

(1*S*, 3*S*)-3-Amino-4-difluoromethylenyl-1-cyclopentanoic Acid (CPP-115), a Potent γ -Aminobutyric Acid Aminotransferase Inactivator for the Treatment of Cocaine Addiction

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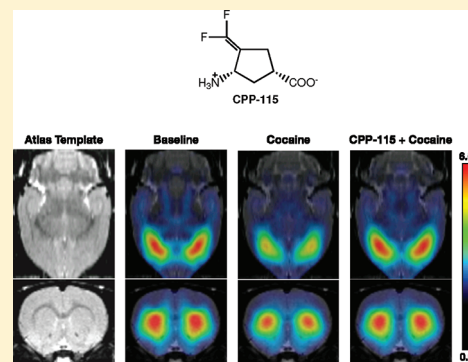
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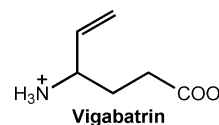
S Supporting Information

ABSTRACT: Vigabatrin, a GABA aminotransferase (GABA-AT) inactivator, is used to treat infantile spasms and refractory complex partial seizures and is in clinical trials to treat addiction. We evaluated a novel GABA-AT inactivator (1*S*, 3*S*)-3-amino-4-difluoromethylenyl-1-cyclopentanoic acid (CPP-115, compound **1**) and observed that it does not exhibit other GABAergic or off-target activities and is rapidly and completely orally absorbed and eliminated. By use of *in vivo* microdialysis techniques in freely moving rats and microPET imaging techniques, **1** produced similar inhibition of cocaine-induced increases in extracellular dopamine and in synaptic dopamine in the nucleus accumbens at $1/_{300}$ to $1/_{600}$ the dose of vigabatrin. It also blocks expression of cocaine-induced conditioned place preference at a dose $1/_{300}$ that of vigabatrin. Electroretinographic (ERG) responses in rats treated with **1**, at doses 20–40 times higher than those needed to treat addiction in rats, exhibited reductions in ERG responses, which were less than the reductions observed in rats treated with vigabatrin at the same dose needed to treat addiction in rats. In conclusion, **1** can be administered at significantly lower doses than vigabatrin, which suggests a potential new treatment for addiction with a significantly reduced risk of visual field defects.



■ INTRODUCTION

The neurochemical response to cocaine and other drugs of abuse is well characterized by a rapid elevation in the release of dopamine in the nucleus accumbens (NAc).¹ This increase in dopamine, and associated behaviors, can be antagonized by an increase in the concentration of γ -aminobutyric acid (GABA), which has been shown to occur with use of the FDA-approved epilepsy drug vigabatrin (also known as CPP-109), a known mechanism-based inactivator^{2,3} of γ -aminobutyric acid aminotransferase (GABA-AT).⁴ Vigabatrin is currently marketed for the treatment of infantile spasms (West's syndrome) and refractory complex partial seizures.



Vigabatrin has been used in the treatment of addiction,^{5,6} specifically in animal models for cocaine,¹ nicotine,⁷ methamphetamine, heroin, ethanol,⁸ and combination addictions.⁹ In

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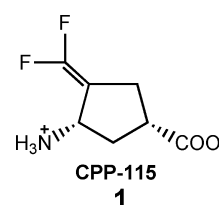
human studies, vigabatrin treatment is effective for addiction,^{10,11} including a recently reported randomized, double-blind, placebo-controlled trial of 103 subjects,¹² in which 28% of subjects treated with vigabatrin achieved abstinence compared to 7.5% of subjects treated with a placebo.

The acceptance of vigabatrin for the treatment of both epilepsy and as a potential treatment for addiction has been hampered primarily by concerns about abnormalities of the peripheral visual field (visual field defects or VFDs) in 25–50% of patients following chronic administration of vigabatrin.^{13,14} As a result, an unmet medical need remains for a GABA-AT inhibitor with improved potency and reduced, or eliminated, visual field defects for the treatment of both addiction and epilepsy.

The cellular mechanisms leading to the visual field defects are not known; however, it remains an active area of research. Visual field defects could be from (1) a direct toxic effect of vigabatrin, (2) elevated GABA levels, (3) inactivation of GABA-AT, (4) the consequence of an enzymatically produced by-product from one of the enzyme inactivation mechanisms, (5) off-target activities, or (6) a combination of these potential modes of action. In albino rats acute vigabatrin exposure damages the outer retina by a GABA-independent and vigabatrin-specific mechanism, resulting in sensitization of photoreceptors to light-induced damage.¹⁵ In this case, it is probable that reactive oxygen species are involved, since they participate in light-mediated retinal toxicity.¹⁶ Recently, the visual field defects in an animal model were reported to result from drug related toxic effects that lead to a reduction in taurine, an essential amino acid related to retinal function.¹⁷

A new synthetic compound, (1*S*,3*S*)-3-amino-4-difluoromethylenyl-1-cyclopentanoic acid (**1**, CPP-115), was designed as a mechanism-based inactivator of GABA-AT, which could generate a more reactive intermediate along the pathway to attachment to the active site of GABA-AT via a Michael addition.¹⁸ In contrast to the high K_i value reported for vigabatrin as an inactivator of GABA-AT (3.2 mM¹⁸ or 10 mM⁴), **1** has a K_i value (at suboptimal pH) of 31 μ M.¹⁸ A comparison of the k_{inact}/K_i values, a measure of the efficiency of the inactivator, indicated that **1** is 187 times more effective as an inactivator of GABA-AT than vigabatrin under suboptimal conditions (at optimal conditions for substrate turnover the rate of inactivation by **1** is too rapid to measure; these values were obtained at a pH and temperature well below the optimum). Despite irreversibility of the inhibition, the low potency and poor blood–brain barrier penetration of vigabatrin translate into treatment doses of 1–3 g/day.¹⁹ Because **1** displayed superior enzyme inactivation properties compared to vigabatrin, we have carried out pharmacological and preclinical studies with **1**. Because of the preponderance of data indicating vigabatrin is effective for the treatment of addiction, the effect of **1** was investigated on cocaine-induced conditioned place preference in rats, an established animal model for effectiveness of addiction treatments. We also used microPET imaging to measure the ability of **1** to antagonize cocaine-induced increases in synaptic nucleus accumbens dopamine. Because of the higher potency of **1** relative to vigabatrin, we hypothesized that **1** would exhibit less retinal toxicity either by reducing the formation of toxic enzyme inactivation byproduct or by a reduction of direct toxic effects of the drug as a result of the lower dose needed to inactivate GABA-AT. Therefore, we used electroretinography (ERG) in an animal

model to evaluate the retinal toxicity potential of **1** compared to vigabatrin.^{17,20}



RESULTS

Effects of (1*S*,3*S*)-3-Amino-4-difluoromethylenyl-1-cyclopentanoic Acid (**1**) at GABA Transporters and Receptors.

