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Benzyloxybenzylammonium chlorides: Simple amine salts that display anticonvulsant activity

Hyosung Lee [a,†], Alexander S. Gold [a], Xiao-Fang Yang [b], Rajesh Khanna [b,c,d], Harold Kohn [a,e,*]

[a] Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7568, USA
[b] Department of Pharmacology & Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[c] Department of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[d] Program in Medical Neuroscience, Paul and Carole Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[e] Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599-3290, USA

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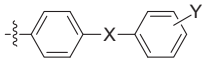
ABSTRACT

Several antiepileptic drugs exert their activities by inhibiting Na^+ currents. Recent studies demonstrated that compounds containing a biaryl-linked motif (Ar-X-Ar′) modulate Na^+ currents. We, and others, have reported that compounds with an embedded benzyloxyphenyl unit ($ArOCH_2Ar'$, OCH_2 = X) exhibit potent anticonvulsant activities. Here, we show that benzyloxybenzylammonium chlorides ($^+H_3NCH_2C_6H_4OCH_2$ Ar′ Cl^-) displayed notable activities in animal seizure models. Electrophysiological studies of 4-(2′-trifluoromethoxybenzyloxy)benzylammonium chloride (**9**) using embryonic cortical neurons demonstrated that **9** promoted both fast and slow inactivation of Na^+ channels. These findings suggest that the potent anticonvulsant activities of the earlier compounds were due, in part, to the benzyloxyphenyl motif and provide support for the use of the biaryl-linked pharmacophore in future drug design efforts.

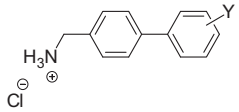
1. Introduction

Epilepsy is a group of chronic neurological disorders that arise from dysregulations in neuronal function, including hyperexcitability and hypersynchronous firing.[1] They affect up to 1% of the world's population.[1,2] Despite the many antiepileptic drugs (AEDs)[‡] currently in clinical use, epilepsy in nearly 30% of patients is pharmacoresistant and does not respond to at least two of the first-line AEDs.[3] Furthermore, adverse side effects (e.g., drowsiness, dizziness, nausea) are experienced by nearly 40% of patients with epilepsy.[4] These shortcomings have prompted a search for new agents.

We have shown, using whole-cell, patch–clamp electrophysiology, that compounds containing the biaryl-linked motif **A** modulated Na^+ channel slow- and fast-inactivation processes and, in some cases, promoted frequency (use)-dependent blockage of Na^+ currents.[5–8] These three processes are proven pathways that reduce neuronal hyperexcitability and constitute important mechanisms of action for AEDs.[6,9–11] Knowing this, we demonstrated that (biphenyl-4-yl)methylammonium chlorides [8] (compound class **B**), where a single bond linked the two aryl units in motif **A**, displayed activity in the maximal electroshock seizure (MES),[12] psychomotor 6 Hz seizure (6 Hz),[13] and scMetrazol seizure (scMet)[14] models.



Biaryl-linked Motif **A**



Compound Class **B**

Compounds **1**[15] and **2**[16] exhibit pronounced anticonvulsant activities. These compounds contain an embedded benzyloxyphenyl unit **C**, thus having a biaryl-linked motif **A** in which an oxymethylene (OCH_2) group separates the two aryl units. To further document the potential of the biaryl-linked **A** motif to elicit anticonvulsant activity, we show, herein, that the readily accessible and structurally simple benzyloxybenzylammonium chlorides (compound class **D**) exhibited notable activity in pre-clinical seizure models.

* Corresponding author. Tel.: +1 919 843 8112; fax: +1 919 966 0204.
E-mail address: hkohn@email.unc.edu (H. Kohn).
† Cell Dynamics Research Center, Gwangju Institute of Science and Technology, Gwangju, South Korea.
‡ *Abbreviations:* AED, antiepileptic drug; ASP, Anticonvulsant Screening Program; ED_{50}, effective dose (50%); ip, intraperitoneally; MES, maximal electroshock seizure; NINDS, National Institutes of Neurological Disorders and Stroke; po, orally; scMet, scMetrazol; TD_{50}, neurological impairment (toxicity, 50%).

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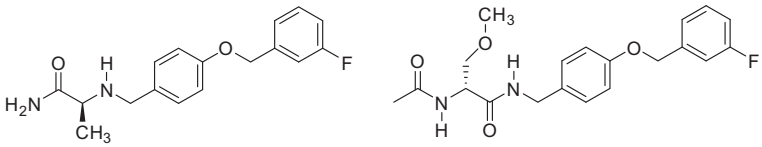
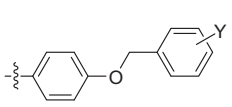
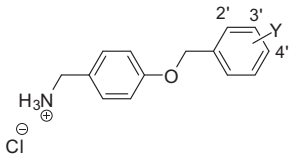


1 2



Benzyloxyphenyl Unit **C**



Compound Class **D**

2. Results and discussion

2.1. Selection of compounds

We prepared nine compounds from the class **D** (**3**–**11**), varying the terminal aryl substituent (Y) and the site of modification. We restricted our choices for the Y substituent to electron-withdrawing groups since these moieties provided the highest anticonvulsant activities for compound class **B**. The Y substituent was placed in the 2′, 3′ or 4′ position of the benzyloxy aromatic ring. Finally, in **D**, we retained the methyleneamino ($CH_2N(H)$) unit, which was found in **1** and **2** in the form of the methyleneammonium ($CH_2NH_3^+$ Cl^-) group to increase the compounds' water solubility.

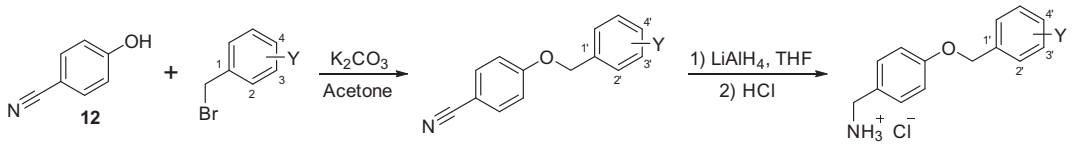
2.2. Synthesis

Compounds **3**–**11** were prepared in two steps (Scheme 1). Treating 4-cyanophenol (**12**) with the appropriate substituted benzyl bromide (**13**–**21**) and base gave the Williamson ether coupled products **22**–**30**. $LiAlH_4$ reduction of the nitrile group in **22**–**30** afforded the amines, which were immediately converted to the corresponding amine hydrochlorides **3**–**11**. In the Experimental (Section 4), we report the synthesis and physical and spectral data for all new intermediates and final compounds, while in the Supplementary data, we provide this information for previously reported compounds. Salts **3**–**11** were soluble in water when tested at concentrations of 300 μM.

