW14. The fact that this band was observed following photolysis in both solvents suggests it is produced by an intramolecular reaction. Since the TPD group cannot undergo a simple rearrangement following carbene formation, this suggests that the carbene may react with a proximate group on the neurosteroid, perhaps the 17-acetyl group. A similar pattern of photolabel incorporation was observed with CW12, with formation of a cyclohexane adduct as a major product and a common product formed in both ethanol and cyclohexane suggesting an intramolecular reaction product. Importantly, these data show that UV-irradiation of both CW14 and CW12 forms a carbene capable of intermolecular insertion in a C–H bond, indicating that these reagents should be efficient and unbiased photolabeling reagents.

Biological activity of CW12 and CW14

The GABA_A receptor is affected by a diverse range of chemical compounds that almost certainly bind to a variety of distinct sites (Franks 2008). It is therefore imperative to demonstrate that the attachment of a photoreactive group does not substantially alter the interaction of the drug with its targets and that the photolabeling reagent closely mimics the effects of the parent compound. CW12 and CW14 were both shown to be efficacious anesthetics in pre-limb-bud stage tadpoles (a widely accepted assay for anesthetic action) with EC₅₀ values very similar to those of the parent compounds (Figure 4B1 and 4B2), indicating that the photolabeling reagents retain the *in vivo* pharmacologic effect of neurosteroids. To assay the actions of CW12 and CW14 on GABA_A receptors, we examined their effects on [³⁵S]TBPS binding in rat brain membranes, because neurosteroids have a characteristic bimodal effect on [³⁵S]TBPS binding. CW12 and CW14 both showed the characteristic pattern of modulation in the absence of GABA, with enhancement of binding at low steroid concentrations and complete inhibition of binding at higher concentrations (Figure 4A1 and 4A2). The low concentration enhancement of [³⁵S]TBPS is hypothesized to result from neurosteroid binding to the site(s) responsible for potentiation of GABA action and the inhibition observed at higher concentrations is hypothesized to result from binding to the site(s) that mediate direct activation of the GABA_A receptor (Evers et al. 2010). In the presence of GABA, CW12 and CW14 both showed single-component inhibition of [³⁵S]TBPS binding, characteristic of neurosteroid effect (Figure 4A1 and 4A2). These results indicate that CW12 and CW14 replicate the effects of the parent neurosteroids and suggest that the photolabeling reagents bind to both the potentiating and direct activation sites.

Electrophysiological effects of CW12 and CW14 were studied in *Xenopus* oocytes expressing the α_1 , β_2 and γ_{2L} subunits of the GABA_A receptor. Both CW12 and CW14 were potent and efficacious potentiators of GABA-elicited current. The slow washout of potentiating effects observed with CW12 and CW14 (Figure 3A) suggests that their high potency may arise, at least in part, from the hydrophobicity of CW12 and CW14 which results in a high membrane/aqueous partition coefficient, enhancing the apparent potency of the drug by presenting high concentrations to neurosteroid binding sites in the transmembrane domains (Akk et al. 2005; Chisari et al. 2010; Chisari et al. 2009). The ability of CW12 and CW14 to potentiate GABA-elicited current was markedly reduced by a mutation in the α_1 subunit (Q241L) that has been shown to selectively prevent neurosteroid

modulation of GABA_A receptor currents (Figure 3B1 and 3B2), providing further evidence that these neurosteroid analogues act via the same mechanism as endogenous neurosteroids. CW12 directly activated GABA_A currents (Figure 3C) quantitatively similar to its parent compound, allopregnanolone. CW14, like its parent compound pregnanolone, was a weak direct activator of GABA_A currents (Figure 3C). The combined results of the [³⁵S]TBPS binding studies and electrophysiological studies suggest that CW12 and CW14 interact with all of the pharmacologically relevant neurosteroid binding sites on the GABA_A receptor.

Photolabeling GABA_A receptors

While CW12 and CW14 appear to interact with neurosteroid binding sites on the GABA_A receptor, they may still not covalently insert at these sites following UV irradiation. This could occur if the photolabeling moiety is not positioned sufficiently close to the walls of the binding pocket or if photolabeling is not adequately efficient. To determine if CW12 and CW14 can covalently label GABA_A receptors, photolabeling studies were performed in native rat brain membranes and in membranes from TSA cells transfected with GABA_A receptor subunits.

