

1 NBI-921352, a First-in-Class, Na_v1.6 Selective,
2 Sodium Channel Inhibitor That Prevents
3 Seizures in *Scn8a* Gain-of-Function Mice, and
4 Wild-Type Mice and Rats
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7 JP Johnson Jr², Thilo Focken⁴, Kuldip Khakh², Parisa Karimi Tari¹, Celine Dube¹, Aaron Williams²,
8 Jean-Christophe Andrez⁴, Girish Bankar¹, David Bogucki⁴, Kristen Burford⁴, Elaine Chang², Sultan
9 Chowdhury⁴, Richard Dean², Gina de Boer⁵, Shannon Decker⁴, Christoph Dehnhardt⁴, Mandy
10 Feng², Wei Gong⁴, Samuel J Goodchild², Michael Grimwood⁴, Abid Hasan⁴, Angela Hussainkhel²,
11 Qi Jia⁴, Stephanie Lee⁵, Jenny Li², Sophia Lin², Andrea Lindgren⁵, Verner Lofstrand⁴, Janette
12 Mezeyova², Rostam Namdari⁶, Karen Nelkenbrecher¹, Noah Gregory Shuart², Luis Sojo⁵, Shaoyi
13 Sun⁴, Matthew Taron⁴, Matthew Waldbrook¹, Diana Weeratunge², Steven Wesolowski⁴,
14 Michael Wilson⁴, Zhiwei Xie², Rhena Yoo², Clint Young², Alla Zenova⁴, Wei Zhang⁴, Alison J
15 Cutts³, Robin P Sherrington⁷, Simon N Pimstone⁷, Raymond Winqvist⁷, Charles J Cohen⁷, James
16 R Empfield⁷

17
18 Xenon Pharmaceuticals Inc.

19 In Vivo Biology¹, In Vitro Biology², Scientific Affairs³, Chemistry⁴, Compound Properties⁵,
20 Translational Drug Development⁶, Executive Team⁷

21

22 Abstract

23 NBI-921352 (formerly XEN901) is a novel sodium channel inhibitor designed to specifically
24 target Nav1.6 channels. Such a molecule provides a precision-medicine approach to target
25 *SCN8A*-related epilepsy syndromes (*SCN8A*-RES), where gain-of-function (GoF) mutations lead
26 to excess Nav1.6 sodium current, or other indications where Nav1.6 mediated hyper-excitability
27 contributes to disease (Gardella and Moller, 2019; Johannesen et al., 2019; Veeramah et al.,
28 2012). NBI-921352 is a potent inhibitor of Nav1.6 (IC₅₀ 0.051 μM), with exquisite selectivity over
29 other sodium channel isoforms (selectivity ratios of 756X for Nav1.1, 134X for Nav1.2, 276X for
30 Nav1.7, and >583X for Nav1.3, Nav1.4, and Nav1.5). NBI-921352 is a state-dependent inhibitor,
31 preferentially inhibiting activated (inactivated or open) channels. The state dependence leads
32 to potent stabilization of inactivation, inhibiting Nav1.6 currents, including resurgent and
33 persistent Nav1.6 currents, while sparing the closed/rested channels. The isoform-selective
34 profile of NBI-921352 led to a robust inhibition of action-potential firing in glutamatergic
35 excitatory pyramidal neurons, while sparing fast-spiking inhibitory interneurons, where Nav1.1
36 predominates. Oral administration of NBI-921352 prevented electrically induced seizures in a
37 *Scn8a* GoF mouse, as well as in wild-type mouse and rat seizure models. NBI-921352 was
38 effective in preventing seizures at lower brain and plasma concentrations than commonly
39 prescribed sodium channel inhibitor antiseizure medicines (ASMs) carbamazepine, phenytoin,
40 and lacosamide. NBI-921352 was well tolerated at higher multiples of the effective plasma and
41 brain concentrations than those ASMs. NBI-921352 is entering phase II proof-of-concept trials
42 for the treatment of *SCN8A*-developmental epileptic encephalopathy (*SCN8A*-DEE) and adult
43 focal-onset seizures.