2.3. Pharmacological evaluation

Compounds **3**–**11** were evaluated for anticonvulsant activity at the National Institute of Neurological Disorders and Stroke's (NINDS) Anticonvulsant Screening Program (ASP). Screening was performed using the procedures described by Stables and Kupferberg.[17] The anticonvulsant activity data from the MES,[12] 6 Hz,[13] and scMet[14] tests are summarized in Table 1 along with similar results obtained for the AEDs lacosamide,[18,19] phenytoin,[20] valproate,[20] and phenobarbital.[20] The compounds were administered intraperitoneally (ip) to mice and ip or orally (po) to rats. In the initial qualitative studies, the compounds were tested at the ASP at preset dosages (e.g., MES (mice, ip): 50 mg/kg; 100 mg/kg; 30, 50, 100 mg/kg). For compounds that showed significant activity, we report the 50% effective dose (ED_{50}) values obtained in quantitative screening evaluations. Also provided are the median doses for 50% neurological impairment (TD_{50}) in mice, using the rotorod test,[21] and in rats, using the behavioral toxicity effects.[22]

Benzyloxybenzylammonium chlorides **3**–**11** showed anticonvulsant activities in either the MES or the 6 Hz seizure models in mice (ip) at doses below 100 mg/kg. For compounds **4**, **7**–**11**, we observed activity in both models. Compounds **7**, **9** and **10** were the most potent in the MES and gave MES ED_{50} values of 54, 20, and 28 mg/kg, respectively. These values were similar to phenobarbital (MES ED_{50} = 22 mg/kg)[20] but greater than lacosamide (MES ED_{50} = 4.5 mg/kg)[18] and phenytoin (MES ED_{50} = 9.5 mg/kg).[20] The activities of **3**–**11** (>30 mg/kg) in the 6 Hz model (32 mA) were less than that of lacosamide (ED_{50} = 10 mg/kg).[19] Compounds **7**, **9**, and **10** did not exhibit activity in the scMet model at the doses tested (>80 mg/kg). In rats, **6**, **7**, **9**, **10–12** exhibited activity in the MES model upon ip administration at a dosage of 30 mg/kg, with **9** (MES ED_{50} = 18 mg/kg) and **10** (MES ED_{50} = <30 mg/kg) being the most active. Of these compounds, only **9** provided partial protection upon po administration at 30 mg/kg. The promising results for **7**, **9**, and **10**, led us to test these compounds in the corneally kindled mouse seizure assay (ip).[23,24]



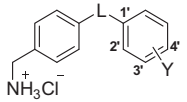
13 Y = H
14 Y = 2-F
15 Y = 3-F
16 Y = 4-F
17 Y = 3-Cl
18 Y = 4-Cl
19 Y = 2-OCF_3
20 Y = 3-OCF_3
21 Y = 4-OCF_3

22 Y = H
23 Y = 2′-F
24 Y = 3′-F
25 Y = 4′-F
26 Y = 3′-Cl
27 Y = 4′-Cl
28 Y = 2′-OCF_3
29 Y = 3′-OCF_3
30 Y = 4′-OCF_3

3 Y = H
4 Y = 2′-F
5 Y = 3′-F
6 Y = 4′-F
7 Y = 3′-Cl
8 Y = 4′-Cl
9 Y = 2′-OCF_3
10 Y = 3′-OCF_3
11 Y = 4′-OCF_3

Scheme 1. Synthesis of substituted benzyloxybenzylammonium chlorides (**D**).

Table 1
Anticonvulsant activities of substituted benzyloxybenzylammonium chlorides (**D**)[a] and (biphenyl-4-yl)methylammonium chlorides (**B**)[a]



		L = –OCH_2– (Compound class **D**)									L = single bond (Compound class **B**)				
Compd no.		Mice (ip)[b] [hour] (confidence Interval)				Rat (ip)[c] [hour] (confidence Interval)		Rat (oral)[c] [hour] (confidence Interval)		Compd no.	Mice (ip)[b] [hour] (confidence Interval)			
		MES[d]	6 Hz[e]	scMet[f]	Tox[g]	MES[d]	Tox[h]	MES[d]	Tox[h]		MES[d]	6 Hz[e]	scMet[f]	Tox[g]
Y = H	**3**	>100 [0.5–2.0]	30–100 [0.5]	ND[i]	100–300 [0.5–2.0]	>30 [0.25–4.0]	>50 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**31**[j]	30–100 [0.25–0.5]	~100 [0.5–1.0]	ND[i]	>100 [0.25–4.0]
Y = 2′-F	**4**	30–100 [0.5]	30–100 [0.5]	ND[i]	100–300 [0.5–2.0]	>30 [0.25–4.0]	>50 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	Not prepared				
Y = 3′-F	**5**	<100 [0.25–0.5]	>50 [0.25–4.0]	ND[i]	>100 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**32**[j]	>50 [0.25–4.0]	~50 [1.0]	~50 [2.0]	>100 [0.25–4.0]
Y = 4′-F	**6**	<100 [0.25]	>100 [0.25–4.0]	ND[i]	>100 [0.25–4.0]	~30 [0.25]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**33**[j]	~50 [4.0]	<100 [1.0–4.0]	>50 [0.25–4.0]	>50 [0.25–4.0]
Y = 3′-Cl	**7**	54 [0.25](49–61)	78 [2.0] (69–91)	>180 [0.25]	160 [0.25] (130–180)	~30 [0.25]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**34**[j]	<100 [0.25, 1.0]	<100 [0.5–4.0]	<100 [0.5–2.0]	>100 [0.25–4.0]
Y = 4′-Cl	**8**	<100 [0.25]	<100 [2.0]	ND[i]	>100 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**35**[j]	>50 [0.25–4.0]	<100 [1.0–4.0]	>50 [0.25–4.0]	>50 [0.25–4.0]
Y = 2′-OCF_3	**9**	20 [0.25] (17–25)	<100 [0.25–4.0]	>80 [0.25]	64 [0.25] (55–77)	18 [0.5] (14–21)	81 [0.5](60–108)	~30 [1.0]	>30 [0.25–4.0]	**36**[j]	40 [0.25] (28–59)	<100 [0.25–4.0]	>120 [0.25]	72 [0.25] (59–84)
Y = 3′-OCF_3	**10**	28 [0.5] (24–38)	~50 [1.0]	>170 [0.5]	92 [0.25] (70–100)	<30 [0.25–0.5]	>30 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**37**[j]	25 [0.5] (20–34)	43 [1.0] (35–53)	81 [4.0] (60–118)	81 [6.0] (58–99)
Y = 4′-OCF_3	**11**	<100 [0.5]	30–100 [2.0]	ND[i]	>100 [0.25–4.0]	~30 [0.25]	>50 [0.25–4.0]	>30 [0.25–4.0]	>30 [0.25–4.0]	**38**[j]	~50 [0.5–2.0]	<100 [2.0]	>50 [0.25–4.0]	>50 [0.25–4.0]
Lacosamide[k,l]		4.5 [0.5] (3.7–5.5)	10 [0.5]		27 [0.25] (26–28)			3.9 [2.0] (2.9–6.2)	>500					
Phenytoin[m]		9.5 [2.0] (8.1–10)			66 [2.0] (53–72)			30 [40] (22–39)						
Phenobarbital[m]		22 [1.0] (15–23)			69 [0.5] (63–73)			9.1 [5.0] (7.6–12)	61 [0.5] (44–96)					
Valproate[m]		270 [0.25] (250–340)			430 [0.25] (370–450)			490 [0.5] (350–730)	280 [0.5] (190–350)					