Interaction of **1 with GABA Transporters.** Unlike vigabatrin, which inhibits the hGAT1 transporter, compound **1** displayed no inhibitory activity at 1 mM at each of the four human or mouse GABA transporter subtypes, in neurons, astrocytes, or mammalian cells recombinantly expressing human or mouse transporter subtypes (see Supporting Information Table 1).²¹

Interaction of **1 with GABA Receptors.** To investigate a possible interaction of **1** with GABA receptors, we tested the compound for its ability to displace [³H]GABA binding to ionotropic GABA_A receptors or metabotropic GABA_B receptors in rat brain cortical homogenates.²² At 100 μ M, no inhibition of binding was observed at either receptor tested, whereas 1 μ M cold GABA inhibited radioligand binding as expected (see Supporting Information Table 2). Additionally, we tested **1** for activity at recombinant human ρ 1 GABA_C receptors expressed in oocytes, and it was found to exhibit no effect as an agonist or antagonist at 100 μ M (see Supporting Information Figure 1).

Effects of (1*S*,3*S*)-3-Amino-4-difluoromethylenyl-1-cyclopentanoic Acid (**1**) at Non-GABAergic Off-Targets.

Off-target tests of 111 different biological targets were carried out at Cerep (Poitiers, France). There was no significant effect of **1** on any of these off-targets at 10 μ M concentration (see Supporting Information Tables 3 and 4 for a summary of testing). There also was no in vitro effect at 6 mM concentration on aspartate aminotransferase, alanine aminotransferase, or succinic semialdehyde dehydrogenase, unlike vigabatrin, which is known to reduce activities of alanine aminotransferase^{23,24} and succinic semialdehyde dehydrogenase²⁵ in vivo. Compound **1** also did not inhibit or induce CYP1A2, CYP2B6, and CYP3A4/5 or inhibit CYP2C8, CYP2C9, CYP2D6, which are the more common human liver microsomal cytochrome P450 enzymes (100 μ M). The in vitro effects of **1** (300 μ M) on the human ether-a-go-go-related gene (hERG) channel current at near-physiological temperature was determined. Compound **1** inhibited hERG current by (mean \pm SEM) 1.1 \pm 0.2% at 10 μ M (n = 3) and 1.5 \pm 0.2% at 300 μ M (n = 3) versus 0.8 \pm 0.3% (n = 3) in control. At both test concentrations hERG inhibition was not statistically significant (p < 0.05) when compared to vehicle control values, indicating a minimal risk for **1** induced cardiac arrhythmias.

Pharmacokinetics and Metabolic Stability of **1.** Compound **1** is rapidly orally absorbed (1.7 h⁻¹ for rats and 2.3 h⁻¹ for beagle dogs), exhibits excellent bioavailability (79% for rats and >100% for dogs), and is rapidly eliminated ($T_{1/2}$ is 1 h for rats and 2.3 h for dogs). The volume of distribution was about 1.1 and 0.8 L/kg for rats and dogs, respectively, indicating the drug is widely distributed. The clearance rate was about 0.8 and 0.2 L/h/kg for rats and dogs, respectively, with

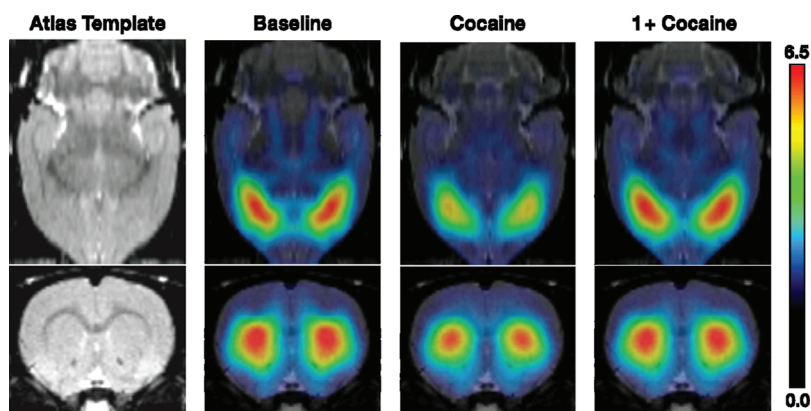


Figure 1. Stimulatory effects of cocaine by pretreatment with **1**. In vivo microPET imaging shows that the stimulatory effects of cocaine are reduced by pretreatment with **1**. [^{11}C]Raclopride is sensitive to competition with synaptic dopamine, so its binding (higher levels in red) is inversely related to dopamine levels. Increases in dopamine produced by a cocaine challenge (20 mg/kg, cocaine) displace [^{11}C]raclopride binding. This effect is blocked by pretreatment with **1** (0.5 mg/kg, **1** + cocaine) 2.5 h prior to cocaine.

the lower clearance rate for dogs accounting for the longer elimination half-life observed in dogs. Rats received a 10 mg/kg iv dose or a 30 mg/kg oral dose. Dogs received a 5 mg/kg iv dose or a 10 mg/kg oral dose. The greater than 100% bioavailability observed for dogs may be due to random variability (only three animals were employed) or due to nonlinear dose–response relationships in the rates of absorption and/or elimination for the iv and oral doses.

The metabolic stability of **1** was evaluated in cryopreserved human hepatocytes (1 000 000 cells/mL, in triplicate), and 16% loss was observed after 4 h ($T_{1/2}$ is 20 h), which is consistent with a drug that would survive first pass metabolism and through several half-lives in the bloodstream. The calculated $T_{1/2}$ is well past the end of the incubation period and is only approximate.

Toxicity of 1. Compound **1** was tested in the in vitro mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of an Aroclor-induced rat liver S9 metabolic activation system. At 10 mM, the percentage of cells with structural or numerical aberrations in the test-article-treated groups was not significantly increased relative to solvent control ($p > 0.05$, Fisher's exact test). Results of the bacterial reverse mutation assay (*Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* tester strain WP2 *uvrA*) indicate that **1** (234 mM, 5000 $\mu\text{g}/\text{plate}$) did not cause a positive response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

Vigabatrin also has been shown to cause microvacuolization in the white matter of the CNS, which has been characterized as intramyelinic edema.²⁶ This histopathological finding resolves in a few weeks after discontinuation of therapy. There are currently no histological data for **1** to confirm whether or not this effect occurs with **1** and to what degree.