44

45 Introduction

46 Nav1.6 voltage-gated sodium channels are widely expressed in the brain and are important
47 contributors to neural excitability (Meisler, 2019; Royeck et al., 2008). Mutations in the *SCN8A*
48 gene result in malfunction of Nav1.6 sodium channels and cause a spectrum of *SCN8A*-related
49 syndromes in humans, and disruptions of mouse Nav1.6 likewise disrupt normal physiology
50 (Burgess et al., 1995; Gardella and Moller, 2019; Johannesen et al., 2019; Meisler, 2019;
51 Veeramah et al., 2012; Wagnon et al., 2015). Variants of Nav1.6 channels can result in either
52 gain or loss of function. Loss-of-function (LoF) variants in humans are generally associated with
53 autism spectrum disorders with cognitive and developmental delay without epilepsy (Inglis et
54 al., 2020; Liu et al., 2019), but, in some cases, can lead to late-onset seizures. In mice, LoF
55 variants of Nav1.6 lead to motor impairment but increase seizure resistance (Hawkins et al.,
56 2011; Martin et al., 2007). Gain-of-function (GoF) variants in human *SCN8A* generally result in
57 early-onset *SCN8A*-related epilepsy syndromes (*SCN8A*-RES). The most severe of these epilepsy
58 syndromes is *SCN8A* developmental and epileptic encephalopathy (*SCN8A*-DEE) (Gardella and
59 Moller, 2019; Hammer et al., 2016; Johannesen et al., 2019). Most *SCN8A*-RES patients carry de
60 novo heterozygous missense variants that lead to a gain of function of the Nav1.6 channel,
61 though inherited and bi-allelic variants have been reported (Gardella and Moller, 2019;
62 Wengert et al., 2019). *SCN8A*-DEE patients present early in life with seizure onset usually
63 occurring in the first year of life. After seizure onset, patients begin to miss developmental
64 milestones and display additional symptoms, including cognitive and motor delay, hypotonia

65 and cortical blindness. *SCN8A*-DEE individuals are predisposed to early death, including sudden
66 unexplained death in epilepsy (SUDEP). While *SCN8A*-RES patients often have treatment-
67 resistant seizures, many can achieve seizure reduction or seizure freedom upon treatment with
68 anti-seizure medicines (ASMs) that non-selectively inhibit voltage-gated sodium channels, like
69 phenytoin (Boerma et al., 2016; Braakman et al., 2017). *SCN8A*-RES patients may require doses
70 that are higher than those prescribed for most epilepsy patients and, as a result, can be more
71 prone to drug-related adverse events (Boerma et al., 2016; Gardella and Moller, 2019). Even
72 with high doses and multiple ASMs, many patients continue to have uncontrolled seizures as
73 well as extensive comorbidities. The aggressive pharmacotherapy required to protect *SCN8A*
74 patients from life-threatening seizures often comes with attendant side effects that would not
75 be tolerated in less severely impacted populations.

76 Existing sodium channel inhibitor ASMs are nonselective, blocking all voltage-gated sodium
77 channel isoforms at similar plasma or brain concentrations. This lack of selectivity likely limits
78 the benefits of sodium channel inhibitors since LoF variants of $Na_v1.1$ are known to impair
79 inhibitory interneuron function and cause generalized epilepsy with seizures plus (GEFS+) and
80 *SCN1A*-DEE (Dravet Syndrome) (Catterall et al., 2010; Claes et al., 2001; Escayg et al., 2000;
81 Gennaro et al., 2003). Thus, inhibiting $Na_v1.1$ may counter the benefit of inhibiting the sodium
82 channels of excitatory neurons. Inhibiting $Na_v1.4$ and $Na_v1.5$ currents is also undesirable since
83 those channels are critical for facilitating contraction of skeletal and cardiac muscles,
84 respectively (Chen et al., 1998; Ptacek et al., 1991; Rojas et al., 1991).