[a] The compounds were tested through the auspices of the NINDS ASP.
[b] The compounds were administered intraperitoneally to mice. ED_{50} and TD_{50} values are in milligrams per kilogram. Numbers in parentheses are 95% confidence intervals. A dose–response curve was generated for all compounds that displayed sufficient activity. The dose–effect data for these compounds was obtained at the 'time of peak effect' (indicated in hours in the brackets).
[c] The compounds were administered either intraperitoneally or orally to rats as shown in parentheses.
[d] MES = maximal electroshock seizure test.
[e] 6 Hz = 6 Hz psychomotor seizure test (32 mA).
[f] scMet = subcutaneous pentylenetetrazol (Metrazol) test.
[g] Tox = rotorod test.
[h] Tox = behavioral toxicity.
[i] ND = not determined.
[j] Ref. 8.
[k] Ref. 18.
[l] Ref. 19.
[m] Ref. 20.

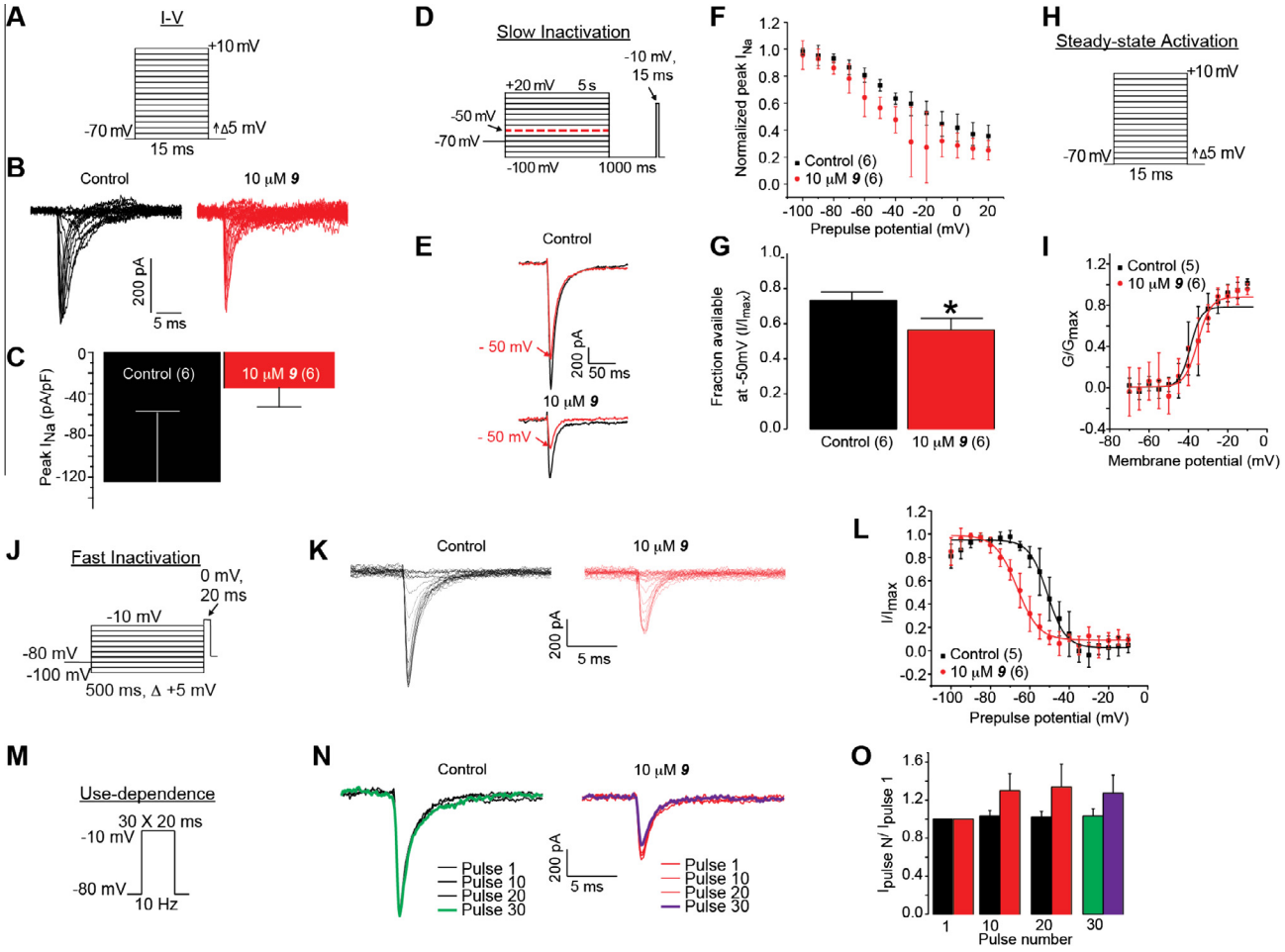


Figure 1. Evaluation of **9** on biophysical properties of Na^+ currents in rat embryonic cortical neurons. (A) The voltage protocol used to evoke macroscopic Na^+ currents. (B) Representative families of current responses of cortical neurons cells treated with 0.01% DMSO control (control; black traces) or 10 μM **9** (red traces). (C) Peak current density (pA/pF) measured at −10 mV for the two conditions are shown (n = 6 each). (D) Slow inactivation protocol: currents were evoked by 5 s prepulses between −100 mV and +20 mV and then fast-inactivated channels were allowed to recover for 1000 ms at a hyperpolarized pulse to −100 mV. The fraction of channels available at −10 mV was analyzed. (E) Representative current traces from cortical neurons in the absence (control, 0.1% DMSO) or presence of 10 μM **9**. The black and red traces represent the current at −50 mV. (F) Summary of steady-state slow activation curves for cortical neurons treated with DMSO (control) or 10 μM **9**. (G) Summary of the fraction of current available at −50 mV for cortical neurons treated with DMSO (control) or 10 μM of **9**. Steady-state activation (H) and fast inactivation (J) protocols. Values for $V_{1/2}$, the voltage of half-maximal activation and steady-state fast inactivation and the slope factors (k) were derived from Boltzmann distribution fits to the individual recordings (examples of fast inactivation recordings illustrated in K) and averaged to determine the mean (±SEM) voltage dependence of activation and fast inactivation (see Table 2). Representative Boltzmann fits for steady-state activation (I) and fast inactivation (L) for cortical neurons treated with 0.1% DMSO (control) and 20 μM of **9** are shown. Fast-inactivation was significantly affected by 10 μM of **9**. (M–O) The frequency dependence of block was examined by holding cells at the hyperpolarized potential of −80 mV and evoking currents at 10 Hz by 20-ms test pulses to −10 mV. Representative overlaid traces are illustrated by pulses 1, 10, 20, and 30 for control and in the presence of **9** (10 μM). (O) Summary of average frequency-dependent decrease in current amplitude (±SEM) produced by control or by the presence of **9** (10 μM) (p >0.05, one-way ANOVA with Dunnett's post-hoc test). Data are from 5–6 cells per condition.

This model has been advanced as a sensitive screening model for partial epilepsy in man.[23] We observed that **7** (78 mg/kg), **9** (20 mg/kg), and **10** (28 mg/kg) provided protection in 3 of the 4 animals and led to a reduction of the average seizure score from 5 to 2–3.5.

We compared the anticonvulsant activities of compound class **D** (i.e., **3**, **5–11**) with compound class **B**[8] (i.e., **31–38**) in mice (ip) and found similar results (Table 1). Many compounds in both series exhibited antiseizure properties at 100 mg/kg. For **D**, like **B**, the trifluoromethoxy compounds (compound class **D**: **9**, **10**; compound class **B**: **36**, **37**) were among the most active agents. Interestingly, we found only one compound in class **D** (**9**, ED_{50} = ~30 mg/kg) and one in class **B** (**37**, ED_{50} = 8.7 mg/kg)[8] that exhibited activity in the rat upon po administration at 30 mg/kg or less.

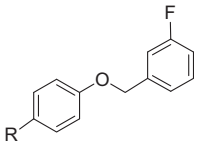