Effects of 1 on Cocaine Induced CNS Neurochemical Changes. *MicroPET Imaging of the Effects of 1 on Cocaine-Induced Lowering of [^{11}C]Raclopride.* To compare the pharmacological effects of **1** to that previously reported for vigabatrin, we determined the effect of cocaine administration on NAc-released dopamine (Figure 1). In these microPET imaging studies, cocaine reduced [^{11}C]raclopride binding by an average of 22%, consistent with an increase in synaptic dopamine.¹ However, when treated with **1**, cocaine had no effect on [^{11}C]raclopride binding. That is, [^{11}C]raclopride

binding was similar to the control data, consistent with **1** (at 0.5 mg/kg) producing a blockade of cocaine-induced increases in synaptic dopamine at a dose 600 times lower (0.5 mg/kg) than the required 300 mg/kg dose of vigabatrin that was effective previously.²⁷ In two microPET imaging studies, **1** administered 2.5 h prior to radiotracer injection did not produce a change in [^{11}C]raclopride binding.

Effects of 1 on Cocaine-Induced Increases in Extracellular Dopamine. To compare the pharmacological effects of vigabatrin and **1**, we measured the effect of cocaine stimulation on extracellular dopamine (Figure 2). Control rats pretreated

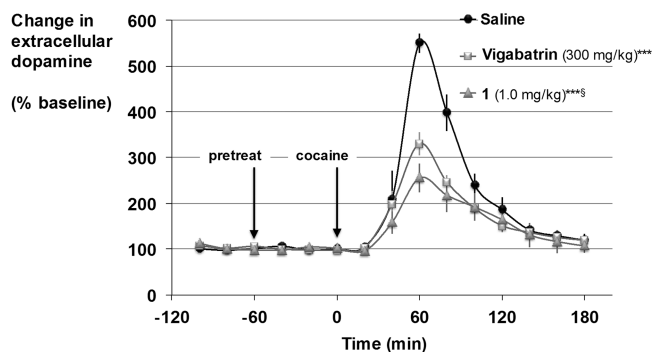


Figure 2. Effect of **1** on cocaine-induced increases in nucleus accumbens dopamine in freely moving rats. Compound **1** preferentially attenuates cocaine-induced increases in nucleus accumbens dopamine in freely moving rats. All drugs (saline, vigabatrin, and **1**) were given by intraperitoneal injection 60 min prior to cocaine (20 mg/kg). Significant differences in peak effect were determined using a Student's *t* test: (***) $p < 0.001$ from saline controls, (§) $p < 0.05$ from vigabatrin.

with saline prior to cocaine showed an increase in dopamine concentration of $550 \pm 21\%$ relative to untreated rats. Sixty minutes prior to the cocaine challenge, rats were pretreated with either 300 mg/kg vigabatrin or 1 mg/kg **1**. Pretreatment with vigabatrin attenuated this response by 40%, or an increase in dopamine of $331 \pm 32\%$. In contrast, pretreatment with **1** attenuated the response to cocaine by 54%, a cocaine-induced increase in dopamine of only $256 \pm 26\%$. Thus, in freely moving, unrestrained animals, **1** at $1/300$ the dose used for vigabatrin produced a greater inhibition of the stimulatory effects of cocaine than vigabatrin, without altering baseline

dopamine levels. The 10–20 min delay in response in Figure 2 occurs because of a 10 min sampling interval. In vivo microdialysis techniques, unlike microPET, require that extracellular samples are collected over some period of time and injected into an HPLC. This period of time is directly related to the sensitivity of the electrochemical detector, in this case 10 min.

Effect of 1 on the Expression of Conditioned Place Preference, a Model for Cocaine Addiction. Increases in NAc dopamine following administration of cocaine produces a dose-dependent and profound effect on the expression of conditioned place preference in rats. Conditioned place preference is a well-documented model that assesses the saliency of drugs of abuse in a drug-free state.¹ The ability to pharmacologically block the expression of a cocaine-induced conditioned place preference suggests that these compounds might have an indication for treating cocaine addiction.

Cocaine produced a dose-dependent conditioned place preference response, with the most reliable and robust response occurring at 20 mg/kg. Therefore, a 20 mg/kg cocaine dose was chosen to examine the effect of the administration of 1 on the expression of a cocaine-induced conditioned place preference (Table 1). The results clearly indicate that 1.0 mg/kg

Table 1. Compound 1 Alone Is Not Rewarding and Blocks the Expression of Preference to Cocaine^a

treatment group	no. of animals	time in chamber (min)	
		paired	unpaired
saline/saline ^b	9	7.3 ± 0.5	7.7 ± 0.6
saline/cocaine ^b	9	11.8 ± 0.4	3.2 ± 0.4
saline/1	8	8.1 ± 0.2	6.9 ± 0.9
1/cocaine	8	7.8 ± 0.5	7.2 ± 0.9
vigabatrin/cocaine ^b	10	7.9 ± 0.5	7.1 ± 0.6

^a1 (1 mg/kg) and vigabatrin (300 mg/kg) produced comparable inhibition of cocaine-induced place preference, $p < 0.001$, Student's t -test. ^bDewey et al.⁷

1 blocked the expression of cocaine-induced conditioned place preference. By itself, 1 produced neither a conditioned place preference nor a conditioned aversive response, indicating that 1 exhibits no abuse potential. These data are particularly exciting in that similar findings with vigabatrin required a dose of 300 mg/kg, while the effects of 1 were obtained using a dose of 1.0 mg/kg. Specifically, in the saline/saline pairings, animals spent an equal amount of time in both chambers (7.3 ± 0.5 versus 7.7 ± 0.6 min). However, in the saline/cocaine pairings, animals spent a significantly greater amount of time in the cocaine-paired chamber, 11.8 ± 0.4 versus 3.2 ± 0.4 min ($p < 0.01$, Student's two-tailed t test). In the saline/compound 1 pairings, animals spent an equal amount of time in both chambers (8.1 ± 0.2 versus 6.9 ± 0.9 min), suggesting that 1 did not produce a conditioned place preference on its own (i.e., is not addictive). In the cocaine/saline + compound 1 pairings, animals again spent an equal amount of time in both chambers (7.8 ± 0.5 versus 7.2 ± 0.9 min), demonstrating that at a dose of 1.0 mg/kg, 1 completely blocked the expression of a cocaine-induced conditioned place preference with 300 times greater potency than that of vigabatrin.

