Photodynamic Effects of Steroid-Conjugated Fluorophores on GABA_A Receptors

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ABSTRACT

We have shown that fluorescent, 7-nitro-2,1,3-benzoxadiazol-4-yl amino (NBD)-conjugated neurosteroid analogs photopotentiate GABA_A receptor function. These compounds seem to photosensitize a modification of receptor function, resulting in long-lived increases in responses to exogenous or synaptic GABA. Here we extend this work to examine the effectiveness of different fluorophore positions, conjugations, steroid structures, and fluorophores. Our results are generally in agreement with the idea that steroids with activity at GABA_A receptors are the most potent photopotentiators. In particular, we find that an unnatural enantiomer of an effective photopotentiating steroid is relatively weak, excluding the idea that membrane solubility alone, which is identical for enantiomer pairs, is solely responsible for potent photopotentiation. Furthermore, there is a significant correlation between baseline GABA_A receptor activity and photopotentiation. Curiously, both sulfated steroids, which

bind a presumed external neurosteroid antagonist site, and hydroxysteroids, which bind an independent site, are effective. We also find that a rhodamine dye conjugated to a 5 β -reduced 3 α -hydroxy steroid is a particularly potent and effective photopotentiator, with minimal baseline receptor activity up to 10 μ M. Steroid conjugated fluorescein and Alexa Fluor 546 also supported photopotentiation, although the Alexa Fluor conjugate was weaker and required 10-fold higher concentration to achieve similar potentiation to the best NBD and rhodamine conjugates. Filling cells with steroid-conjugated or free fluorophores via whole-cell patch pipette did not support photopotentiation. FM1-43, another membrane-targeted, structurally unrelated fluorophore, also produced photopotentiation at micromolar concentrations. We conclude that further optimization of fluorophore and carrier could produce an effective, selective, light-sensitive GABA_A receptor modulator.

There has been recent experimental interest in the control of neuronal activity by light. This approach offers promise as

a relatively noninvasive and highly controlled method to stimulate or inhibit neuronal activity experimentally or therapeutically. These techniques include transmitter uncaging, heterologous ligand-gated channel expression, and more recent optogenetic techniques (Deisseroth et al., 2006; Sjulson and Miesenböck, 2008). Although exciting, these techniques suffer from a number of limitations, including the necessity for ultraviolet light or for expensive multiphoton excitation

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ABBREVIATIONS: NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; CW17, (2 β ,3 α ,5 α)-3-hydroxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-pregnan-20-one; CW18, *N*-[(2 β ,3 α ,5 α)-3-hydroxy-20-oxopregnan-2-yl]-3-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-propanamide; CW19, (2 β ,3 α ,5 α)-3-hydroxy-2-[methyl(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-pregnan-20-one; CW21, *N*-(3',6'-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9'*H*]xanthen]-5-yl)-*N'*-[(2 β ,3 α ,5 α)-3-hydroxy-20-oxopregnan-2-yl]-thiourea; CW22, *N*-[(2 β ,3 α ,5 α)-3-hydroxy-20-oxopregnan-2-yl]-*N'*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-formamide; CW38, (2 β ,3 β ,5 α)-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-3-(sulfoxy)-pregnan-20-one; CW41, (2 β ,3 α ,5 α ,17 β)-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-17-phenylandrostan-3-ol; CW23, (3 α ,5 α ,11 β)-3-hydroxy-11-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-pregnan-20-one; CW24, *N*-[(3 α ,5 α ,11 β)-3-hydroxy-20-oxopregnan-11-yl]-*N'*-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-thiourea; CW26, (3 α ,5 α ,11 β)-3-hydroxy-11-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl]-pregnan-20-one; CW25, (3 α ,5 α ,17 β)-17-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl]-androstane-3-ol; *ent*-CW25, (3 β ,5 β ,8 α ,9 β ,10 α ,13 α ,14 β ,17 α)-17-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl]-androstane-3-ol; CW28, 2,3,5-trichloro-6-(1,3,4,8,9,10-hexahydro-2,2,4,8,10,10-hexamethyl-12,14, disulfo-2*H*-pyrano[3,2-*g*:5,6-*g'*]diquinolin-6-yl)-4-[[2-[[6-[[[3 α ,5 α ,17 β)-3-hydroxyandrostan-17-yl]methyl]amino]-6-oxoethyl]amino]-2-oxoethyl]thio]-benzoic acid monosodium salt; AKB2, (3 α ,5 β ,17 β)-17-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl]-androstane-3-ol; AKB5, (3 α ,5 β ,17 β)-17-[[[chi[4-[3,6-(bisdimethylamino)xanthylum-9-yl]-3-carboxyphenyl]amino]thioxomethyl]amino]methyl]-androstane-3-ol inner salt; 3 α 5 α P, (3 α ,5 α)-3-hydroxypregnan-20-one; LRR, loss of righting reflex; LSR, loss of swimming reflex; DMSO, dimethyl sulfoxide; TBPS, *t*-butylbicyclophosphorothionate.

methods, and/or the need for heterologous protein introduction (e.g., with transfection or viral transduction).

We have discovered recently that the fluorophore NBD supports robust, neurosteroid/barbiturate-like photopotential of GABA_A receptors (Eisenman et al., 2007). This phenomenon outlives the presence of fluorophore and GABA agonist suggesting that it is mediated by a structural modification. Photopotential is observed over a range of light intensities, the strongest light levels producing more rapid photopotential onset (Eisenman et al., 2007). Fluorophore-assisted photopotential of GABA_A receptors might offer an additional, advantageous method of optical control over neuronal activity. One could envision experimental or clinical situations in which light could be used to inhibit neuronal activity under spatiotemporal control. An otherwise inert drug could be administered systemically and would be activated only in specific brain areas. The potential utility of light for control of neural activity has recently been demonstrated in rodent and primate models (Gradinaru et al., 2009; Han et al., 2009).