In an earlier study, we showed that **39** and **40** potently promoted Na^+ channel slow inactivation, affected the fast inactivation process, and induced frequency (use)-dependent inhibition of Na^+ currents at 100 μM concentrations.[5] Compound **40** is the corresponding free amine of hydrochloride salt **5**. These findings suggest that compounds of class **D**, such as **5**, exert their anticonvulsant activity, in part, by affecting Na^+ channel processes. Accordingly, we determined the Na^+ channel properties of **9** using rat embryonic cortical neurons. These neurons typically express CNS Na^+ channel isoforms $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, and $Na_V1.6$.[25] Total macroscopic currents, slow inactivation, steady-state inactivation, fast inactivation, and use-dependence of Na^+ currents in cortical neurons grown for 7–10 days in vitro (Fig. 1A, D, H, J, and M) were examined using protocols described earlier.[8] Current–voltage relationships in control (0.01% DMSO-treated) or **9**-treated cells were examined by the application of 15-ms step depolarizations ranging from −70 mV to +80 mV (in +5 mV increments) from a holding potential of −70 mV (Fig. 1A). The transient inward current in cortical neurons activated between −40 and −30 mV and reached its peak at −10 mV to +0 mV. Peak

Table 2
Comparative Boltzmann parameters of voltage-dependence of channel activation and steady-state fast-inactivation curves for control (0.01% DMSO) or **9** (10 μM) treated cortical neurons

Condition	Voltage-dependence of activation		Voltage-dependence of fast-inactivation	
	$V_{1/2}$ (mV)	Slope (mV/e-fold)	$V_{1/2}$ (mV)	Slope (mV/e-fold)
Control	−39.0 ± 7.8 (5)	2.8 ± 1.3 (5)	−51.1 ± 1.4 (5)	4.6 ± 0.8 (5)
10 μM **9**	−35.3 ± 3.2 (6)	3.3 ± 2.0 (6)	−66.4 ± 0.8 (6)*	5.5 ± 0.7 (6)

Values for $V_{1/2}$, the voltage of half-maximal activation, and slope, were derived from Boltzmann distribution fits to the individual recordings and averaged to determine the mean and standard error of the mean (±SEM). *N* Values are indicated in parentheses. Asterisks represent statistically significant differences as compared to control (i.e., DMSO condition) (p <0.05, ANOVA with Dunnett's post-hoc test).

inward Na^+ currents were measured and expressed as peak current density (pA/pF) to account for variations in cell size. Initial experiments were performed with a single concentration (20 μM) of **9** so as to directly compare with compound **37**.[8] Compound **9** is structurally related to **37** differing only in the composition of the bridging unit in the bi-aryl motif (**9**: oxymethylene; **37**: single bond) and the regional position of the trifluoromethoxy unit (**9**: 2′; **37**: 3′). However, at 20 μM concentration, Na^+ currents could not be evaluated due to acute toxicity to the cortical cells and loss of cell membrane integrity. Therefore, all electrophysiology experiments were performed using a 10 μM of **9**. At this concentration, total macroscopic Na^+ currents were not different between control (0.01% DMSO)-treated versus **9**-treated (Fig. 1B and C). The extent of slow inactivation induced by **9** was significantly greater than neurons treated with the vehicle 0.01% DMSO: 0.27 ± 0.03 (n = 6) versus 0.44 ± 0.05 (n = 6), respectively (p >0.05, one-way ANOVA Fig. 1F and G). Steady-state activation was unchanged between the two conditions with the $V_{1/2}$ and k values being statistically similar (Table 2, Fig. 1H and I). Steady-state, fast inactivation curves of Na^+ currents from DMSO-treated and 10 μM **9**-cortical neurons were well fitted with a single Boltzmann function (R^2 >0.931 for both conditions) and are illustrated in Figure 1L. The $V_{1/2}$ value for inactivation of 0.01% DMSO-treated cells was −51.1 ± 1.4 mV (n = 6), which was significantly different from the $V_{1/2}$ value of −66.4 ± 0.8 mV (n = 6) for **9**-treated neurons (p <0.05; ANOVA with a post-hoc Dunnett's test). The 10 μM concentration of **9** caused a significant hyperpolarizing shift of ∼15.3 mV with no commensurate significant changes in slope values compared with control cells. Finally, we found that **9** did not exhibit frequency (use)-dependent inhibition of Na^+ currents in cortical neurons (Fig. 1M–O). Thus, **9**, like **37**,[8] promoted slow and fast inactivation, but did not affect frequency (use)-dependent block of Na^+ currents. Together, these results with previous findings,[5–8] provide support that the biaryl-linked motif **A** can modulate Na^+ channel function.