Retinal Toxicity of 1. We collected electroretinograms (ERG) for scotopic (rod), mesopic (standard combined), photopic (cone), and 10 and 15 Hz flicker ERGs for both eyes from each animal in the study. One of the animals in the

vigabatrin group at the 45-day time point exhibited a larger difference between the right and left eyes for all ERGs. Histological examination of both eyes confirmed that the eye producing the lower ERG results showed inflammation of both the vitreous and retina and was indicative of a major infection. Therefore, all results from this animal (both eyes) were omitted from further consideration. During the course of the study, because of the use of a high maximum tolerated dose (MTD) for both drugs (20 mg/kg for 1 and 200 mg/kg for vigabatrin), four animals were euthanized or found dead (two female and two male) in the 1 group and two deaths (one of each sex) in the vigabatrin group, out of 15 animals in each group. The need for the euthanizations resulted from the sedative effects of both drugs in the first 3 weeks of treatment, which led to malnourishment, dehydration, and general distress. The MTD of 20 mg/kg for 1 was determined directly by a dose ranging study (unpublished results). The MTD for vigabatrin used in this study was determined empirically from previously published results of vigabatrin toxicity,^{26,28} the 250 mg kg⁻¹ day⁻¹ dose that was used in the previously reported retinal toxicity study upon which this study is based¹⁷ and in consideration of the longer duration of this study. Doses several times larger of both 1 and vigabatrin are needed for a given pharmacological effect when given repeatedly over extended times compared to when the drug is administered as a single dosage.^{28,29} As a result, the single dosages previously described for cocaine addiction animal models used a larger dosage than the retinal toxicity study.

The implicit times of the ERGs were generally unremarkable and showed little or no drug effect for either drug, which is consistent with past reports for vigabatrin in this model.^{17,20} However, in females the rod, mesopic, and cone b-wave implicit times were significantly increased with vigabatrin compared to controls or 1 (data not shown). The a-wave amplitudes, when present, generally showed the same trends described below, but with less statistical power than the b-wave amplitudes as a result of the smaller size of these signals. We also collected flicker ERGs at both 10 and 15 Hz. The 10 Hz flicker ERGs were significantly lower with vigabatrin compared to both 1 and controls in both sexes at both time points (data not shown). Only the 15 Hz flicker ERG is discussed because the 15 Hz ERG responses exhibit smaller rod contributions because of the higher stimulus frequency and, therefore, would be more indicative of cone photoreceptor recovery time. Vigabatrin showed a larger decrease in the scotopic, mesopic, and photopic b-wave ERG and flicker ERG average amplitude responses when compared to 1. Vigabatrin and 1 treatment in females resulted in a greater reduction in all ERG measurements compared to males (see Table 2 and Figure 3) with vigabatrin generally resulting in more pronounced reductions in amplitude.

DISCUSSION

Because of the importance of GABAergic effects on a variety of neurological disorders and the inherent complexity of this system resulting from multiple subtypes of receptors and transporters, it is crucial that potential therapeutic compounds are selective for specific components of the GABAergic system. In this study, we evaluated the selectivity profile of a recently described GABA-AT inhibitor, 1.¹⁸ We observed that 1 does not affect GABA uptake in recombinantly expressed human and mouse GABA transporters or in mouse cortical astrocytes or neurons (Supporting Information Table 1). Therefore, unlike

Table 2. Number of Eyes Examined and ERG Mean and Standard Errors of Comparisons to the Matching Control Values

		female			male		
		<i>n</i>	mean	SEM	<i>n</i>	mean	SEM
Scotopic (Rod) ERG							
control	45 d	10	290.3	39.0	10	274.6	41.7
	90 d	20	294.4	31.5	20	302.1	46.0
1	45 d	8	260.1	51.7	8	235.1	37.9
	90 d	18	236.3	32.1	16	232.5	23.6
vigabatrin	45 d	8	93.9	33.1	6	225.2	44.2
	90 d	20	110.1	32.4	20	193.5	34.8
Standard Combined ERG							
control	45 d	10	393.1	56.4	10	397.1	58.9
	90 d	20	379.2	32.6	20	362.4	62.2
1	45 d	8	337.8	67.8	8	311.6	49.3
	90 d	18	280.6	44.3	16	309.1	30.5
vigabatrin	45 d	8	169.8	36.2	6	280.9	69.4
	90 d	20	129.6	39.8	20	235.7	44.3
Photopic (Cone) ERG							
control	45 d	10	62.6	8.2	10	73.7	14.3
	90 d	20	62.2	5.1	20	66.0	5.6
1	45 d	8	62.8	13.9	8	52.8	7.2
	90 d	18	51.7	7.9	16	52.9	4.6
vigabatrin	45 d	8	37.8	3.5	6	57.6	9.5
	90 d	20	26.4	5.4	20	47.6	6.9
15 Hz Flicker ERG							
control	45 d	10	33.7	3.5	10	44.4	6.3
	90 d	20	33.4	3.2	20	36.2	3.6
1	45 d	8	34.5	10.8	8	27.8	4.2
	90 d	18	24.1	4.7	16	27.8	3.9
vigabatrin	45 d	8	8.6	1.4	6	26.4	7.3
	90 d	20	12.1	3.5	20	21.8	4.8

vigabatrin, which is a GABA reuptake inhibitor,³⁰ **1** does not interfere with GABA transport. Furthermore, **1** displays no affinity for GABA_A or GABA_B receptors (Supporting Information Table 2) and is neither an agonist nor an antagonist for GABA_C receptors (Supporting Information Figure 1). The principal GABAergic site of action of **1** appears to be GABA-AT, the enzyme that catabolizes GABA.³¹ Because of the effectiveness of vigabatrin, an irreversible inactivator of GABA-AT, on the reversal of specific addiction-associated biochemical and behavioral measures to a variety of drugs of abuse, **1** was investigated for its ability to block cocaine-induced increases in NAc dopamine concentrations by microPET in sedated animals, an indicator of addictive behavior. Further, we extended these biochemical findings to a behavioral measure, the expression of a cocaine-induced conditioned place preference.