Both [³H]CW12 and [³H]CW14 labeled multiple proteins in rat brain membranes, with prominent labeling of a band at ≈52-kDa (Figure 5A). While this band is consistent with labeling of a GABA_A receptor subunit, this identification is uncertain. To confirm that the GABA_A receptor is photolabeled, we analyzed [³H]CW14 photolabeled rat brain membranes by blue-native gel electrophoresis. A photolabeled band at ≈242-kDa was observed on the blue native gel, consistent with intact GABA_A receptor pentamers (Figure 6A). The identity of this photolabeled protein as a GABA_A receptor was confirmed by immunoblots showing that the α₁, β_{2/3} and γ_{2L} subunits of the GABA_A receptor all co-migrated with this photolabeled band. Photolabeling of the GABA_A receptor was stereospecific as indicated by the finding that the 3β-epimer of [³H]CW14 did not label GABA_A receptors (Figure 6B).

As a final confirmation that CW12 and CW14 are effective photolabeling reagents for the GABA_A receptor, membranes from TSA cells expressing either α₁β₂ or β₃-homomeric GABA_A receptors were photolabeled with [³H]CW14 and analyzed by SDS-PAGE with gel slicing. Bands at ≈52- and ≈100-kDa were observed in the transfected, but not in wild-type TSA cell membranes (Figure 7). These data support [³H]CW14 photolabeling of GABA_A receptor β-subunits, with the 100-kDa band presumably reflecting a dimer of the β-subunit. It cannot be determined from these results if [³H]CW14 also labels α₁ subunits.

In summary, CW12 and CW14 are novel photolabeling analogues of the endogenous neurosteroids allopregnanolone and pregnanolone, respectively. The photochemistry of these compounds is favorable; they can undergo intermolecular carbene insertion, allowing photolabeling of any proximate amino acid residue. CW12 and CW14 closely mimic the actions of their parent neurosteroids, both as anesthetics and as modulators of GABA_A receptor function, fulfilling an essential criterion for a photolabeling reagent to identify neurosteroid binding sites. Finally, CW12 and CW14 photolabel β and possibly other subunits of the GABA_A receptor. These reagents will be useful for identifying the specific

amino acid residues with which neurosteroids interact on GABA_A receptors, enabling the identification and modeling of their sites of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grant PO1-GM47969 from the National Institute of General Medical Sciences to A.S.E., D.F.C., J.H.S and C.F.Z and by grant MH099658 to S.M. from the National Institute of Mental Health. Washington University receives income and equity based on a license of related technology to Sage Therapeutics, Inc. D.F.C., C.F.Z. and A.S.E. have equity holdings in Sage Therapeutics, Inc. Sage Therapeutics, Inc. did not support this research or have any other role in the research or its conclusions.