85 We hypothesized that a selective inhibitor of $Na_v1.6$ could provide a safer and more effective
86 treatment for patients with *SCN8A*-RES and might also be more broadly efficacious in more

87 common forms of epilepsy. An extensive medicinal-chemistry effort produced NBI-921352, the
88 first potent and selective inhibitor of Nav1.6 channels (Neurocrine, 2019). We explored the
89 profile of NBI-921352 in vitro, ex vivo and in three preclinical in vivo rodent seizure models,
90 including electrically induced seizure assays in genetically engineered mice bearing
91 heterozygous *Scn8a* GoF Nav1.6 channels (N1768D), as well as in wild-type mice and rats.

92

93 Results

94 In vitro Nav potency and selectivity

95 Human Nav channel isoforms hNav1.1, hNav1.2, hNav1.3, hNav1.4, hNav1.5, hNav1.6, and hNav1.7 were
96 heterologously expressed in HEK-293 cells, and the potency and isoform selectivity of NBI-921352
97 (Figure 1, Table 1) was determined by automated patch-clamp techniques. NBI-921352 potently
98 inhibited hNav1.6 channel currents with an inhibitory concentration 50% (IC₅₀) of 0.051 μM (95% CI:
99 0.030 to 0.073 μM; N=3) calculated from 3 biological replicates. Inhibition of other human Nav1.X
100 isoforms required higher concentrations of NBI-921352 with IC₅₀'s of 39 μM (95% CI: 31 to 47 μM; N=3)
101 for hNav1.1, 6.9 μM (95% CI: 1.6 to 12 μM; N=3) for hNav1.2, >30 μM for hNav1.3, >30 μM for hNav1.4,
102 >30 μM for hNav1.5, and 14 μM (95% CI: 6.4 to 22 μM; N=3) for hNav1.7. These potencies provide
103 selectivity ratios for hNav1.6 versus the other hNav isoforms (IC₅₀ hNav1.X / IC₅₀ hNav1.6) of 756
104 (Nav1.1), 134 (Nav1.2), 276 (Nav1.7) and >583 (Nav1.3, Nav1.4, Nav1.5).

105 Since we intended to evaluate in vivo effects of NBI-921352 in mouse seizure models, we also
106 assessed the potency of NBI-921352 in the mouse Nav isoforms that are most highly expressed
107 in the brain, Nav1.6, Nav1.1, and Nav1.2. The potency and selectivity in mouse Nav channels

108 closely paralleled that seen in the human orthologues with IC_{50} 's of 0.058 μM (95% CI: 0.046 to
109 0.070 μM ; N=3) for mNav1.6, 41 μM (95% CI: 30 to 52 μM ; N=3) for mNav1.1, and 11 μM (95%
110 CI: 8.2 to 14 μM ; N=3) for mNav1.2. Selectivity ratios (IC_{50} mNav1.X / IC_{50} mNav1.6) were 709
111 (Nav1.1), and 191 (Nav1.2). These data indicate that NBI-921352 potently inhibits both human
112 and mouse Nav1.6 channels, and that it does so at concentrations \geq 134-fold lower than for any
113 of the other channel isoforms tested.

114 [NBI-921352 inhibited patient-identified variants of Nav1.6 channels](#)