The microdialysis studies demonstrated that pretreatment with vigabatrin or **1** reduced the magnitude of the cocaine challenge-induced increase, or “surge”, in NAc dopamine levels (Figure 2). This reduced NAc surge in dopamine levels correlated with elimination of cocaine induced conditioned place preference (Table 1) and an increase in raclopride binding to dopamine receptors (Figure 1), which is known to correlate with reduced dopamine receptor occupancy by dopamine. In fact, we observed that a dose of **1** that is 1/300 (1.0 mg/kg) to 1/600 (0.5 mg/kg) that of vigabatrin (300 mg/kg) reversed cocaine-induced increases in synaptic dopamine as well as in the expression of a cocaine-induced conditioned place preference. The effects of vigabatrin, an irreversible GABA-AT inhibitor, are thought to be activity dependent. That is, while

vigabatrin increases presynaptic GABA stores, these stores are not released into the synaptic space (and thus the extracellular environment) unless stimulated by an increase in neuronal cell firing rates. The fact that we do not see changes in either extracellular (microdialysis) or synaptic (microPET) dopamine following vigabatrin treatment is consistent with this reported mechanism of action and suggests that **1** might also be activity dependent. Given the effectiveness of vigabatrin in clinical trials for the treatment of cocaine and/or methamphetamine addiction,^{10–12} in combination with its preclinical efficacy for the treatment of nicotine,⁷ methamphetamine, heroin, and ethanol⁸ abuse, it is reasonable that **1** also will be effective in treating these addictive behaviors in humans.

The effects of a 20 mg/kg dose, compared to the 0.5–1 mg/kg addiction treatment dose, of **1** on retinal function were evaluated by electroretinography. This dose is effective at reducing or eliminating cocaine induced CNS changes associated with addiction and represents a safety factor of 20- to 40-fold over the treatment dose. In contrast, the retinal function effects of vigabatrin, the positive control, evaluated at a 200 mg/kg dose, compared to its effective dose for cocaine addiction of 100–300 mg/kg, represents a safety factor of only about 1, which could account for its serious visual field defects in humans. Despite the high dose of **1** used in the retinal toxicity studies relative to its therapeutic dose, the effects on ERG parameters were less than those found for vigabatrin. It is also interesting that the vigabatrin induced reductions in ERG response plateau at 45 days (see Figure 3) in the female animals. There is insufficient data from this study to determine

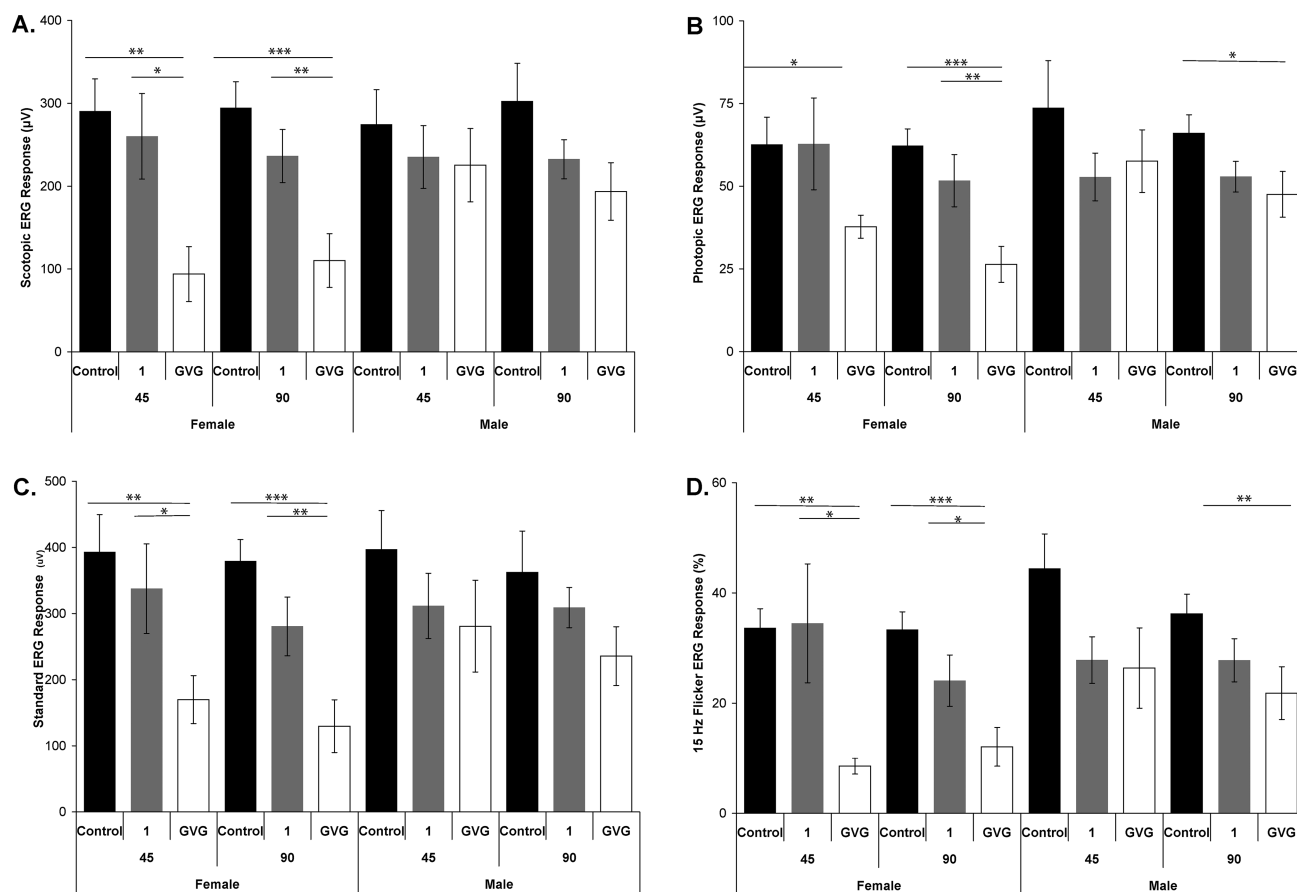


Figure 3. Effect of **1** and vigabatrin (GVG in the figure) on the ERG responses. The b-wave amplitudes for the control, **1**, and vigabatrin groups, for both males and females, for the scotopic (rod) (A), photopic (cone) (B), standard combined (C), and the 15 Hz flicker (D) ERG average response at 45 and 90 days are presented, with the standard error of the mean of each measurement. Statistically significant differences, where observed, are also indicated. Significant *p*-values are indicated with *** for extremely significant ($p < 0.0001$), ** for very significant (p of 0.0001–0.01), and * for significant (p of 0.01–0.05).

the rate profile at which vigabatrin approaches this plateau of reduced retinal function. However, on the basis of the magnitude of the reduced ERG response values for both drugs at both 45 and 90 days, we conclude that the rate at which vigabatrin affects retinal function is substantially faster than that of **1**.