References

- Akk G, Bracamontes JR, Covey DF, Evers A, Dao T, Steinbach JH. Neuroactive steroids have multiple actions to potentiate GABA_A receptors. *J Physiol.* 2004; 558:59–74. [PubMed: 15146041]
- Akk G, Covey DF, Evers AS, Mennerick S, Zorumski CF, Steinbach JH. Kinetic and structural determinants for GABA-A receptor potentiation by neuroactive steroids. *Current neuropharmacology.* 2010; 8:18–25. [PubMed: 20808543]
- Akk G, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S. Mechanisms of neurosteroid interactions with GABA(A) receptors. *Pharmacol Ther.* 2007; 116:35–57. [PubMed: 17524487]
- Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH. Mutations of the GABA-A receptor alpha1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. *Mol Pharmacol.* 2008; 74:614–27. [PubMed: 18544665]
- Akk G, Shu HJ, Wang C, Steinbach JH, Zorumski CF, Covey DF, Mennerick S. Neurosteroid access to the GABA_A receptor. *J Neurosci.* 2005; 25:11605–13. [PubMed: 16354918]
- Atkinson RM, Davis B, Pratt MA, Sharpe HM, Tomich EG. Action of some steroids on the central nervous system of the mouse. II. *Pharmacology. Journal of medicinal chemistry.* 1965; 8:426–32. [PubMed: 5883705]
- Brunner J. New photolabeling and crosslinking methods. *Annu Rev Biochem.* 1993; 62:483–514. [PubMed: 8352595]
- Brunner J, Senn H, Richards FM. 3-Trifluoromethyl-3-phenyldiazirine. A new carbene generating group for photolabeling reagents. *J Biol Chem.* 1980; 255:3313–8. [PubMed: 7364745]
- Bureau MH, Olsen RW. GABA_A receptor subtypes: ligand binding heterogeneity demonstrated by photoaffinity labeling and autoradiography. *J Neurochem.* 1993; 61:1479–91. [PubMed: 8397295]
- Chen ZW, Chen LH, Akentieva N, Lichti CF, Darbandi R, Hastings R, Covey DF, Reichert DE, Townsend RR, Evers AS. A neurosteroid analogue photolabeling reagent labels the colchicine-binding site on tubulin: a mass spectrometric analysis. *Electrophoresis.* 2012a; 33:666–74. [PubMed: 22451060]
- Chen ZW, Manion B, Townsend RR, Reichert DE, Covey DF, Steinbach JH, Sieghart W, Fuchs K, Evers AS. Neurosteroid analog photolabeling of a site in the third transmembrane domain of the $\beta 3$ subunit of the GABA_A receptor. *Mol Pharmacol.* 2012b; 82:408–19. [PubMed: 22648971]
- Chiara DC, Jayakar SS, Zhou X, Zhang X, Savechenkov PY, Bruzik KS, Miller KW, Cohen JB. Specificity of intersubunit general anesthetic-binding sites in the transmembrane domain of the human $\alpha 1\beta 3\gamma 2$ gamma-aminobutyric acid type A (GABAA) receptor. *J Biol Chem.* 2013; 288:19343–57. [PubMed: 23677991]
- Chisari M, Eisenman LN, Covey DF, Mennerick S, Zorumski CF. The sticky issue of neurosteroids and GABA(A) receptors. *Trends in neurosciences.* 2010; 33:299–306. [PubMed: 20409596]
- Chisari M, Eisenman LN, Krishnan K, Bandyopadhyaya AK, Wang C, Taylor A, Benz A, Covey DF, Zorumski CF, Mennerick S. The influence of neuroactive steroid lipophilicity on GABA_A receptor