115 Patients with *SCN8A*-RES carry missense variants in the Nav1.6 channel. A great number of
116 variants have been identified, with a range of biophysical defects. Since most variants are de
117 novo, many have been identified in only one or a few patients. For this reason, we determined
118 the effectiveness of NBI-921352 to inhibit 9 patient identified variants spread across the
119 channel ([Figure 2](#), [Table 2](#)) (Gardella and Moller, 2019; Wagon and Meisler, 2015). The 9
120 variants studied have all been identified in *SCN8A*-RES patients and are in Domains II, III, and IV.
121 Inhibition of the mutant channel constructs was evaluated by automated patch-clamp
122 electrophysiological techniques after transient transfection of the human Nav1.6 variant
123 construct of interest into Expi293F™ cells. All the variants were sensitive to inhibition by NBI-
124 921352. Observed IC_{50} s for inhibition were 0.051 μM (WT mean from [Figure 1](#)), 0.031 μM (95%
125 CI: 0.027 to 0.037 μM) (T767I), 0.021 μM (95% CI: 0.017 to 0.026 μM) (R850Q), 0.032 μM (95%
126 CI: 0.029 to 0.036 μM) (N984K), 0.035 μM (95% CI: 0.029 to 0.043 μM) (I1327V), 0.039 μM (95%
127 CI: 0.031 to 0.050 μM) (N1466K), 0.34 μM (95% CI: 0.26 to 0.44 μM) (R1617Q), 0.055 μM (95%
128 CI: 0.046 to 0.064 μM) (N1768D), 0.068 μM (95% CI: 0.054 to 0.085 μM) (R1872W), and 0.035
129 μM (95% CI: 0.029 to 0.041 μM) (N1877S). We found that 8 of the 9 variants were inhibited

130 with a potency similar to that of the wild-type channel, with most being slightly more potently
131 inhibited. Only one variant, N1617Q, required markedly higher concentrations of NBI-921352
132 for inhibition, with an IC_{50} for inhibition 6.6-fold higher than that of the wild-type Nav1.6
133 channel. The reduced potency for N1617Q is consistent with the variant residing in the
134 predicted binding site of NBI-921352 in the domain IV voltage sensor, see discussion.

135 [NBI-921352 is a state-dependent inhibitor](#)

136 Many small molecule inhibitors of Nav channels bind preferentially to open and or inactivated
137 states (Bean et al., 1983; Courtney et al., 1978; Strichartz, 1976). To query the state
138 dependence of NBI-921352, we measured the apparent potency with two different voltage
139 protocols that favor either the closed (rested) state or activated (open and inactivated) states
140 ([Figure 3](#)). Holding the membrane potential at -120 mV induces most channels to reside in the
141 resting state. Brief depolarizations to measure Nav1.6 current enabled the determination of an
142 IC_{50} of 36 μ M (95% CI: 29 to 47 μ M) for rested-state channels. Holding the membrane potential
143 at -45 mV encourages channels to transition into open and inactivated states. Brief
144 hyperpolarizations allow rapid recovery from inactivation for channels that are not bound to
145 drug followed by a short 20 ms test pulse to -20 mV to measure currents from unbound
146 channels (see methods for details). Measuring the ability of NBI-921352 to inhibit reopening of
147 activated channels leads to an apparent IC_{50} of 0.051 μ M ([Figures 1 and 3](#)). Thus, NBI-921352
148 strongly prefers activated (open or inactivated) channels, inhibiting them at concentrations
149 more than 750-fold less than those needed to inhibit rested or “peak” sodium currents.

150 [NBI-921352 inhibited persistent and resurgent currents from mutant Nav1.6 channels](#)

151 The state-dependent nature of inhibition is also revealed in other types of voltage-clamp
152 protocols, including those designed to measure persistent or resurgent sodium currents. Some
153 drugs or candidate drugs, like PRAX-330 and Riluzole, have been touted based on their
154 preference for persistent currents, but, in fact, this is a feature of all the compounds in Nav
155 inhibitor class. Apparent differences in persistent current selectivity are driven by differential
156 kinetics and concentration dependences in combination with the electrophysiological protocols
157 chosen for the measurements.