A substantial sex-based bias in the effect of vigabatrin on retinal function was also noted that has not been previously reported for this model. However, the statistical analysis of the ERG results for **1** and the control showed no statistically significant sex-based differences.

Compound **1** exhibits remarkable pharmacokinetic characteristics: rapid and complete oral absorption, excellent metabolic stability, and rapid elimination, which are all highly favorable for orally delivered, irreversible inhibitor drugs. The half-life for the bioactivity of vigabatrin, also an irreversible inhibitor, is much greater than its elimination half-life.⁴⁵

A remarkable advantage of **1** is its much greater potency relative to vigabatrin, which should markedly reduce its daily dose relative to that of vigabatrin (1–3 g/day). Furthermore, given the greatly reduced retinotoxicity of **1** and the greater safety factor at which this retinotoxicity was evaluated, it is reasonable to conclude that **1** will exhibit greater visual safety in clinical use for both addiction and epilepsy. Since the predominant cause for visual field defects in vigabatrin therapy is believed not to be the result of elevated GABA,¹⁵ the mechanistic differences between vigabatrin and **1** with regard to

their potential inactivation of GABA-AT would contribute to less visual field defects in the case of **1**.

Many clinical studies have examined visual deficits associated with vigabatrin treatment for epilepsy. Because of these potential side effects, an official registry has been initiated to closely monitor and follow up with patients being treated with vigabatrin.³² Clinical ophthalmic visual testing, including for ERG responses to examine cone function with flicker analysis, is an essential component for defining visual toxicity in vigabatrin treatment.³³ In a newly released pediatric study, a subgroup of patients had vigabatrin-induced visual field loss and progressive cone ERG deficits.³⁴ Our animal ERG studies demonstrate that **1** is an alternative, and potentially safer, pharmacological agent to vigabatrin for targeting the deficits in the GABA pathway and in other clinical studies that require visual testing. Compound **1** is currently in Phase I clinical trials.

EXPERIMENTAL SECTION

Materials. Vigabatrin (Sabril), (R)-baclofen, GABA, isoguvacine, sodium pyruvate, theophylline, gentamycin, and all buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Vigabatrin for the electroretinography study was supplied by Divi's Laboratories, Andhra Pradesh, India. (1S,3S)-3-Amino-4-difluoromethylenyl-1-cyclopentanoic acid (**1**) was synthesized as reported previously. Anal. Calcd for $C_7H_9NO_2F_2$: C, 47.46; H, 5.12; N, 7.91. Found: C, 47.16; H, 4.79; N, 7.68.¹⁸ **1** was >95% pure. [³H]GABA (35 or 40.0 Ci/mmol) and [³H]muscimol (36.6 Ci/mmol) were purchased from PerkinElmer

(Boston, MA, U.S.). All reagents for cell culture techniques were purchased from Invitrogen (Paisley, U.K.). Cocaine USP was provided by the National Institute on Drug Abuse (NIDA). All animals were adult male Sprague–Dawley rats (200–225 g, supplied by Taconic Farms, Germantown, NY), with the exception of those animals used for the electroretinography study, as described below. The general care and housing of the animals in all experiments met or exceeded the AAALAC standards. The animals were euthanized in accordance with the AVAMA Guidance on Euthanasia.

GABA Uptake Assay. [^3H]GABA Uptake Assay at Human GABA Transporters. The tsA201 cells were cultured in GlutaMAX-I DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 . The plasmids encoding hGAT-1, hBGT-1, hGAT-2, and hGAT-3,³⁵ respectively, were transfected into tsA201 cells using PolyFect according to the protocol of the manufacturer (Qiagen, West Sussex, U.K.). The next day, the tsA201 cells transiently expressing each of the four human GABA transporter subtypes were split into poly-D-lysine-coated white 96-well plates (PerkinElmer). The pharmacological assays were performed 36–48 h after transfection exactly as described previously.³⁶ In short, assay buffer supplemented with 30 nM [^3H]GABA and test compounds was added to the cells, and the uptake of [^3H]GABA was determined after incubation at 37 °C for 3 min. Quantification was performed using Microscint 20 scintillation fluid (PerkinElmer) and a Packard TopCount microplate scintillation counter.

[^3H]GABA Uptake Assay at Mouse GABA Transporters. Cortical astrocytes were cultured essentially as previously described.³⁷ The neopallium was removed from new born NMRI mice (Taconic, Denmark) and passed through an 80 μm nylon sieve and cultured in modified Dulbecco's modified Eagle's medium with fetal calf serum. The calf serum was lowered from 20% to 10% over 3 weeks, and finally the astrocytes were allowed to differentiate using 0.25 mM dibutyryl cyclic AMP during the last week of growth.

Cortical neurons were cultured essentially as previously described³⁸ by removing the neopallium of 15-day-old NMRI embryos by dissection followed by mild trypsinization. The neurons were cultured in 10% fetal calf serum, and after 48 h, cytosine arabinoside was added to a final concentration of 20 μM to prevent glial proliferation. Four cultures of stably transfected human embryonic kidney (HEK)-293 cells expressing mGAT1–4 were prepared by the method previously reported.³⁹ The stable cell lines are under the selection pressure of blasticidin-S at 5 $\mu\text{g}/\text{mL}$. Determinations of the IC_{50} values were conducted as described earlier.⁴⁰ In brief, [^3H]GABA uptake was assessed at 37 °C for 3 min on desired cells in PBS buffer containing 1 μM GABA, 13 nM [^3H]GABA, and test compound. Radioactivity was measured using Microscint 20 scintillation fluid (PerkinElmer) and a Packard TopCount microplate scintillation counter.

GABA Receptor Binding Assays. Receptor Preparations. GABA_A and GABA_B binding assays were performed using rat brain synaptic membranes of cortex and the central hemispheres from adult male Sprague–Dawley rats with tissue preparation as earlier described.⁴¹ On the day of the assay, the membrane preparation was quickly thawed, suspended in 40 volumes of ice cold 50 mM Tris-HCl buffer (pH 7.4) using an UltraTurrax homogenizer and centrifuged at 48000g for 10 min at 4 °C. This washing step was repeated four times. The final pellet was resuspended in incubation buffer and the binding assay carried out as detailed below.