For the promise of this technology to be realized, conditions must be found that optimize the effects on GABA receptors. Therefore, in this work, we investigated structure requirements for fluorophore and for steroids that support the fluorophore-assisted photopotential. This work confirms the idea that steroids with structures supporting either positive or negative modulation of GABA_A receptors most potently facilitate photopotential. The present work also shows that a range of fluorophores (e.g., fluorescein, rhodamine, Alexa Fluor 546, FM1-43) produces fluorophore-assisted photopotential. These include red-shifted fluorophores, which would also allow for activation by light frequencies with less light scattering and better tissue penetration (Helmchen and Denk, 2005; Gradinaru et al., 2009).

Materials and Methods

Compound Synthesis. The fluorescent steroids were made by multistep synthetic procedures that will be published elsewhere. The compounds were chromatically pure and had spectroscopic properties consistent with the structures reported. Other reagents were obtained from Sigma (St. Louis, MO) except for FM1-43, which was obtained from Invitrogen (Carlsbad, CA). Steroids were dissolved in dimethyl sulfoxide (DMSO) stocks and stored in the dark until use. Final DMSO concentration did not exceed 0.1%.

Oocyte Electrophysiology. Stage-V to -VI oocytes were harvested from sexually mature female *Xenopus laevis* (Xenopus One, Northland, MI) under 0.1% 3-aminobenzoic acid ethyl ester (Tricaine) anesthesia. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 mg/ml) dissolved in calcium-free solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES at pH 7.4. Capped mRNA encoding rat GABA_A receptor $\alpha 1$, $\beta 2$, and $\gamma 2L$ subunits was transcribed in vitro using the mMESSAGE mMachin Kit (Ambion, Austin, TX) from linearized pBlueScript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20–40 ng of total RNA) 8 to 24 h after defolliculation. Oocytes were incubated up to 5 days at 18°C in ND96 medium containing 96 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 5 mM HEPES at pH 7.4, supplemented with 5 mM pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamicin. GABA currents were potentiated by lorazepam (1 μ M), indicating that a large fraction of receptors successfully incorporated the $\gamma 2$ subunit. In addition, we do not expect that lack of γ subunit should qualitatively affect screening results, because $\alpha\beta$ complexes

are steroid responsive (Belelli et al., 2002; Wohlfarth et al., 2002). The cDNAs for the rat GABA_A-receptor subunits were originally provided by A. Tobin (University of California, Los Angeles) ($\alpha 1$); P. Malherbe (Hoffman-La Roche, Switzerland) ($\beta 2$); and C. Fraser (National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD) ($\gamma 2L$).

GABA currents were measured from oocytes 2 to 5 days after RNA injection. Cells were bathed in unsupplemented ND96 medium and clamped using a two-electrode voltage-clamp amplifier (OC725; Warner Instruments, Hamden, CT). Intracellular recording pipettes had a resistance of ~ 1 M Ω when filled with 3 M KCl. Test compounds were simultaneously coapplied with GABA (2 μ M, which should yield a response that is $<1\%$ of maximum; Wang et al., 2002) using a gravity-flow perfusion system. Holding potential was -70 mV, and peak current during 20-s drug applications was used for quantifica-

Fig. 1. Examples of the data and protocols used for the evaluation of compound activity. A, responses from voltage clamped oocytes (-70 mV) expressing $\alpha 1\beta 2\gamma 2L$ GABA receptor subunits and challenged with 2 μ M GABA and GABA plus increasing concentrations of test compounds. Compound CW17 has been used in our previous publication on photopotential (Eisenman et al., 2007) and therefore serves as our exemplar here. B, example of inhibition of [³⁵S]TBPS binding by compound CW17. The IC₅₀ is given in Table 1. C, photopotential assay in hippocampal neurons using CW17 as an example. The horizontal bars denote application times. Excitation light was 480 nm. The dotted line in this and subsequent traces indicates initial holding current. Note that at 0.1 μ M compound CW17, no baseline GABA_A receptor activity is detected. Note that light induced current is largely lost upon GABA removal, indicating that the outward light generated current is potentiated GABA current with some small directly gated residual outward current.

tion. Data were acquired and analyzed with pCLAMP software (Molecular Devices, Sunnyvale, CA). Statistical differences were determined using a two-tailed Student's *t* test.

Hippocampal Cultures. Primary cultures were prepared from postnatal day 0 to 3 rat pups as described previously (Mennerick et al., 1995). Hippocampi were removed from isoflurane-anesthetized rats and were cut into 500- μ m slices. The hippocampal slices were digested with 1 mg/ml papain in oxygenated Leibovitz L-15 medium (Invitrogen), then mechanically triturated in modified Eagle's medium (Invitrogen) containing 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μ M glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were plated in modified Eagle's medium at a density of $\sim$ 650 cells/mm² as mass cultures (onto 25-mm cover glasses for imaging experiments). Cultures were maintained at 37°C in a humidified incubator with 5% CO₂/95% air. The antimetabolic agent cytosine arabinoside (6.7 μ M) was added 3 to 4 days after plating to stop glial proliferation. At 4 to 5 days after plating, half the culture medium was replaced with Neurobasal medium (Invitrogen) plus B27 supplement (Invitrogen).