39 R = H

40 R = CH_2NH_2

The Na^+ channel properties of **39** and **40** led us test these compounds for anticonvulsant activity at 100 mg/kg in the MES model in mice (ip). We observed seizure protection for **40** but not **39** (data not shown). The factors that contribute to **39**'s lack of activity have not been determined.

Since the biaryl-linked motif (**A**) is found in several biologically active agents,[15,26–29] we determined the receptor binding profile of class **D** compounds **3–11** against 43 receptors at UNC's Psychoactive Drug Screening Program (PDSP) at pH 7.4.[30] We observed appreciable binding at 10 μM, for most compounds, to several serotonin (e.g., 5-HT2A, 5-HT2B) and adrenergic (e.g., alpha 2A, 2B, 2C) receptors, the DAT, NET, and SERT transporters, and the sigma-1 and -2 receptors (Supplementary Table 1). A similar binding profile was seen for class **B** compounds.[8] The binding of class **B** and class **D** compounds to a variety of receptors should be investigated further (i.e., extent of binding, functional activity) as it is possible that these additional activities may represent either an opportunity to treat common co-morbidities of epilepsy such as depression and anxiety or limit the antiepileptic potential of these agents due to adverse effects.

3. Conclusion

Our findings demonstrated that appending the benzyloxyphenyl motif (**C**) to a methyleneammonium unit yielded compounds with notable anticonvulsant activities in several established animal models. The seizure protection is likely associated, in part, with the ability of this pharmacophore to modulate Na^+ currents. The activities of class **B** and class **D** compounds suggest that compounds with a biaryl motif with short bridging unit (e.g., oxygen, vinylic, acetylenic) may also exhibit noteworthy anticonvulsant activities. Finally, these results suggest that medicinal agents containing unit **C**, and the more general biaryl-linked motif (**A**), should be tested for their Na^+ channel properties[31] and that this pharmacophore may be useful in future drug design efforts.

4. Experimental

4.1. General methods

The general methods used in this study are identical to those previously reported.[16] The compounds were checked by TLC, ^{1}H NMR, and ^{13}C NMR, and MS, and for the final compounds by elemental analyses. The analytical results are within ±0.40% of the theoretical value. The NMR and analytical data confirmed the purity of the products was ⩾95%.

4.1.1. 4-(2′-Trifluoromethoxybenzyloxy)benzonitrile (28)

4-Cyanophenol (**12**) (1.03 g, 8.6 mmol) and Na_2CO_3 (3.32 g, 31.3 mmol) were mixed in acetone (80 mL) and 2-trifluoromethoxybenzyl bromide (**19**) (2.00 g, 7.8 mmol) was added. The resulting mixture was stirred (16 h) at reflux and evaporated in vacuo. The resulting residue was diluted in CH_2Cl_2 (120 mL), washed with H_2O (2 × 120 mL), dried (Na_2SO_4), and concentrated in vacuo to give **28** (1.94 g, 84%) as a white solid: R_f = 0.91 (hexanes/EtOAc 7:1); mp 70–74 °C; ^{1}H NMR ($CDCl_3$) δ 5.20 (s, OCH_2), 7.02 (d, J = 7.6 Hz, 2 ArH), 7.31–7.41 (m, 3 ArH), 7.49–7.55 (m, 3 ArH); ^{13}C NMR ($CDCl_3$) δ 64.8 (OCH_2), 104.9, 115.7 (2 ArC), 119.2 (CN),

120.8 (q, J = 256.4 Hz, OCF_3), 120.9, 127.4, 128.7, 129.6, 130.0, 134.3, 147.0, 161.8 (8 Ar*C*); LRMS (ESI^+) 332.03 $[M+H]^+$ (calcd for $C_{15}H_{11}F_3KNO_2^+$ 332.03); HRMS (ESI^+) 316.0571 $[M+Na]^+$ (calcd for $C_{15}H_{10}F_3NO_2Na^+$ 316.0561).

4.1.2. 4-(3′-Trifluoromethoxybenzyloxy)benzonitrile (29)

Employing the procedure for **28** and using **12** (1.03 g, 8.6 mmol), Na_2CO_3 (3.32 g, 31.3 mmol), acetone (80 mL), and 3-trifluoromethoxybenzyl bromide (**20**) (2.00 g, 7.8 mmol) gave **29** (1.84 g, 80%) as a colorless oil: R_f = 0.90 (hexanes/EtOAc 7:1); ^1H NMR ($CDCl_3$) δ 5.10 (s, OCH_2), 6.99 (d, J = 8.8 Hz, 2 Ar*H*), 7.16–7.20 (m, Ar*H*), 7.28–7.41 (m, 3 Ar*H*), 7.55 (d, J = 8.8 Hz, 2 Ar*H*); ^{13}C NMR ($CDCl_3$) δ 69.5 (OCH_2), 104.8, 115.8 (2 Ar*C*), 119.2 (*C*N), 119.4, 120.0 (2 Ar*C*), 120.7 (q, J = 256.3 Hz, OCF_3), 120.9, 130.4, 134.3, 138.4, 149.8, 161.8 (6 Ar*C*); LRMS (ESI^+) 332.03 $[M+K]^+$ (calcd for $C_{15}H_{11}F_3KNO_2^+$ 332.03); HRMS (ESI^+) 316.0573 $[M+Na]^+$ (calcd for $C_{15}H_{10}F_3NNaO_2^+$ 316.0561).

4.1.3. 4-(4′-Trifluoromethoxybenzyloxy)benzonitrile (30)

Employing the procedure for **28** and using **12** (1.03 g, 8.6 mmol), Na_2CO_3 (3.32 g, 31.3 mmol), acetone (80 mL), and 4-trifluoromethoxybenzyl bromide (**21**) (2.00 g, 7.8 mmol) gave **30** (2.02 g, 88%) as a white solid: R_f = 0.91 (hexanes/EtOAc 7:1); mp 59–61 °C; ^1H NMR ($CDCl_3$) δ 5.11 (s, OCH_2), 7.02 (d, J = 8.8 Hz, 2 Ar*H*), 7.25 (d, J = 8.8 Hz, 2 Ar*H*), 7.45 (d, J = 8.8 Hz, 2 Ar*H*), 7.60 (d, J = 8.8 Hz, 2 Ar*H*); ^{13}C NMR ($CDCl_3$) δ 69.6 (OCH_2), 104.8, 115.7 (2 Ar*C*), 119.2 (*C*N), 119.4 (q, J = 256.4 Hz, OCF_3), 121.5, 129.1, 134.3, 134.6, 149.4, 161.9 (6 Ar*C*); LRMS (ESI^+) 332.03 $[M+H]^+$ (calcd for $C_{15}H_{11}F_3KNO_2^+$ 332.03); HRMS (ESI^+) 316.0549 $[M+Na]^+$ (calcd for $C_{15}H_{10}F_3NO_2Na^+$ 316.0561).