- modulation: evidence for a low-affinity interaction. *J Neurophysiol.* 2009; 102:1254–64. [PubMed: 19553485]
- Covey DF, Nathan D, Kalkbrenner M, Nilsson KR, Hu Y, Zorumski CF, Evers AS. Enantioselectivity of pregnanolone-induced gamma-aminobutyric acid(A) receptor modulation and anesthesia. *J Pharmacol Exp Ther.* 2000; 293:1009–16. [PubMed: 10869405]
- Darbandi-Tonkabon R, Hastings WR, Zeng CM, Akk G, Manion BD, Bracamontes JR, Steinbach JH, Mennerick SJ, Covey DF, Evers AS. Photoaffinity labeling with a neuroactive steroid analogue. 6-azi-pregnanolone labels voltage-dependent anion channel-1 in rat brain. *J Biol Chem.* 2003; 278:13196–206. [PubMed: 12560326]
- Darbandi-Tonkabon R, Manion BD, Hastings WR, Craigen WJ, Akk G, Bracamontes JR, He Y, Sheiko TV, Steinbach JH, Mennerick SJ, Covey DF, Evers AS. Neuroactive steroid interactions with voltage-dependent anion channels: lack of relationship to GABA(A) receptor modulation and anesthesia. *J Pharmacol Exp Ther.* 2004; 308:502–11. [PubMed: 14593090]
- Evers AS, Chen ZW, Manion BD, Han M, Jiang X, Darbandi-Tonkabon R, Kable T, Bracamontes J, Zorumski CF, Mennerick S, Steinbach JH, Covey DF. A synthetic 18-norsteroid distinguishes between two neuroactive steroid binding sites on GABA_A receptors. *J Pharmacol Exp Ther.* 2010; 333:404–13. [PubMed: 20124410]
- Franks NP. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. *Nature reviews Neuroscience.* 2008; 9:370–86.
- Gyermek L, Soyka LF. Steroid anesthetics. *Anesthesiology.* 1975; 42:331–44. [PubMed: 236704]
- Harrison NL, Majewska MD, Harrington JW, Barker JL. Structure-activity relationships for steroid interaction with the γ -aminobutyric acid A receptor complex. *J Pharmacol Exp Ther.* 1987; 241:346–53. [PubMed: 3033209]
- Hawkinson JE, Kimbrough CL, McCauley LD, Bolger MB, Lan NC, Gee KW. The neuroactive steroid 3 alpha-hydroxy-5 beta-pregnan-20-one is a two-component modulator of ligand binding to the GABA_A receptor. *Eur J Pharmacol.* 1994; 269:157–63. [PubMed: 7851492]
- Hosie AM, Clarke L, da Silva H, Smart TG. Conserved site for neurosteroid modulation of GABA_A receptors. *Neuropharmacology.* 2009a; 56:149–54. [PubMed: 18762201]
- Hosie AM, Clarke L, da Silva H, Smart TG. Conserved site for neurosteroid modulation of GABA_A receptors. *Neuropharmacology.* 2009b; 56:149–54. [PubMed: 18762201]
- Hosie AM, Wilkins ME, da Silva HM, Smart TG. Endogenous neurosteroids regulate GABA_A receptors through two discrete transmembrane sites. *Nature.* 2006; 444:486–9. [PubMed: 17108970]
- Hosie AM, Wilkins ME, Smart TG. Neurosteroid binding sites on GABA(A) receptors. *Pharmacol Ther.* 2007; 116:7–19. [PubMed: 17560657]
- Li P, Bandyopadhyaya AK, Covey DF, Steinbach JH, Akk G. Hydrogen bonding between the 17 β -substituent of a neurosteroid and the GABA(A) receptor is not obligatory for channel potentiation. *Br J Pharmacol.* 2009; 158:1322–9. [PubMed: 19702782]
- Li P, Bracamontes J, Katona BW, Covey DF, Steinbach JH, Akk G. Natural and enantiomeric etiocholanolone interact with distinct sites on the rat α 1 β 2 γ 2L GABA_A receptor. *Mol Pharmacol.* 2007; 71:1582–90. [PubMed: 17341652]
- Pratt MB, Husain SS, Miller KW, Cohen JB. Identification of sites of incorporation in the nicotinic acetylcholine receptor of a photoactivatable general anesthetic. *J Biol Chem.* 2000; 275:29441–51. [PubMed: 10859324]
- Selye H. Anesthetic effect of steroid hormones. *Proc Soc Exp Biol Med.* 1941; 46:116–121.
- Shu HJ, Zeng CM, Wang C, Covey DF, Zorumski CF, Mennerick S. Cyclodextrins sequester neuroactive steroids and differentiate mechanisms that rate limit steroid actions. *Br J Pharmacol.* 2007; 150:164–75. [PubMed: 17160009]
- Srinivasan S, Sapp DW, Tobin AJ, Olsen RW. Biphasic modulation of GABA(A) receptor binding by steroids suggests functional correlates. *Neurochem Res.* 1999; 24:1363–72. [PubMed: 10555776]
- Wittig I, Braun HP, Schagger H. Blue native PAGE. *Nat Protoc.* 2006; 1:418–28. [PubMed: 17406264]

- Wittmer LL, Hu Y, Kalkbrenner M, Evers AS, Zorumski CF, Covey DF. Enantioselectivity of steroid-induced gamma-aminobutyric acidA receptor modulation and anesthesia. *Mol Pharmacol.* 1996; 50:1581–6. [PubMed: 8967980]
- Ziebell MR, Nirthanan S, Husain SS, Miller KW, Cohen JB. Identification of binding sites in the nicotinic acetylcholine receptor for [³H]azietomidate, a photoactivatable general anesthetic. *J Biol Chem.* 2004; 279:17640–9. [PubMed: 14761946]

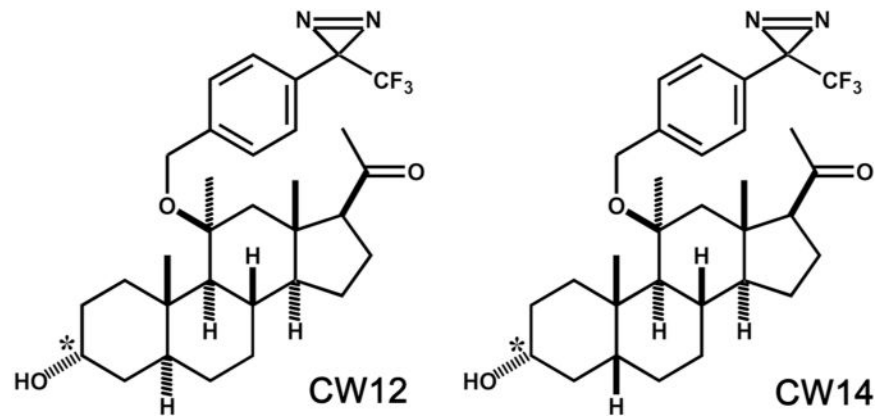


Figure 1.
Structures of CW14 and CW12.