Whole-cell recordings from hippocampal neuron cultures (microisland) were performed 7 to 12 days after plating using an Axopatch 200B amplifier (Molecular Devices). Cells were transferred from culture medium to an extracellular recording solution containing 138 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 0.001 mM 2,3-dihydroxy-6-nitro-7-sulfonyl-benzof[*q*]quinoline, and 0.01 mM D-2-amino-5-phosphonovalerate (to block ionotropic glutamate receptors), pH 7.25. Patch pipettes were filled with an internal solution containing 130 mM cesium methanesulfonate, 4 mM NaCl, 5 mM EGTA, 0.5 mM CaCl₂, and 10 mM HEPES at pH 7.25. When filled with this solution, pipette tip resistance was 4 to 6 M Ω . Cells were clamped at -20 mV. Access resistance (8–20 M Ω) was not compensated. Drug applications were made with a multibarreled, gravity-driven local perfusion system. The estimated solution exchange times were 120 ± 14 ms (10–90% rise), measured by the change in junction currents at the tip of an open patch pipette. Experiments were performed at room temperature.

For typical compound evaluation (e.g., Fig. 1C), GABA (0.5 μ M), which should yield a response that is $<1\%$ of maximum for GABA responses in hippocampal cultures; Shu et al., 2004) was applied for 5 s, followed by coapplication with 0.1 μ M test compound for 20 s. During the subsequent 30 s of coapplication, light at the appropriate wavelength was generated by a metal halide lamp (120 W X-Cite

lamp; EXFO Life Sciences, Mississauga, ON, Canada) through an appropriate fluorescence filter cube and delivered through the 40 $\times$, 0.6 numerical aperture objective. Photographs were taken at light onset with a Photometrics CoolSnap ES2 camera (Roper Scientific, Tucson, AZ). Cellular fluorescence intensity was measured from intracellular regions of interest in these images using MetaMorph image analysis software (Molecular Devices). Uniform light and camera settings were used for all experiments. For NBD and fluorescein dyes, a filter cube with a 480/40 excitation filter was used. For Alexa Fluor 546 and rhodamine dyes, a filter cube with a 535/50 excitation filter was used. Neutral density filtering was not used for these experiments. Light intensity at the preparation was approximately 150 mW/mm². In vitro fluorescence of the compounds was evaluated at 0.1 μ M in ethanol solvent.

***t*-Butylbicyclophosphorothionate Binding.** Rat brains were purchased from Pel-Freez (Rogers, AK) and stored until use at -20°C . Cerebella and brain stem were trimmed from the frozen brains and the cerebral hemispheres were used. Experiments were performed using minor modifications of the method of Hawkinson et al. (1994), as described previously (Covey et al., 2000). In brief, aliquots of membrane suspension (final protein concentration in assay, 0.5 mg/ml) were incubated with 1 to 2 nM [³⁵S]TBPS (60 to 100 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) and 5- μ l aliquots of steroid in DMSO solution (final steroid concentrations, 0.001 to 10 μ M) in a total volume of 1 ml of assay buffer. The assay buffer was 200 mM NaCl and 50 mM potassium phosphate buffer, pH 7.5. GABA (5 μ M) was added to all screening assays. Control binding was defined as binding observed in the presence of 0.5% DMSO and the absence of steroid; all assays contained 0.5% DMSO. Nonspecific binding was defined as binding observed in the presence of 200 μ M picrotoxin and ranged from 12.4 to 32.6% of total binding. Assay tubes were incubated for 2 h at room temperature. A Brandel (Gaithersburg, MD) cell harvester was used for filtration of the assay tubes through Whatman glass fiber (GF/C) filter paper. Filter paper was rinsed with 4 ml of ice-cold buffer three times and dissolved in 4 ml of ScintiVerse II (Thermo Fisher Scientific, Waltham, MA). Radioactivity bound to the filters was measured by liquid scintillation spectrometry and data were fit to the Hill equation using Sigma Plot (Systat Software, Inc., San Jose, CA), assuming complete maximum inhibition.

Tadpole Anesthesia. Tadpole loss of righting reflex (LRR) was measured as described previously (Wittmer et al., 1996). In brief,

TABLE 1

Results of screening assays

Average values for the indicated compounds from the various screening assays

The TBPS column gives the IC₅₀ value from fits to TBPS displacement assays, as exemplified in Fig. 1B. The 10 μ M oocyte column gives average potentiation values for 10 μ M test compound in the presence of 2 μ M GABA in oocyte screening assays like that shown in Fig. 1A. For errors around the average values, see the various summary figures. LRR and LSR columns give loss of righting reflex and loss of swimming reflex EC₅₀ estimates in the tadpole anesthesia assay. Estimated values are given when fewer than 100% of tadpoles exhibited loss of reflex at the highest concentration tested (10 μ M compound). In these cases, a maximum of 100% for fitting purposes was assumed. "None" indicates that there was no loss of reflex at the highest concentration tested. For comparison, the last column gives the degree of photopotential in hippocampal neurons from bar graphs in figures. AKB5 and CW28 were illuminated with green light; blue light was used for all other compounds.