4.1.4. 4-(2′-Fluorobenzyloxy)benzylammonium chloride (4)

A solution of **23** (2.15 g, 9.5 mmol) in THF (10 mL) was added dropwise to a suspension of $LiAlH_4$ (1.08 g, 28.4 mmol) in THF (85 mL) at 0 °C and stirred at room temperature (16 h). H_2O (1.2 mL) was added dropwise to the resulting mixture at 0 °C followed by an aqueous NaOH solution (0.6 mL, 15% w/w), and then H_2O (1.2 mL). The mixture was stirred at room temperature (2 h). The precipitate was filtered and washed with CH_2Cl_2. The combined organic layers were concentrated in vacuo and diluted with CH_2Cl_2 (100 mL). To the resulting solution a HCl solution in dioxane was added dropwise (3 mL, 4 N) and stirred at room temperature (30 min). The precipitate was filtered and washed with hexanes and dried in vacuo to give **4** (1.46 g, 66%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 242–243 °C; ^1H NMR (DMSO-d_6) δ 3.94 (s, NCH_2), 5.16 (s, OCH_2), 7.07 (d, J = 2.2 Hz, 2 Ar*H*), 7.23–7.25 (m, 2 Ar*H*), 7.39–7.45 (m, 3 Ar*H*), 7.52–7.58 (br t, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 42.4 (NCH_2), 64.3 (OCH_2), 115.5 (C_3), 116.2 (d, J = 20.5 Hz, $C_{3'}$), 124.5 (d, J = 14.4 Hz, $C_{4'}$), 125.3 ($C_{5'}$), 127.3 (C_1), 131.2 (d, J = 8.3 Hz, $C_{1'}$), 131.4 (C_2), 159.0 (C_4), 161.2 (d, J = 254.2 Hz, $C_{2'}$), one aromatic resonance was not detected and is believed to overlap with a nearby peak; LRMS (ESI^+) 232.07 $[M+H]^+$ (calcd for $C_{14}H_{15}FNO^+$ 232.11); Anal. $C_{14}H_{15}ClFNO{\cdot}0.5H_2O$: (C, H, Cl, F, N).

4.1.5. 4-(4′-Fluorobenzyloxy)benzylammonium chloride (6)

Employing the procedure used for **4** and using a solution of **25** (2.05 g, 9.0 mmol) in THF (10 mL) and a suspension of $LiAlH_4$ (1.03 g, 27.1 mmol) in THF (90 mL) gave **6** (2.09 g, 100%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 256–258 °C; ^1H NMR (DMSO-d_6) δ 3.90–3.95 (m, NCH_2), 5.11 (s, OCH_2), 7.03 (d, J = 8.0 Hz, 2 Ar*H*), 7.20–7.25 (m, 2 Ar*H*), 7.42–7.53 (m, 4 Ar*H*); ^{13}C NMR (DMSO-d_6) δ 41.6 (NCH_2), 68.4 (OCH_2), 114.8 (C_3), 115.2 (d, J = 21.2 Hz, $C_{3'}$), 126.3 (C_1), 129.9 (d, J = 8.0 Hz, $C_{2'}$), 130.6 (C_2), 133.8 ($C_{1'}$), 158.2 (C_4), 161.7 (d, J = 241.7 Hz, $C_{4'}$); LRMS (ESI^+) 232.07 $[M-Cl]^+$ (calcd for $C_{14}H_{15}FNO^+$ 232.11); Anal. $C_{14}H_{15}Cl_2NO$ (C, H, Cl, F, N).

4.1.6. 4-(3′-Chlorobenzyloxy)benzylammonium chloride (7)

Employing the procedure used for **4** and using a solution of **26** (2.10 g, 8.6 mmol) in THF (10 mL) and a suspension of $LiAlH_4$ (0.98 g, 25.9 mmol) in THF (80 mL) gave **7** (1.70 g, 80%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 240–242 °C; ^1H NMR ($CDCl_3$) δ 3.90–3.96 (br s, NCH_2), 5.16 (s, OCH_2), 7.04 (d, J = 8.0 Hz, 2 Ar*H*), 7.38–7.51 (m, 6 Ar*H*); ^{13}C NMR ($CDCl_3$) δ 41.6 (NCH_2), 68.2 (OCH_2), 114.8, 126.1, 126.4, 127.2, 127.7, 130.4, 130.6, 133.1, 139.6, 158.0 (10 Ar*C*); LRMS (ESI^+) 248.03 $[M+H]^+$ (calcd for $C_{14}H_{15}ClNO^+$ 248.08); Anal. $C_{14}H_{15}Cl_2NO$ (C, H, Cl, N).

4.1.7. 4-(4′-Chlorobenzyloxy)benzylammonium chloride (8)

Employing the procedure used for **4** and using a solution of **27** (2.26 g, 9.3 mmol) in THF (10 mL) and a suspension of $LiAlH_4$ (1.06 g, 27.9 mmol) in THF (80 mL) gave **8** (1.74 g, 76%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 262–264 °C; ^1H NMR (DMSO-d_6) δ 3.92 (s, NCH_2), 5.14 (s, OCH_2), 7.03 (d, J = 8.4 Hz, 2 Ar*H*), 7.42–7.50 (m, 6 Ar*H*); ^{13}C NMR (DMSO-d_6) δ 41.6 (NCH_2), 68.3 (OCH_2), 114.8, 126.4, 128.4, 129.4, 130.5, 132.4, 136.0, 158.1 (8 Ar*C*); LRMS (ESI^+) 248.03 $[M+H]^+$ (calcd for $C_{14}H_{15}ClNO^+$ 248.08); Anal. $C_{14}H_{15}Cl_2NO$ (C, H, Cl, N).