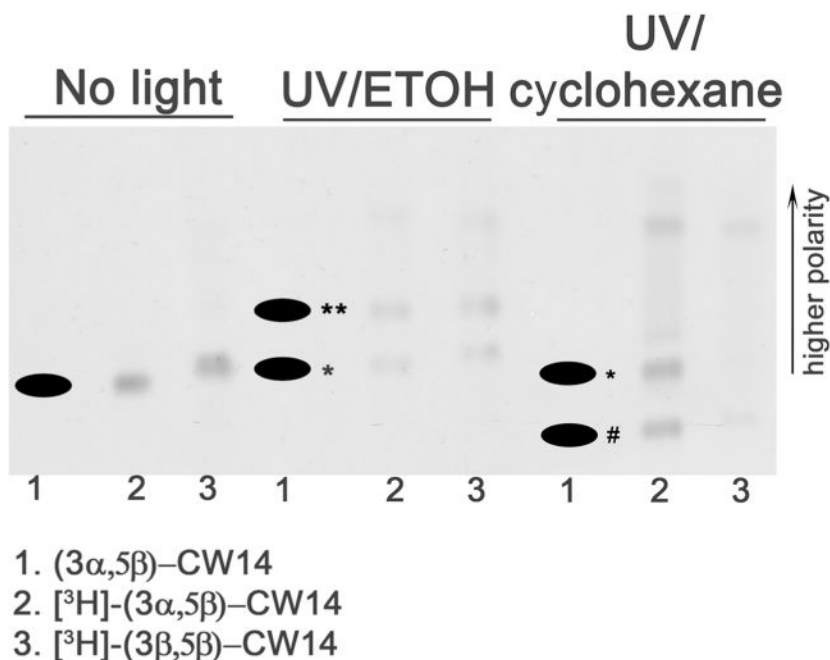


Figure 2. Photolysis products of CW14, [3 H]CW14 and [3 H]3 β -CW14

The figure shows a reverse-phase TLC (RP-TLC) plate with the origin at the bottom. More polar compounds migrate further up the plate. CW14 (**1**), [3 H]-CW14 (**2**), and the 3 β -epimer of [3 H]CW14 (**3**) irradiated with ultraviolet light (UV) in either ethanol (ETOH, **center**) or cyclohexane (**right**) were analyzed by RP-TLC. Non UV-irradiated compounds **1**, **2** and **3** were also analyzed by RP-TLC (**left**). An autoradiogram of the plate was produced to visualize radiolabeled compounds, followed by charring of the plate to visualize non-radiolabeled compounds. The image is an overlay of the autoradiogram with the charred plates (the black dots indicate the charred spots). The photolysis product slightly more polar than the parent compound (presumed intramolecular reaction) is denoted by *. The CW14-ethanol adduct is denoted by ** and the CW14-cyclohexane adduct is denoted by #.