GABA_A Receptor Activity Assay. Rat brain synaptic membranes (100 μg of protein/aliquot) prepared above in Tris-HCl buffer (50 mM, pH 7.4) were incubated with [^3H]muscimol (5 nM) and 100 μM **1** at 0 °C for 60 min in a total volume of 250 μL . GABA (1 mM) was used to define nonspecific binding. The binding reaction was terminated by rapid filtration through GF/B unilters (PerkinElmer) using a 96-well Packard FilterMate cell harvester, followed by washing with 3 \times 250 μL of ice cold binding buffer, drying, and adding scintillation fluid, as described for the [^3H]GABA uptake assay.

GABA_B Receptor Binding Assay. For [^3H]GABA binding to the GABA_B receptors, rat brain synaptic membranes (200 μg of protein/aliquot) were suspended in Tris-HCl buffer (50 mM + 2.5 mM CaCl_2 ,

pH 7.4) and incubated with [^3H]GABA (5 nM), isoguvacine (40 μM), and 100 μM **1** at 25 °C for 45 min in 1 mL total volume. Isoguvacine serves to saturate GABA_A receptors.⁴² Nonspecific binding was determined using 100 μM (*R*)-baclofen. Binding was terminated by filtration through Whatman GF/C filters, using a Brandell M-48R cell harvester. Filters were washed with 3 \times 3 mL of ice cold buffer, and filter-bound radioactivity was counted in a Packard Tricarb 2100 liquid scintillation analyzer using 3 mL of Opti-fluor scintillation fluid (PerkinElmer).

Electrophysiology. Expression of $\rho 1$ in *Xenopus laevis* Oocytes. Human $\rho 1$ cDNA encapsulated in pcDNA1.1 was linearized with *Not*I restriction endonuclease. Linearized cDNA was transcribed to mRNA using the T7 “mMESSAGE mMACHINE” kit (Ambion Inc., Austin, TX, U.S.) as previously described.⁴³ GABA_C receptor activity assays were performed in oocytes harvested from *Xenopus laevis* (housed in the Department of Veterinary Science at the University of Sydney, Australia) and defolliculated. The oocytes were stored in ND96 solution (in mM), NaCl (96), KCl (2), MgCl_2 (1), CaCl_2 (1.8), HEPES (hemi-Na salt, 5) supplemented with sodium pyruvate (2.5), theophylline (0.5), and 50 $\mu\text{g}/\text{mL}^{-1}$ gentamycin, for 2–5 days after injection.

GABA_C Receptor Electrophysiological Assay. Electrophysiological methods were performed as previously described.³⁶ Stages V–VI oocytes were injected with 10 ng 50 nL $^{-1}$ $\rho 1$ mRNA and then stored at 16 °C. Recordings of receptor activity were obtained for 2–5 days by a two-electrode voltage clamp by means of a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia), and Chart, version 3.6.3 program. Oocytes were voltage clamped at –60 mV, and the preparation was continually perfused with ND96 solution at room temperature. Compound **1** (100 μM) dissolved in ND96 was applied in the absence and presence of GABA, respectively, until maximum current was reached, at which time the oocytes were washed for 5–10 min to allow complete recovery of response to GABA (1 μM). Compound **1** was tested on three oocytes from at least two harvests.

Off-Target Assays. Tests of inhibition of human liver cytochrome P450s by **1** were carried out at XenoTech (Lenexa, KS). The hERG channel test was done at ChanTest (Cleveland, OH). Carcinogenicity tests (Ames and chromosomal aberration tests) were done at BioReliance (Rockville, MD). The blood sample collection for the PK analyses was done by Sinclair Research (Columbia, MO), and the sample analysis was done by Cyanta Analytical Services (Maryland Heights, MO).

Aspartate aminotransferase or alanine aminotransferase (0.2 units) was incubated in a total volume of 2000 μL with 6.6 mM α -ketoglutarate, malic dehydrogenase (for Asp-AT) or lactate dehydrogenase (for Ala-AT) (5 units), 3.5 μM NADH, and various concentrations of **1** (0–4 mM) in 100 mM potassium phosphate, pH 7.4, for 1 h at room temperature.

L-Aspartate or L-alanine (125 mM) was added to the incubation mixture, and changes in absorbance at 340 nm were determined for 30 min, monitoring NADH degradation.

Succinic semialdehyde dehydrogenase (SSADH) (5 nM) was incubated in a total volume of 500 μL with an excess amount of **1** (5 mM) in 100 mM potassium phosphate, pH 7.4, at room temperature. At several time points, (1, 2, 4, 8, and 20 h) a 100 μL aliquot was removed and added to 500 μL of potassium phosphate buffer (100 mM) containing 2 mM succinic semialdehyde and 3 mM NADP⁺. Changes in absorbance were measured at 340 nm for 10 min.

Cocaine-Induced Conditioned Place Preference. A nonbiased approach was used for all conditioned place preference studies. Specifically, animals were pretested in the conditioned place preference chambers for a pre-existing chamber bias. Any animals that spent more than 70% of their time in any chamber were eliminated from the study. Thus, only animals that demonstrated no pre-existing chamber bias were used in the study.

In all rodent studies ($n = 8/\text{group}$) animals were allowed to acclimate to the animal housing facility for at least 5 days prior to beginning the experiments. Conditioned place preference chambers

were used as previously described⁴⁴ except instead of one chamber being entirely white and the other black, one chamber was entirely light blue with a stainless steel floor, and the second chamber was light blue with horizontal black stripes (2.5 cm wide) spaced 3.8 cm apart with a smooth Plexiglas floor. In all conditioned place preference studies with **1**, the saline volume was 1 mL/kg, the cocaine doses were 20 mg/kg, and the dose of **1** was 1.0 mg/kg. The saline, cocaine, and compound **1** were all injected intraperitoneally (ip). The conditioning procedure for the acquisition phase consisted of 12 sessions carried out consecutively over 12 days. The conditioned place preference pairings were (1) saline/saline, (2) saline/cocaine, (3) **1**/saline, and (4) saline/cocaine + **1**. Animals in each group were randomly assigned to a 2 × 2 factorial design with one factor being the pairing chamber and the other factor being the order of conditioning.

Animals that received either saline or cocaine were injected and confined to the appropriate compartment for 30 min. Compound **1** injections were given 2.5 h prior to saline or cocaine injections. This was done, as it has been shown that GABA levels reach maximal values 3–4 h following the administration of **1**. On the test day (day 12) neither saline nor drugs were administered, and the animals were allowed to move freely between both chambers for 15 min. The amount of time spent in each chamber was recorded using an automated infrared beam electronically coupled to a timer. For the expression phase of conditioned place preference to cocaine, the animals were habituated and conditioned to cocaine as described in the acquisition studies, but no animals in the expression studies were given **1** on conditioning days. On the test day, the animals being tested received either saline or **1** 2.5 h prior to their being placed in the apparatus and allowed free access to both chambers for 15 min. A time period of 2.5 h was selected because previous studies¹ demonstrated that this was the optimal pretreatment interval allowing for a maximal increase in GABA concentrations.