	TBPS	10 μ M Oocyte	LRR	LSR	Light Potentiation
	μ M	% control	μ M		% control
CW17	0.9	165	2.1	None	430
CW18	6.1	37	None	None	390
CW19	4.4	86	8 ^a	None	650
CW21	3.7	57	10 ^a	None	840
CW22	9.0	90	None	None	190
CW38	1.8	18	None	None	510
CW41	100.0	63	None	None	130
CW23	0.3	1008	2.7	None	1430
CW24	1.0	177	100 ^a	None	390
CW26	0.9	474	3 ^a	None	1550
CW25	0.1	477	3	None	600
ent-CW25	5.1	101	None	None	230
AKB2	0.1	167	0.4	1.2	1250
AKB5	1.5	91	10 ^a	None	1290
CW28		94			140

^a Estimated values.

groups of 10 early pre-limb-bud stage *Xenopus laevis* tadpoles (Nasco, Fort Atkinson, WI) were placed in 100 ml of oxygenated Ringer's stock solution containing various concentrations of compound. Compounds were added from a 10 mM DMSO stock (final concentration of DMSO in test solutions was 0.1%). After equilibrating at room temperature for 3 h, tadpoles were evaluated with the LRR and loss of swimming reflex (LSR) behavioral endpoint. LRR was defined as failure of the tadpole to right itself within 5 s after being flipped by a smooth glass rod. LSR was defined by a failure of purposeful tail movement within 10 s of gently sliding the tadpole around the beaker with a glass rod for 5 s. In most cases, the tadpoles regained their reflexes when placed in fresh oxygenated Ringer's solution. Exceptions were CW19 and AKB2, where at high concentrations (10 μ M), only 50 to 70% of affected tadpoles regained righting reflex after overnight incubation in steroid-free solution. Control beakers containing up to 0.6% DMSO produced no LRR in tadpoles. Concentration-response curves were fit with Sigma Plot to the Hill equation.

Results

We previously found that a pregnane steroid with a 3 α -hydroxy,5 α -reduced stereochemistry and a fluorescent NBD attached via a 2 β -amino group on the steroid (labeled CW17 in the present report) supports robust photopotential of GABA_A receptors (Eisenman et al., 2007). In this study, we explored structural specificity using several screening methods for baseline and light-activated activity at GABA_A receptors and for fluorescence. Figure 1 and Table 1 demonstrate the assays used to investigate properties of the fluorescent steroid analogs. We used GABA-gated currents from a *X. laevis* oocyte expression system, inhibition of TBPS binding in rat brain membranes, and a tadpole anesthesia screen to evaluate baseline GABA_A receptor activity and behavioral effects of the fluorescent analogs. In both of the electrophysiology assays, GABA concentration was below 1% of maxi-

mum, allowing wide dynamic range for potentiation of oocyte and hippocampal neuron GABA responsiveness. An example of one of the prototype compounds, compound CW17, is demonstrated in Fig. 1. CW17 had weak but detectable potentiating activity on GABA induced gating of rat α 1 β 2 γ 2L receptor subunits in oocytes. Maximum potentiation obtained at 10 μ M compound CW17 was $165 \pm 19\%$ ($n = 4$, Fig. 1, 3). For comparison, (3 α ,5 α)-3-hydroxypregnan-20-one (3 α 5 α P), a naturally occurring nonfluorescent neurosteroid, exhibits an EC_{50} of $\sim 1 \mu$ M in oocytes (Wang et al., 2002). In the present studies, 0.5 μ M 3 α 5 α P potentiation was $1288 \pm 209\%$ in a sample of seven oocyte batches. This probably represents both a shift in the GABA EC_{50} with steroid and a change in GABA efficacy (Maksay et al., 2000; Wohlfarth et al., 2002). Although CW17 is clearly weaker than 3 α 5 α P, because of limited solubility, we were unable to distinguish whether the weakness results from low potency, low efficacy, or both. TBPS binding assays suggested that potency is probably affected (Fig. 1B). CW17 exhibited nearly complete TBPS inhibition and an IC_{50} of 0.9 μ M (Fig. 1B, Table 1). The 3 α 5 α P IC_{50} in the TBPS assay is 0.074 μ M (Zeng et al., 2005). Tadpole assays also demonstrated weak activity of compound CW17 at GABA_A receptors; the EC_{50} for loss of righting reflex was 2.1 μ M (Table 1). By contrast, 3 α 5 α P in the LRR assay exhibits an EC_{50} of 0.39 μ M (Wittmer et al., 1996). Therefore, by all GABA receptor assays, compound CW17 exhibits lower activity than its parent compound, 3 α 5 α P.

We screened CW17 at 0.1 μ M in primary cultures of hippocampal neurons with 480 nm light, near the peak of NBD's excitation spectrum (Eisenman et al., 2007). As we have observed previously, CW17 (0.1 μ M) had a minimal effect on GABA responses from hippocampal neurons in the absence of light, but during 480 nm wavelength light excitation, GABA responses gradually potentiated (Fig. 1C). For quantification

Fig. 2. Compound fluorescence. A, in vitro fluorescence of the compounds (0.1 μ M) tested in this work (ethanol solvent). Each mean and S.E.M. is calculated from three measurements. B, cellular fluorescence of the compounds, taken from images acquired during the photopotential assay. Note that the charged compounds CW38, CW28 (Alexa Fluor 546 conjugate), and AKB5 exhibit strong in vitro fluorescence but weak intracellular fluorescence, presumably because the negative charge on these compounds prevents good cellular penetration. Number of cells tested is the same as that given in summary graphs of functional effects shown in Figs. 3 to 5 and 7.

purposes, measurement of light-independent potentiation in figures was obtained by normalizing to current at the end of a 5-s exposure to GABA alone. Measurements of photopoten-

tiation compared the current at the end of light stimulation with the current just before light stimulation.