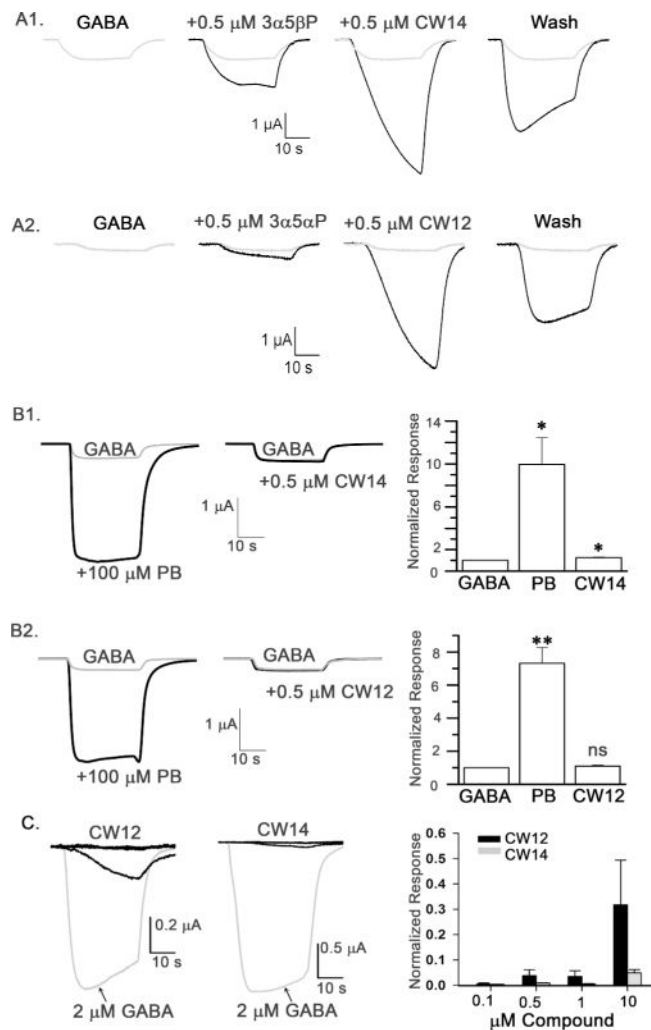


Figure 3. CW14 and CW12 modulate GABA_A receptor function

A. CW14 and CW12 potentiate GABA-elicited currents in *Xenopus laevis* oocytes expressing $\alpha_1\beta_2\gamma_2$ GABA_A receptors. Representative traces of currents elicited by 2 μM GABA in the absence and presence of 0.5 μM pregnanolone (3α5βP) and CW14 (panel A1), or 0.5 μM allopregnanolone (3α5αP) and CW12 (panel A2). Membrane potential was -70 mV. **B.** CW14 and CW12 have minimal effect on GABA-induced currents in oocytes expressing $\alpha_1(Q241L)\beta_2\gamma_2L$ GABA_A receptors. Representative traces of currents elicited by 0.2 μM GABA in the absence and presence of 100 μM pentobarbital (PB, positive control) and 0.5 μM CW14 (panel B1) or 100 μM PB and 0.5 μM CW12 (panel B2). Summary graphs (right) show the effects (mean ± SEM) of PB and CW14 of (panel B1; n = 5 oocytes) or PB and CW12 (panel B2; n = 4 oocytes) relative to the current produced by 0.2 μM GABA alone. * = P < 0.05 compared to GABA alone; **P < 0.01 compared to GABA alone; ns = not significant compared to GABA alone. **C.** Direct activation of GABA_A receptors by CW12 and CW14 in oocytes expressing $\alpha_1\beta_2\gamma_2L$ GABA_A receptors. Representative traces (left) of currents elicited by 0.1, 0.5, 1.0 and 10 μM CW12 and CW14 or 2 μM GABA (grey trace). Bar graph shows concentration-dependent activation of current by CW12 and CW14 (n = 4 for each concentration) expressed as a fraction of the current elicited by 2 μM GABA.