MicroPET Imaging Studies. Using separate adult animals (male Sprague–Dawley rats, $n = 2$) microPET studies were performed using a Concorde Microsystems R4. Baseline [¹¹C]raclopride binding was examined in anesthetized (ketamine/xylazine) animals. [¹¹C]-Raclopride (20.4 min half-life) is selective for the dopamine family of receptors and competes directly with dopamine for receptor binding. Thus, drug-induced increases in brain dopamine produce a decrease in [¹¹C]raclopride binding while dopamine depletion produces an increase in binding. Approximately 2 h following these baseline scans, animals received an intravenous injection of cocaine (5 mg/kg) followed 5 min later by a second injection of [¹¹C]-raclopride. Approximately 2 h following this scanning session, animals received **1** (0.5 mg/kg). Approximately 2.5 h following the administration of **1**, animals received a second intravenous dose of cocaine (5.0 mg/kg) followed 5 min later with a third injection of [¹¹C]raclopride (this was the procedure used for vigabatrin so that they could be compared). Imaging studies show that in an acute, drug naive setting, where animals (adult female baboons and/or rats) are tested up to 4 times with cocaine, methyl phenidate, or methamphetamine, the reproducibility of the response (<8%) is consistent with the test/retest variability in [¹¹C]raclopride binding.

In Vivo Microdialysis Studies of Extracellular Dopamine Levels in Freely Moving Rats. In vivo microdialysis studies were performed as previously described.⁸ All drugs (saline, vigabatrin, and **1**) were administered by intraperitoneal injection 60 min prior to cocaine (20 mg/kg; $n = 8$ /group).

Evaluation of Retinotoxicity by Electroretinography (ERG). *Materials and Methods for ERG.* These studies were carried out at the Sinclair Research Center, LLC (Columbia, MO). Forty-five male and female Wistar albino rats (Charles River Laboratories), 9 weeks of age at the start of dosing, were acclimated, and placed into one of three treatment groups (vehicle, **1**, or vigabatrin). Animals received a single intraperitoneal injection of vehicle or test formulations once daily for either 45 or 90 consecutive days at 0, 20, or 200 mg/kg for the vehicle, **1**, and vigabatrin treatment groups, respectively. The formulations for each treatment group were prepared fresh weekly in 0.9% normal saline.

All animals were acclimated for 7 days to the test facility prior to the start of dosing and were housed in individual polycarbonate cages. The cage environment uses a standard 12 h/12 h light/dark cycle, with light intensity of 7.7 lum/ft² or 82.9 lx during the light cycle. During the course of the study, the animals were monitored for mortality, moribundity, clinical signs of illness, feed intake, and body weight change. Any animals found in distress for more than 24 h were humanely euthanized. Because of the sedative effects of both **1** and vigabatrin, some animals received special food supplements and/or intraperitoneal fluids in the first 3 weeks of dosing. By the end of the third week of the dosing, the sedative effects of both drugs decreased, and no special food supplements or ip fluids were required. At the conclusion of the dosing phase (5 of each sex for 45 days and 10 of each sex for 90 days), the animals entered a 5–7 day “washout” period, after which electroretinograms (ERGs) were measured for both eyes. The animals were then humanely euthanized by CO₂ asphyxiation, in accordance with AVMA guidelines on euthanasia, for post-mortem pathology examinations.

Electroretinogram Recordings. Following a dark adaptation period of at least 12 h, each eye was dilated with tropicamide (1 drop of 1% solution) and phenylephrine (1 drop of 10% solution), an anesthetic dose of ketamine HCl (up to 55 mg/kg) and xylazine HCl (up to 12 mg/kg) was administered im or ip, and just prior to the ERG, a topical anesthesia (0.5% proparacaine HCl) was applied to the eye. The types of ERG measurements made and the ERG testing parameters are found in Table 3. The ERGs were measured with an

Table 3. Electroretinography (ERG) Experimental Parameters

ERG type	flashes at interval	flash mode	pulse period (ms)	intensity (cd·s/m ²)
rod	5 at 500 ms	pulse	4	0.02
standard combined	5 at 40000 ms	pulse	4	7
light adaptation	0 at 900 s	N/A	N/A	25
single flash, cone	5 at 15000 ms	pulse	4	6
10 Hz flicker	20	continuous	4	3
15 Hz flicker	20	continuous	4	3

Espion² ERG system with ColorDome Ganzfeld type illuminator by Diagnosys, Inc. All light stimuli were white light. The ground electrode was a Grass needle electrode inserted subcutaneously at the base of the tail. The reference electrode was a Grass gold disk electrode placed on the tongue. The eye electrode was a gold wire. Data were collected on a dual channel system (1 channel per eye) at 1000 samples per second with a second order 0.3–300 Hz band-pass Bessel filter and also a 60 Hz notch filter to remove interference from the line power.

The ERG data were analyzed with Prism Sigmaplot, version 5.2. It is generally well-known that ERG data are not normally distributed. Therefore, Kruskal–Wallis one-way ANOVA by ranks and Dunn’s post test were employed. Significant p values are indicated with *** for extremely significant ($p < 0.001$), ** for very significant ($0.001 < p < 0.01$), * for significant ($0.01 < p < 0.05$), and ns ($p > 0.05$) as not significant in Table 2.

■ ASSOCIATED CONTENT

Supporting Information

Table of effects of GABA and **1** on GABA uptake in neurons, astrocytes, and human and mouse GABA transporter-expressing cells, table of the ability of **1** to displace [³H]GABA binding to ionotropic GABA_A receptors or metabotropic GABA_B receptors in rat brain cortical homogenate, table of in vitro off-target pharmacology summary results using binding assays, table of in vitro off-target pharmacology summary results using enzyme assays, and effects of GABA and **1** on GABA_C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS USED

ERG, electroretinographic or electroretinogram or electroretinography; GABA, γ -aminobutyric acid; GABA-AT, γ -aminobutyric acid aminotransferase; hERG, human ether-a-go-go-related gene; HPBL, human peripheral blood lymphocyte; NAc, nucleus accumbens; PET, positron emission tomography; SSADH, succinic semialdehyde dehydrogenase; VFD, visual field defect

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