In addition to functional assays of GABA_A receptor activ-

Fig. 3. Comparisons among steroid analogs with fluorescent groups attached to steroid carbon 2. A1 and B1 show structures of the compounds. A2 and B2 show baseline activity on GABA_A receptors expressed in oocytes at three concentrations (mean $\pm$ S.E.; $n = 4$ oocytes for each compound). The dotted line at 100% represents the response to GABA alone. Assays were performed as in Fig. 1A; A3 and B3 show summary data from hippocampal neurons challenged as in Fig. 1C with the indicated compounds at 0.1 μ M ($n = 3-6$ cells per bar). Black bar, normalized baseline compound activity before light onset; dotted line, response to GABA alone before compound addition; white bar, normalized response in the presence of 480 nm light.

ity, we also examined cellular and in vitro fluorescence of all compounds (see *Materials and Methods*). Figure 2 shows a summary of the fluorescence of the neurosteroid compounds excited with blue light and tested in the present work. Chemical structures of these compounds are shown in the various subsequent figures. We measured in vitro fluorescence at a concentration of 0.1 μ M in ethanol solvent, and we also measured the intracellular fluorescence of the compounds in hippocampal neurons during photopotential screening. The pattern of intracellular fluorescence did not track the in vitro fluorescence pattern particularly well. This is explained at least partly by the lack of cell penetration of some of the compounds.

Figure 3 explores structural and fluorophore manipulations at the steroid 2 position (Fig. 3, A1 and B1), the site of fluorophore modification in compound CW17. We tested compounds for which we had sufficient material in the oocyte, TBPS, tadpole, and hippocampal neuron screens (Fig. 3, Table 1). Summary screening data for oocytes are shown in Fig. 3, A2 and B2, and for hippocampal neurons in Fig. 3, A3 and B3. Summary data for CW17, exemplar traces of which are shown in Fig. 1, are shown in Fig. 3, A2 and A3. Compared with compound CW17, other compounds with an NBD group at the steroid 2 position showed varying degrees of photopotential and baseline GABA receptor activity. CW19 was notable because of weak baseline GABA receptor activity in both oocytes and hippocampal neurons but robust photopotential.

The compounds shown in Fig. 3B exhibited inhibitory actions on GABA_A receptors at baseline. Analog CW38, because it contains an anionic 3 β -sulfate group at carbon 3 would, as observed, be expected to have a strong inhibitory effect on

GABA_A receptors (Park-Chung et al., 1999; Eisenman et al., 2004). However, despite inhibitory actions in the absence of light, CW38 remained an effective photopotentiator. The inhibitory actions of the other three compounds in Fig. 3B, which are all 3 α -hydroxysteroids, was not anticipated, because 3 α -hydroxysteroids generally do not cause inhibition. Nevertheless, two of three of these 3 α -hydroxysteroids were also photopotentiators. The results indicate that structural requirements for potentiation are more stringent than those for photopotential.

Compound CW21, with a fluorescein dye attached via a thiourea linker at steroid position 2, was among those exhibiting a mild inhibitory effect on GABA responses at the highest concentration employed. It exhibited strong photopotential of GABA responses in hippocampal neurons. This indicates that the NBD fluorophore is not required for photopotential and that the effect is likely to be a photodynamic effect capable of being generated by multiple fluorophores.

Compound CW41 (Fig. 3B1) is a fluorescent analog of a steroid antagonist that we developed previously (Mennerick et al., 2004). This compound showed mild, possibly nonspecific (weakly concentration dependent) inhibition of GABA responses in oocytes (Fig. 3B2). In hippocampal neurons, the compound was relatively inert at 0.1 μ M (Fig. 3B3) and showed little intracellular fluorescence, probably indicating poor cellular penetration and weak in vitro fluorescence in ethanol solvent at the wavelengths evaluated (Fig. 2). Photopotential was also very weak (Fig. 3B3).

Figure 4 depicts compounds with the NBD fluorophore attached in various ways to steroid position 11. Compound CW17 is redisplayed for comparison. We found that attach-

Fig. 4. Compounds with NBD fluorophore attached by different linkers to steroid carbon 11. Data from compound CW17 are repeated here for comparison. Summary data are similar in format to Fig. 3. A, structures. B, oocyte responses ($n = 4$ oocytes each compound). C, baseline (black bars) and light-activated (white bars) activity on GABA responses in hippocampal neurons. The inset shows the baseline activity of 0.1 μ M compound at higher resolution. Consistent with oocyte data in B, compounds CW23 and CW26 show most baseline activity and highest photopotential in this series.

ment via an 11 β -amino group produced compounds with more baseline activity on GABA_A receptors than the comparator, compound CW17, which has the NBD group attached via a 2 β -amino group (Fig. 4, B and C). Attaching the NBD group via an 11 β -aminomethyl group also gave a compound (CW26) the photopotential of which was stronger than that of CW17. However, NBD attachment via a thiourea linker at steroid position 11 (compound CW24) produced a compound with weaker baseline and light-activated GABA_A receptor activity, equivalent to that of CW17.