4.1.8. 4-(2′-Trifluoromethoxybenzyloxy)benzylammonium chloride (9)

Employing the procedure used for **4** and using a solution of **28** (2.10 g, 7.2 mmol) in THF (10 mL) and a suspension of $LiAlH_4$ (0.82 g, 21.5 mmol) in THF (70 mL) gave **9** (2.10 g, 88%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 133–136 °C; ^1H NMR (DMSO-d_6) δ 3.95 (s, NCH_2), 5.16 (s, OCH_2), 7.06 (d, J = 8.8 Hz, 2 Ar*H*), 7.41–7.60 (m, 5 Ar*H*), 7.65 (d, J = 7.2 Hz, Ar*H*), 8.30–8.40 (br s, 3H); ^{13}C NMR (DMSO-d_6) δ 41.7 (NCH_2), 64.3 (OCH_2), 114.7 (Ar*C*), 120.6 (q, J = 230.1 Hz, OCF_3), 120.7, 126.5, 127.6, 129.2, 130.3, 130.6, 130.8, 146.7, 158.2 (9 Ar*C*); LRMS (ESI^+) 298.08 $[M-Cl]^+$ (calcd for $C_{15}H_{15}F_3NO_2^+$ 298.10); Anal. $C_{15}H_{15}ClF_3NO_2{\cdot}0.1H_2O$ (C, H, Cl, F, N).

4.1.9. 4-(3′-Trifluoromethoxybenzyloxy)benzylammonium chloride (10)

Employing the procedure used for **4** and using a solution of **29** (1.80 g, 6.1 mmol) in THF (6 mL) and a suspension of $LiAlH_4$ (0.70 g, 18.4 mmol) in THF (52 mL) gave **10** (1.06 g, 52%) as a white solid: R_f = 0.00 (hexanes/EtOAc 1:1); mp 248–251 °C; ^1H NMR (DMSO-d_6) δ 3.91–3.95 (m, NCH_2), 5.20 (s, OCH_2), 7.05 (d, J = 8.8 Hz, 2 Ar*H*), 7.32 (d, J = 7.6 Hz, Ar*H*), 7.41–7.55 (m, 5 Ar*H*); ^{13}C NMR (DMSO-d_6) δ 41.6 (NCH_2), 68.2 (OCH_2), 114.9, 119.4, 119.8, 120.3 (4 Ar*C*), 120.6 (q, J = 256.4 Hz, OCF_3), 123.8, 126.5, 130.6, 139.9, 148.4, 158.1 (6 Ar*C*); LRMS (ESI^+) 298.08 $[M-Cl]^+$ (calcd for $C_{15}H_{15}F_3NO_2^+$ 298.10); Anal. $C_{15}H_{15}ClF_3NO_2$ (C, H, Cl, F, N).

4.1.10. 4-(4′-Trifluoromethoxybenzyloxy)benzylammonium chloride (11)

Employing the procedure used for **4** and using a solution of **30** (2.02 g, 6.9 mmol) in THF (7 mL) and a suspension of $LiAlH_4$ (0.78 g, 20.7 mmol) in THF (63 mL) gave **11** (1.21 g, 55%) as a white solid: R_f = 0.91 (hexanes/EtOAc 7:1); mp 251–254 °C; ^1H NMR (DMSO-d_6) δ 3.93 (s, NCH_2), 5.17 (s, OCH_2), 7.04 (d, J = 8.4 Hz, 2 Ar*H*), 7.38 (d, J = 8.2 Hz, 2 Ar*H*), 7.46 (d, J = 8.2 Hz, 2 Ar*H*), 7.58 (d, J = 8.4 Hz, 2 Ar*H*); ^{13}C NMR (DMSO-d_6) δ 41.6 (NCH_2), 68.2 (OCH_2), 114.8 (Ar*C*), 120.7 (q, J = 255.6 Hz, OCF_3), 121.1, 126.4, 129.5, 130.6, 136.5, 147.8, 158.1 (7 Ar*C*); LRMS (ESI^+) 298.08 $[M-Cl]^+$ (calcd for $C_{15}H_{15}F_3NO_2^+$ 298.10); Anal. $C_{15}H_{15}ClF_3NO_2$ (C, H, Cl, F, N).

4.2. Whole animal pharmacological evaluation

Compounds were screened under the auspices of the NINDS ASP. Experiments were performed in male rodents [albino Carworth Farms No. 1 mice (ip), albino Sprague-Dawley rats (ip, po)]. Housing, handling, and feeding were in accordance with guidelines contained in the *Guide for the Care and Use of Laboratory Animals*. Anticonvulsant activity was established using the MES,[12] 6 Hz,[13] scMet,[14] and the corneal kindled[23] tests according to previously reported methods.[18,23]

4.3. Cortical neurons

Rat cortical neuron cultures were prepared from cortices dissected from embryonic day 19 brains exactly as described.[32,33]

4.4. Electrophysiology

Whole-cell voltage clamp recordings were performed at room temperature on cortical neurons using an EPC 10 Amplifier (HEKA Electronics, Lambrecht/Pfalz Germany).[8] Electrodes were pulled from thin-walled borosilicate glass capillaries (Warner Instruments, Hamden, CT) with a P-97 electrode puller (Sutter Instrument, Novato, CA) such that final electrode resistances were 1–2 MΩ when filled with internal solutions. The internal solution for recording Na^+ currents contained (in mM): 110 CsCl, 5 $MgSO_4$, 10 EGTA, 4 ATP Na_2-ATP, 25 HEPES (pH 7.2, 290–310 mOsm/L). The external solution contained (in mM): 100 NaCl, 10 tetraethylammonium chloride (TEA-Cl), 1 $CaCl_2$, 1 $CdCl_2$, 1 $MgCl_2$, 10 D-glucose, 4 4-AP, 0.1 $NiCl_2$, 10 HEPES (pH 7.3, 310–315 mOsm/L). Whole-cell capacitance and series resistance were compensated with the amplifier. Series resistance error was always compensated to be less than ±3 mV. Cells were considered only when the seal resistance was less than 3 MΩ. Linear leak currents were digitally subtracted by P/4.

4.5. Data acquisition and analysis

Signals were filtered at 10 kHz and digitized at 10–20 kHz. Analysis was performed using Fitmaster and origin8.1 (OriginLab Corporation, MA, USA). For activation curves, conductance (G) through Na^+ channels was calculated using the equation $G = I/(V_m - V_{rev})$, where V_{rev} is the reversal potential, V_m is the membrane potential at which the current was recorded and I is the peak current. Activation and inactivation curves were fitted to a single-phase Boltzmann function $G/G_{max} = 1/\{1+\exp[(V - V_{50})/k]\}$, where G is the peak conductance, G_{max} is the fitted maximal G, V_{50} is the half-activation voltage, and k is the slope factor. Additional details of specific pulse protocols are described in the results text or figure legend.