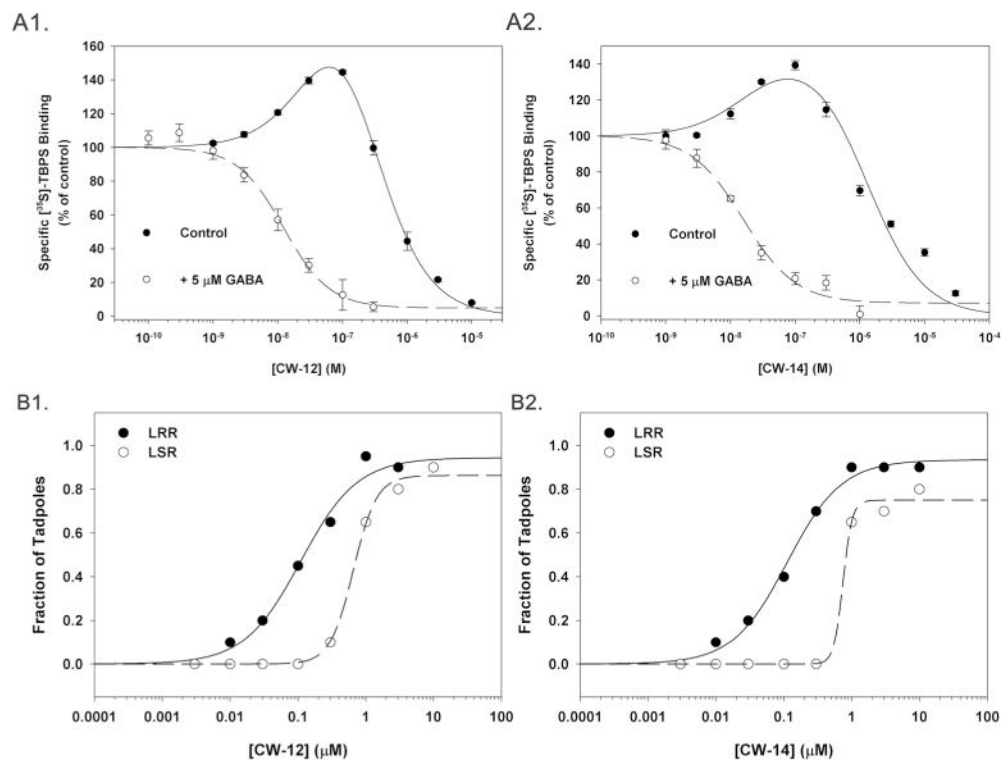


Figure 4. Biological actions of CW12 and CW14

A. CW12 (panel A1) and CW14 (panel A2) modulation of $[^{35}\text{S}]\text{TBPS}$ binding in rat brain membranes. Each curve represents the mean \pm SEM of three experiments with each point performed in triplicate. Closed circles (continuous lines) are from experiments performed in the absence of GABA and open circles with dashed lines from experiments performed in the presence of 5 μM GABA. **B.** Anesthetic effects of CW12 and CW14 in tadpoles. Loss of righting reflex (LRR) and loss of swimming reflex (LSR) in tadpoles induced by CW12 (B1) and CW14 (B2). The EC_{50} values for LRR by CW12 and CW14 were 110 ± 21 nM and 112 ± 14 nM respectively. The EC_{50} values for LSR by CW12 and CW14 were 655 ± 43 nM and 747 ± 364 nM, respectively.

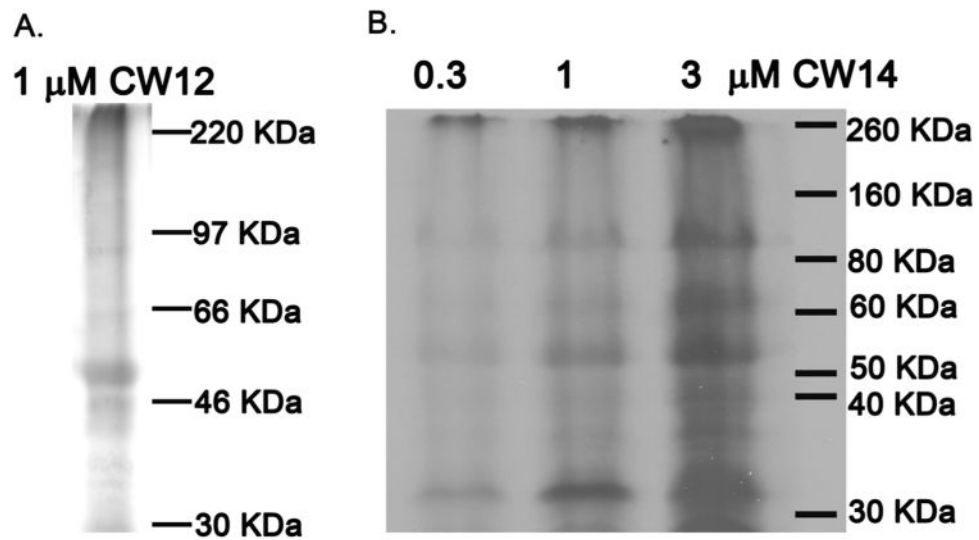


Figure 5. SDS-PAGE autoradiograms of [³H]CW12 and [³H]CW14 photolabeled rat brain membranes

Rat brain membranes (400 μg) were photolabeled with either: (A) 1 μM [³H]CW12 or; (B) 0.3, 1 and 3 μM [³H]CW14. A band at ≈52-kDa is labeled by both [³H]CW12 and [³H]CW14. Photolabeling of the ≈52-kDa band by [³H]CW14 is concentration-dependent.