Figure 5 shows the effect of the NBD substituent attached via an aminomethyl group at steroid position 17 (compound CW25). We found that compound CW25 exhibited reasonably strong GABA receptor actions at baseline (Fig. 5B) and strong photopotential (Fig. 5, C and D). Because a hydrogen bond acceptor at carbon 17 has been considered to be necessary for potent GABA receptor modulation by neurosteroid potentiators (Covey et al., 2001), the baseline potentiation suggests that the NBD rings can serve this role. To determine whether photopotential requires specific chiral protein interactions, we synthesized the enantiomer of compound CW25. We found that at 0.1 μ M, *ent*-CW25 exhibited little photopotential in sibling cultures of those evaluated with compound CW25. However, at 10-fold higher concentra-

tion, *ent*-CW25 effectively photopotentialized GABA currents. We conclude that photopotentialization exhibits enantioselectivity and is therefore facilitated by protein chiral interactions. However, enantioselectivity of photopotentialization is not as strong as enantioselectivity of baseline potentiation of GABA responses (Figure 5B). Therefore, interaction with proteinaceous sites (e.g., the GABA receptor) is not an absolute requirement for photopotentialization.

It seems likely that the chiral interaction relevant to photopotentialization might be with the GABA_A receptor itself. To test the idea that baseline (light-independent) interactions with GABA_A receptors aid and predict photopotentialization, we performed a correlation across all 13 fluorescent compounds excited by 480-nm light used in the present study (containing an NBD or fluorescein). Our analysis included both positive and negative modulation of GABA receptor function (see *Materials and Methods*). We found a significant correlation between baseline activity at GABA receptors and degree of photopotentialization (Fig. 6).

Neurosteroid modulation of GABA_A receptors tolerates a 5 β -reduced steroid structure (Harrison et al., 1987). To determine whether the 5 β -reduced stereochemistry also supports photopotentialization, we synthesized a 5 β -reduced version of compound CW25 (compound AKB2; Fig. 7A). Figure 7, B to

Fig. 5. Effects of enantiomeric steroid *ent*-CW25 on photopotentialization. **A**, structures of compound CW25 and its enantiomer *ent*-CW25. **B**, baseline GABA_A receptor activity is not observed for *ent*-CW25 on oocytes at concentrations up to 10 μ M. **C**, example traces from the photopotentialization protocol in hippocampal neurons (0.1 μ M compound and 480-nm light wavelength). The traces come from different cells. **D**, summary of photopotentialization protocol in hippocampal neurons ($n = 9$ for 0.1 μ M CW25; $n = 10$ for 0.1 μ M *ent*-CW25 and $n = 6$ for *ent*-1 μ M CW25). Black bars, baseline (before light); white bars, excitation with 480 nm light.

D, shows that both baseline GABA receptor activity and light-induced photopotential were supported by the fluorescent 5 β -reduced steroid analog. In fact, photopotential by compound AKB2 was among the most robust observed among tested compounds ($1251 \pm 462\%$ potentiation, $n = 5$).

Because the 5 β -reduced steroid AKB2 produced very robust photopotential, we evaluated the effect of a rhodamine dye attached via a thiourea linker to the 17 β -aminomethyl group of the 5 β -reduced steroid (Fig. 7A, compound AKB5). It is noteworthy that baseline potentiation was lost with this manipulation (Fig. 7B, Table 1). Furthermore, illumination with 480-nm light was relatively ineffective at eliciting compound AKB5 photopotential, consistent with the excitation spectrum of rhodamine (Fig. 7C). By contrast, green excitation light (535 nm) robustly photopotentialized GABA responses in the presence of compound AKB5 (Fig. 7, C and D). This result confirms the idea that multiple fluorophores support robust photopotential.

The completely inert behavior of compound AKB5 in the absence of light seemed to violate the correlation between the best photopotentiators and those compounds with strongest GABA receptor activity at baseline (Fig. 6). We hypothesized that AKB5 might in fact interact with receptors as a steroid site antagonist. In preliminary experiments we examined the effect of AKB5 on 3 α -hydroxy-5 β -pregnan-20-one potentiation. We found evidence that 10 μ M AKB5 strongly and reversibly reduced potentiation by 0.5 μ M 3 α -hydroxy-5 β -pregnan-20-one in two oocytes (data not shown). These preliminary results will be pursued fully in a subsequent publication. For present purposes, AKB5 seems to interact with the receptor, although it is functionally inert unless a modulatory steroid is present. Therefore, it does not necessarily violate the general correlation between baseline receptor interaction and robust photopotential.

We also tested an Alexa Fluor 546 fluorophore attached to the 17 β -aminomethyl group (compound CW28; Fig. 7A) in the context of a 5 α -reduced steroid. This compound was synthesized for our previous work probing the locus of neurosteroid action at GABA_A receptors (Akk et al., 2005). Compound CW28 failed to have significant baseline activity on GABA_A receptors and failed to photopotentialize when exposed to either blue or green light at a concentration of 0.1 μ M (Fig. 7,

Fig. 6. Correlation between GABA_A receptor activity and photopotential. Ranked GABA receptor activity was established by taking potentiation values at 10 μ M compound, or the reciprocal of the fractional response in the case of inhibitory actions, in the oocyte assay. This ranked order was correlated with photopotential values from hippocampal neurons. The correlation was significant (Spearman's $\rho = 0.588$, $p < 0.05$, $n = 13$ NBD/fluorescein-tagged compounds). Compound identifiers are used as symbols. Solid line is a linear regression.

C and D). However, at a concentration of 1 μ M, compound CW28 effectively photopotentialized with excitation by appropriate light wavelength (Fig. 7D, inset).

Although AKB5 did not show strong intracellular fluorescence (see Fig. 2), it clearly permeated cell membranes with longer exposure times (data not shown). By contrast, CW28 failed to stain intracellular compartments with longer exposure, despite strong in vitro fluorescence (Fig. 2). This is likely to arise from the negative charge on the Alexa Fluor, which prevented it from penetrating cells. The ability of the anionic steroid analogs CW38 and CW28 to photopotentialize suggests that cell penetration is not necessary for photopotentialization.