158 Elevated persistent and or resurgent currents are believed to underlie or contribute to the
159 pathology of many sodium channel related pathologies (Mason et al., 2019; Pan and Cummins,
160 2020; Potet et al., 2020; Tidball et al., 2020; Zaman et al., 2019). In most conditions, normal
161 Nav1.6 channels inactivate rapidly and nearly completely after opening. Persistent currents
162 result from channels that are not stably inactivated – a common phenotype for epilepsy-
163 inducing variants in Nav1.6, including N1768D (Tidball et al., 2020; Wagnon et al., 2015). We
164 found that NBI-921352 inhibited N1768D Nav1.6 persistent currents (measured as the non-
165 inactivating current 10 ms after initiation of the depolarizing test pulse) with a similar potency
166 as for open and inactivated wild-type Nav1.6 channels with an IC₅₀ of 0.059 μM (95% CI: 0.044
167 to 0.082 μM) ([Figure 3](#)).

168 Resurgent currents occur after repolarizing following a strong depolarization as channels
169 redistribute between closed, open, and inactivated states (Raman and Bean, 1997). These
170 resurgent currents are enhanced in many SCN8A-RES variants (Pan and Cummins, 2020; Raman

171 et al., 1997). NBI-921352 also effectively inhibited resurgent currents from N1768D channels
172 with apparent IC_{50} of 0.037 μ M (95% CI: 0.025 to 0.060 μ M).

173 NBI-921352 preferentially inhibited excitatory pyramidal neurons and spared inhibitory
174 interneurons.

175 A primary goal of creating Nav1.6 selective inhibitors was to spare Nav1.1, the voltage-gated
176 sodium channel that is most prevalent in inhibitory interneurons. This should allow the
177 selective targeting of excitatory neurons, where Nav1.6 and Nav1.2 are believed to be
178 dominant, over inhibitory interneurons. To test this hypothesis, we performed current-clamp
179 experiments in glutamatergic pyramidal neurons from mouse layer 5 neocortex and from fast
180 spiking interneurons in the same region. Application of 0.250 μ M NBI-921352 decreased the
181 maximum firing rate in all pyramidal neurons tested (Figure 4A). This reduction was significantly
182 different from control values at current injection intensities between 120 pA and 320 pA ($n = 3$,
183 $p < 0.05$, paired 2-tailed student's t-test), except for 310 pA ($p = 0.051$). In contrast, NBI-921352
184 subtly increased the number of action potentials (APs) in fast-spiking inhibitory interneurons
185 (Figure 4B) in a statistically significant manner at multiple current injection levels (see Figure 4B).

186 In contrast, carbamazepine inhibited action-potential firing in both pyramidal neurons and in
187 fast-spiking interneurons to a similar degree. The Figure 4 insets show the paired effects of
188 compound on AP number from a stimulus injection of approximately 3X the control Rheobase
189 for that neuron (Pyramidal NBI-921352: 200 pA, Pyramidal carbamazepine: 160 pA, Interneuron
190 NBI-921352: 220 pA, Interneuron carbamazepine: 300 pA).

191 [NBI-921352 inhibited electrically induced seizures in *Scn8a*^{N1768D/+} mice](#)