4.6. Statistical analyses

Differences between means were compared by either paired or unpaired, two-tailed Student's t-tests or an analysis of variance (ANOVA), when comparing multiple groups (repeated measures whenever possible). If a significant difference was determined by ANOVA, then a Dunnett's post-hoc test was performed. Data are expressed as mean ± SEM, with $p < 0.05$ considered as the level of significance.

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Supplementary data

Supplementary data (experimental procedures for intermediates and final products previously reported, elemental analysis and receptor binding assay profile for compounds **3**–**11** against 43 receptors, and ^1H and ^{13}C NMR spectra for all new compounds (**28**–**30**, **4**, **6**–**11**)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.031.

References and notes

1. Stafstrom, C. E. *J. Cereb. Blood Flow Metab.* **2006**, *26*, 983.
2. CDC Epilepsy; http://www.cdc.gov/epilepsy/basics/fast_facts.htmg (January 16, 2013).
3. Picot, M. C.; Baldy-Moulinier, M.; Daurs, J. P.; Dujols, P.; Crespel, A. *Epilepsia* **2008**, *49*, 1230.
4. Pellock, J. M.; Willmore, L. J. *Neurology* **1991**, *41*, 961.
5. King, A. M.; Yang, X.-F.; Wang, Y.; Dustrude, E. T.; Barbosa, C.; Due, M. R.; Piekarz, A. D.; Wilson, S. M.; White, F. A.; Salomé, C.; Cummins, T. R.; Khanna, R.; Kohn, H. *ACS Chem. Neurosci.* **2012**, *3*, 1037.
6. Wang, Y.; Wilson, S. M.; Brittain, J. M.; Ripsch, M. S.; Salomé, C.; Park, K. D.; White, F. A.; Khanna, R.; Kohn, H. *ACS Chem. Neurosci.* **2011**, *2*, 317.
7. Park, K. D.; Yang, X.-F.; Lee, H.; Dustrude, E. T.; Wang, Y.; Khanna, R.; Kohn, H. *ACS Chem. Neurosci.* **2013**, *4*, 463.
8. Lee, H.; Park, K. D.; Yang, X.-F.; Dustrude, E. T.; Khanna, R.; Kohn, H. *J. Med. Chem.* **2013**, *56*, 5931.
9. Errington, A. C.; Stohr, T. C.; Lees, G. *Curr. Top. Med. Chem.* **2005**, *5*, 15.
10. Errington, A. C.; Stohr, T.; Heers, C.; Lees, G. *Mol. Pharmacol.* **2008**, *73*, 157.
11. Sheets, P. L.; Heers, C.; Stoehr, T.; Cummins, T. R. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 89.
12. Levy, R. H.; Mattson, R.; Meldrum, B. *Antiepileptic Drugs*, 4th ed.; Raven Press: New York, 1995. Chapter 6.
13. Barton, M. E.; Klein, B. D.; Wolff, H. H.; White, H. S. *Epil. Res.* **2001**, *47*, 217.
14. Swinyard, E. A. *Epilepsia* **1969**, *10*, 107.
15. Pevarello, P.; Bonsignori, A.; Dostert, P.; Heidempergher, F.; Pinciroli, V.; Colombo, M.; McArthur, R. A.; Salvati, P.; Post, C.; Fariello, R. G.; Varasi, M. *J. Med. Chem.* **1998**, *41*, 579.
16. Salomé, C.; Salomé-Grosjean, E.; Stables, J. P.; Kohn, H. *J. Med. Chem.* **2010**, *53*, 3756.
17. Stables, J. P.; Kupferberg, H. G. In *Molecular and Cellular Targets for Antiepileptic Drugs*; Avanzini, G., Tanganelli, P., Avoli, M., Eds.; John Libbey: London, 1977; pp 191–198.
18. Choi, D.; Stables, J. P.; Kohn, H. *J. Med. Chem.* **1996**, *39*, 1907.
19. Stoehr, T.; Kupferberg, H. J.; Stables, J. P.; Choi, D.; Harris, R. H.; Kohn, H.; Walton, N.; White, H. S. *Epil. Res.* **2007**, *74*, 147.
20. Porter, R. J.; Cereghino, J. J.; Gladding, G. D.; Hessie, B. J.; Kupferberg, H. J.; Scoville, B.; White, B. G. *Cleveland Clin. Q.* **1984**, *51*, 293.
21. Dunham, N. W.; Miya, T.-S. *J. Am. Pharm. Assoc.* **1957**, *46*, 208.
22. White, H. S.; Woodhead, J. H.; Wilcox, K. S.; Stables, J. P.; Kupferberg, H. J.; Wolf, H. H. In *Antiepileptic Drugs*; Levy, R. H., Mattson, R. H., Meldrum, B. S., Perruca, E., Eds., fifth ed.; Lippincott, Williams and Wilkins: Philadelphia, 2002; pp 36–48.
23. Matagne, A.; Klitgaard, H. *Epil. Res.* **1998**, *31*, 24.
24. Loscher, W. *Epil. Res.* **2002**, *50*, 105.
25. Goldin, A. L. *Ann. Rev. Physiol.* **2001**, *63*, 871.
26. Salvati, P.; Maj, R.; Caccia, C.; Cervini, M. A.; Fornaretto, M. G.; Lamberti, E.; Pevarello, P.; Skeen, G. A.; White, H. S.; Wolf, H. H.; Faravelli, L.; Mazzanti, M.; Mancinelli, M.; Varasi, M.; Fariello, R. G. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 1151.

27. King, A. M.; Salomé, C.; Salomé-Grosjean, E.; De Ryck, M.; Kaminski, R.; Valade, A.; Stables, J. P.; Kohn, H. *J. Med. Chem.* **2011**, *54*, 6417.
28. Horton, D. A.; Bourne, G. T.; Smythe, M. L. *Chem. Rev.* **2003**, *103*, 893.
29. Bleicher, K. H.; Green, L. G.; Martin, R. E.; Rogers-Evans, M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 287.
30. http://pdsp.med.unc.edu/PDSP%20Protocols%20II%202013-03-28.pdf.
31. For a related hypothesis, see: Hudgens, D. P.; Taylor, C.; Batts, T. W.; Patel, M. K.; Brown, M. L. *Bioorg. Med. Chem.* **2006**, *14*, 8366.
32. Brittain, J. M.; Piekarz, A. D.; Wang, Y.; Kondo, T.; Cummins, T. R.; Khanna, R. *J. Biol. Chem.* **2009**, *284*, 31375.
33. Brittain, J. M.; Wang, Y.; Eruvwetere, O.; Khanna, R. *FEBS Lett.* **2012**, *586*, 3813.