To test explicitly the issue of extracellular versus intracellular photodynamic effects of fluorophores on GABA_A receptor activity, we filled cells with two membrane-impermeant fluorescent analogs. We used the sulfated NBD compound (compound CW38; Fig. 8A, C) and the charged Alexa Fluor 546 conjugated analog (compound CW28; Fig. 8, B and C). To compensate for poor steroid filling through whole-cell loading (Akk et al., 2005), we used a high pipette concentration of analogs (50 μ M). Unlike extracellular loading, pipette loading produced detectable intracellular fluorescence (Fig. 8, A and B, insets). However, neither compound produced photopotentialization (Fig. 8, A and B). Furthermore, loading cells with free Alexa Fluor 488 hydrazide (10 μ M) failed to produce photopotentialization in any of three tested cells despite very bright intracellular fluorescence. As an additional test of intracellular fluorescence, we transfected neurons with GFP. We found no evidence that GFP fluorescence excitation photopotentialized GABA responses ($n = 5$ cells, data not shown). Taken together, these results confirm that the photodynamic effect occurs toward the extracellular side of the cell membrane and is not supported by intracellular delivery (Eisenman et al., 2007).

Based on the above analysis, it seems that multiple fluorophores can support GABA_A receptor photopotentialization. To test whether nonsteroid lipophilic fluorophores also support photopotentialization, we examined the membrane probe FM1-43, popularly used in neuroscience research to label cycling synaptic vesicles (Betz and Bewick, 1992). We found that at 0.1 μ M, the concentration at which the fluorescent steroids were screened, FM1-43 supported only weak GABA_A receptor photopotentialization ($80 \pm 46\%$ potentiation, $n = 4$ cells). However, at 5 μ M, we observed robust GABA_A receptor photopotentialization (Fig. 9A1; $850 \pm 4.4\%$ enhancement, $n = 3$ cells). We have previously shown for neurosteroid photopotentialization that the effect is long-lived after removal of light, GABA, and fluorophore. To verify that this is also the case with other fluorophores, we challenged four additional cells with a reapplication of GABA after 1 min of washing after the initial photopotentialization. Figure 9A, right trace, shows that strong potentiation remained after the 1-min wash with saline solution. In four cells, the response to GABA remained potentiated $573 \pm 133\%$ compared with the initial GABA response. As expected, FM1-43 fluorescence was largely limited to the plasma membrane and did not exhibit significant intracellular accumulation. In summary, these results suggest that a wide range of fluorophores can support photopotentialization, although fluorescent neurosteroid analogs are particularly potent photopotentializers.

Discussion

We have analyzed the photodynamic effect of fluorescent steroids on GABA_A receptors. Our goals were to develop a better understanding of the structural requirements for photopotential and to generate a lead compound for use as a tool for optical influence over inhibition in brain cells and tissue. Our major conclusions are that baseline (absence of light) GABA_A receptor binding, resulting in posi-

tive or negative modulation of channel function, is helpful but not necessary for photopotential. Photopotential always occurs at concentrations below those that have direct, detectable effects at baseline on GABA_A receptor function (e.g., 0.1 μ M in hippocampal neurons). Photodynamic effects seem to be limited to the outside of cells; intracellular delivery is ineffective. Finally, multiple fluorophores, including red-shifted fluorophores expected to

Fig. 7. Effects of 5 β -reduced steroid carrier and of red-shifted fluorophores. **A**, structures. **B**, summary of oocyte effects ($n = 4$ oocytes for each compound). **C**, top traces are representative of responses to blue light of the three compounds (0.1 μ M) in hippocampal neurons. The bottom traces are responses from cells evaluated with green light under the same conditions. Each trace is from a separate cell. **D**, summary of evaluations performed as in **C** ($n = 3$ –5 for each bar). Note that optimal photopotential occurs with excitation wavelengths best matched to the fluorophore's excitation spectrum. Compound CW28 did not respond to green light until the concentration was raised to 1 μ M (**D**, inset).

be recruited with less light scattering, can elicit the photodynamic effect.

Of the compounds tested, compound AKB5 has a number of desirable properties. These include baseline inertness at concentrations that yield robust photopotential, high potency, red-shifted excitation, and poor cell permeation. Baseline inertness is desirable to limit activity in the absence of light, thereby improving the spatiotemporal control of GABA receptor modulation. A caveat with regard to AKB5 is that its potential effects as an antagonist at steroid sites on GABA_A receptors need to be considered if endogenous steroids are present. The red-shifted excitation of AKB5 is strongly desired to maximize penetration of relevant wavelengths into tissue. High potency and poor cell permeation should help limit undesired baseline and collateral photodynamic effects (e.g., Antic et al., 1999; Eisenman et al., 2009) of the compound and could improve delivery by limiting cellular uptake and retention. Limiting these effects may be possible with compounds that can be delivered at low enough concentrations (with high enough specificity) to avoid such effects. The Alexa Fluor-tagged compound CW28 had the wavelength, inertness, and cell exclusion benefits but possessed poor potency relative to AKB5 and many of the other compounds.