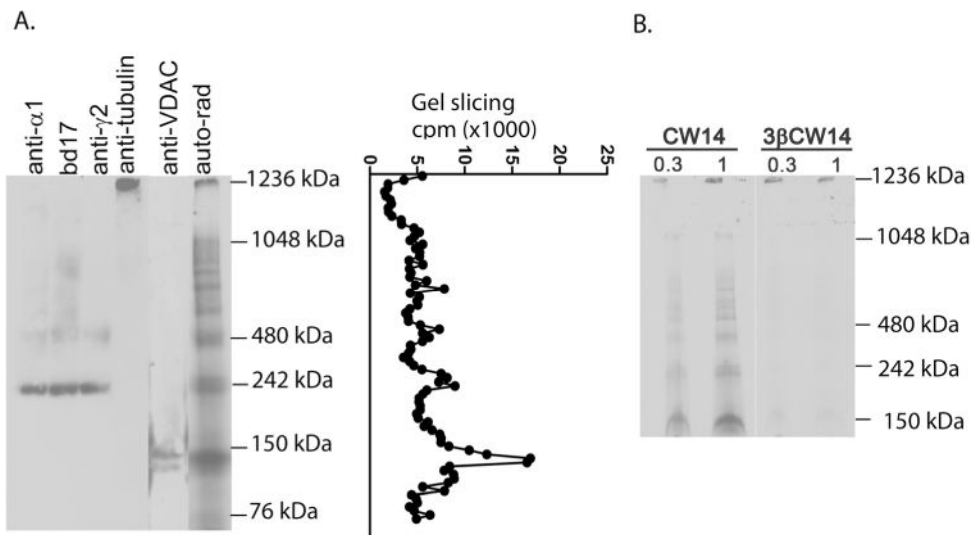


Figure 6. Blue native-PAGE of [3 H]CW 14 photolabeled rat brain membranes

A. Rat brain membranes (400 μ g) photolabeled with 3 μ M [3 H]CW14 were loaded onto a BN-PAGE followed by autoradiography, gel slicing or immunoblotting with antibodies to the α_1 , $\beta_{2/3}$ (bd17) or γ_2 subunits of the GABA_A receptor, to VDAC-1 or to β -tubulin. The photolabeled proteins correspond to GABA_A receptors (\approx 240 and \approx 480-kDa) as shown by anti- α_1 , bd17 and anti- γ_2 antibodies; tubulin (\approx 1236 kDa); and VDAC (\approx 140 kDa). **B.** Autoradiogram of blue native-PAGE from rat brain membranes (400 μ g) photolabeled with 0.3 and 1 μ M [3 H]CW14 or the 3 β -epimer of [3 H]CW14. [3 H]CW14 photolabels GABA_A receptors (\approx 240-kDa) in a concentration-dependent pattern. The photolabeling is stereospecific as evidenced by the minimal photolabeling of GABA_A receptors and VDAC by [3 H]3 β -CW14.

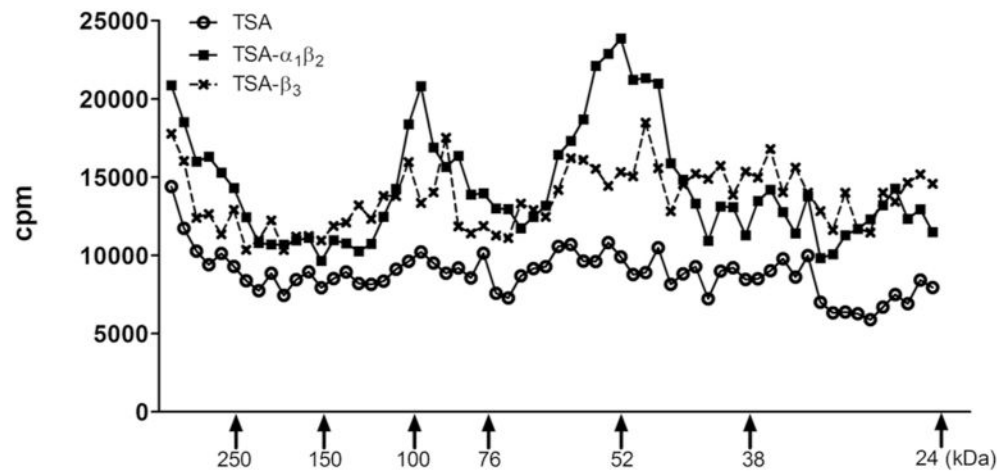


Figure 7. CW14 photolabels GABA_A receptors expressed in TSA cells

Membranes (400 μ g protein) from wild-type TSA cells or TSA cells expressing $\alpha_1\beta_2$ subunits or β_3 subunits of the GABA_A receptors were photolabeled with 10 μ M [³H]CW14. Gel slicing demonstrated photolabeled protein peaks at \approx 52-kDa and \approx 100-kDa in the $\alpha_1\beta_2$ and β_3 containing membranes, corresponding to monomers and dimers of GABA_A receptor subunits. No photolabeled peaks were observed in the wild-type membranes.