Because of apparent structural promiscuity supporting

photopotential, we tested an additional lipophilic fluorescent dye, FM1-43, and found that it also exhibits photopotential. This result raises a caution in the use of FM1-43 for monitoring presynaptic activity and as a general membrane probe. The concentration that we found effective for photopotential (5 μ M) is well within the concentration range used for probing synaptic vesicle exo-/endocytosis. However, it is worth noting that our conditions were atypical of the way FM1-43 is used as a synaptic vesicle probe. We assessed photopotential in the continuous presence of 5 μ M FM1-43 in the bath solutions with continuous, prolonged light exposure. In most studies that use FM1-43 for synaptic vesicle study, bath (and plasma membrane) staining is washed away before assays are performed, leaving only presynaptic vesicle fluorescence. Furthermore, with brief and low-intensity illumination, photopotential should be limited. Thus, with proper precautions and controls, GABA_A receptor photopotential can probably be avoided as a complication in imaging studies.

In our initial report of photopotential of GABA_A receptors, we noted that the potentiation is not a steroid effect, because steroids are not required to elicit photopotential (Eisenman et al., 2007). We speculated that steroids serve as potent carriers as a result of some combination of lipid solu-

Fig. 8. Intracellular loading of fluorophores is not effective. **A**, comparison of CW38 (sulfated steroid) photopotential with extracellular application (left) and intracellular application of 50-fold higher concentration (right). Effective loading was verified by intracellular fluorescence (insets). **B**, comparison of compound CW28 (negatively charged Alexa Fluor 546 fluorophore) extracellularly and intracellularly applied. **C**, summary of effects of the two charged compounds applied to either side of the membrane ($n = 3$ cells for each bar, except for compound CW38 outside, where $n = 24$).

bility and affinity for GABA_A receptors. The present study suggests that interaction with GABA_A receptors is the key feature provided by the steroid carrier molecules. This conclusion is based on several observations. First, the correlation between potency of steroid effects in the absence of light and the potency of photopotentiation (Fig. 6) suggests the importance of direct interaction with the GABA_A receptor rather than or in addition to lipid solubility. Second, the observation that the lipophilic compound FM1-43 is less potent than steroids suggests interaction with the receptor is more important than lipid solubility, although differences that are due to different fluorophores cannot be excluded. Finally, the observation that *ent*-CW25 (Fig. 4) is significantly less potent than CW25 strongly supports interaction with GABA_A receptors as a key feature, because the solubility of these two compounds should be identical. This observation suggests that maintenance of interactions with GABA_A receptors should be a design goal in future work to develop better compounds.

What is the nature of the photodynamic effects on GABA_A receptors? Other photodynamic effects on proteins have been attributed to oxygen radicals and other reactive species generated as a by-product of fluorescence excitation (Sjölund and Miesenböck, 2008). This seems likely in the case of GABA_A receptor photopotentiation also. GABA_A receptors are potentiated by ultraviolet wavelengths in the absence of fluorophore (Chang et al., 2001; Leszkiewicz and Aizenman, 2003), and our work shows that a similar effect is sensitized by a variety of fluorophores. NMDA receptors are also subject to photodynamic effects, and in this case, singlet oxygen has been implicated in ultraviolet light effects and in fluorophore photosensitization (Leszkiewicz and Aizenman, 2002; Eisenman et al., 2009). It seems likely that a similar mechanism applies to GABA_A receptor modulation, although a paradox is that oxidizing agents typically inhibit GABA_A receptor function (Pan et al., 1995; Amato et al., 1999; Pan et al., 2000; Leszkiewicz and Aizenman, 2003). Possibilities that could resolve this paradox are that oxidation of associated proteins

or of annular lipid surrounding the receptor may be important for photopotentiation.

Our work suggests a correlation between receptor binding and potency of photodynamic effect. We interpret this correlation and the observed enantioselectivity as evidence that steroids with the ability to gain close proximity to the receptor (e.g., by binding a site) serve as particularly potent carriers of the photosensitization effect. These observations reinforce the idea that the photodynamic effect involves structural modification of the receptor itself or of a very local partner (annular lipid, associated protein). However, because steroid structures that support both positive and negative modulation are effective, it is possible that multiple receptor target sites are capable of producing photopotentiation. From a practical standpoint, using fluorescently tagged ligands for the receptor (rather than nonspecific fluorophores like FM1-43) maximize prospects for specificity and limiting collateral effects.

Future work will be aimed at further improvements over our best compounds (e.g., AKB5). Especially useful may be a fluorophore with even longer excitation wavelengths. For instance, fluorophores used in humans for photodynamic cancer therapy typically have wavelengths >600 nm to maximize light accessibility to deep tissue (Dolmans et al., 2003). Additional work will focus on delineating the nature of the photodynamic effect so that the effect can be more rationally harnessed. It is also possible that the observation that receptor proximity might be important for photopotentiation could be exploited to yield receptor subtype selective probes. For instance, fluorophores could be conjugated to subunit selective carrier molecules (Rudolph and Möhler, 2006). An ultimate goal would be a compound suitable for in vivo systemic delivery, with implantable light guides that allow local spatiotemporal control over neural activity. Recent applications of light control over ion channels have demonstrated that use of optical techniques in clinical situations may not be far off (Gradinaru et al., 2009; Han et al., 2009).

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Fig. 9. FM1-43 photopotentiation. **A**, robust photopotentiation by 5 μ M FM1-43. GABA concentration was 0.5 μ M. **A**, the standard screening protocol is shown in the left trace. In the right trace, GABA alone was re-applied 1 min after the initial photopotentiation. The dashed line extending from A1 to A2 represents the approximate original GABA response, to highlight the residual potentiation after a 1 min wash. **B**, structure of FM1-43. **C**, fluorescence image of a cell pre-exposed to 5 μ M FM1-43 for 20 s, at the onset of illumination.

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