192 A selective inhibitor of Nav1.6 should lend itself to the treatment of disease states caused by
193 pathologic gain of function of Nav1.6 channels. Hence, we examined the ability of NBI-921352
194 to inhibit electrically induced seizures in mice with a patient-identified GoF variant in the *Scn8a*
195 gene encoding Nav1.6. N1768D is a variant of Nav1.6 identified in the first reported SCN8A-DEE
196 patient (Veeramah et al., 2012). N1768D Nav1.6 channels have impaired voltage-dependent
197 inactivation gating that results in persistent sodium currents and enhanced resurgent currents.
198 Because Nav1.6 channels are highly expressed in the neurons of the brain, increased sodium
199 flux in excitatory neurons leads to seizures. Genetically modified mice bearing the same variant
200 (*Scn8a*^{N1768D/+}) were created and found to be seizure prone, producing a mouse model with a
201 similar phenotype as that observed in SCN8A-DEE patients (Wagnon et al., 2015). Some
202 *Scn8a*^{N1768D/+} mice develop spontaneous seizures at age p60 to p100, but seizure onset and
203 frequency is quite variable, making spontaneous seizure studies challenging. In addition, mice
204 rapidly clear NBI-921352, making it extremely difficult to maintain drug plasma and brain levels
205 in an efficacious range for chronic or subchronic dosing experiments. As an alternative means of
206 assessing NBI-921352's ability to engage Nav1.6 channels in vivo, we designed a modified
207 version of the 6Hz psychomotor seizure assay in *Scn8a*^{N1768D/+} mice (Barton et al., 2001; Focken
208 et al., 2019). A mild current stimulation (12 mA) evoked robust generalized tonic-clonic seizures
209 (GTC) with hindlimb extension in *Scn8a*^{N1768D/+} mice, but not in wild-type littermates.
210 Oral administration of NBI-921352 two hours prior to electrical stimulation prevented induction
211 of GTC with hindlimb extension in *Scn8a*^{N1768D/+} mice in a dose-dependent manner with a 50%
212 effective dose (ED₅₀) of 15 mg/kg (95% CI 9.6 to 23 mg/kg, see [Figure 5A](#)).

213 After seizure assessment, all animals were euthanized, and the concentration of NBI-921352
214 was determined in the plasma and brain tissue from each mouse. The average concentrations
215 for each dose group were used to generate plasma concentration and brain concentration
216 versus efficacy relationships (Figures 5B and 5C, respectively). The plasma 50% effective
217 concentration (EC_{50}) was 0.037 μ M (95% CI 0.018 to 0.090 μ M, see Figure 5B). The brain EC_{50}
218 was 0.064 μ M (95% CI 0.045 to 0.091 μ M, see Figure 5C).

219 [NBI-921352 inhibited electrically induced seizures in wild-type mice](#)

220 $Na_v1.6$ is an important mediator of neuronal excitability even in animals without GoF
221 mutations. For this reason, we wondered whether NBI-921352 might have broader application
222 in epilepsy beyond *SCN8A*-RES and in other syndromes of neural hyperexcitability. To gain
223 insight into this possibility, we assessed NBI-921352 in a MES assay induced by direct-current
224 electrical stimulus (DC-MES, see methods) in wild-type mice. Figures 5D, E & F show that NBI-
225 921352 prevented GTC with hindlimb extension induction in the DC-MES assay in a dose- and
226 concentration-dependent manner. The ED_{50} for NBI-921352 was 23 mg/kg (95% CI 16 to 34
227 mg/kg, see Figure 5D). The efficacy of NBI-921352 was also concentration dependent with a
228 plasma EC_{50} of 0.52 μ M (95% CI 0.25 to 1.2 μ M, see Figure 5E) and a brain EC_{50} of 0.20 μ M (95%
229 CI 0.12 to 0.38 μ M, see Figure 5F).

230 [NBI-921352 inhibited electrically induced seizures in wild-type rats](#)

231 To further explore the preclinical efficacy of NBI-921352, we assessed NBI-921352 in a MES
232 assay induced by direct-current electrical stimulus in wild-type Sprague Dawley rats (see
233 methods). Figures 5G, H & I show that NBI-921352 prevented GTC with hindlimb-extension

234 induction in the rat DC-MES assay in a dose- and concentration-dependent manner. The ED₅₀
235 for NBI-921352 was 3.7 mg/kg (95% CI 2.3 to 7.6 mg/kg, see [Figure 5G](#)). The efficacy of NBI-
236 921352 was also concentration dependent with a plasma EC₅₀ of 0.15 μM (95% CI 0.09 to 0.31
237 μM, see [Figure 5H](#)) and a brain EC₅₀ of 0.037 μM (95% CI 0.028 to 0.054 μM, see [Figure 5I](#)).

238 [Repeated-dosing efficacy in mice and rats](#)

239 We found that repeated dosing tended to increase efficacy at lower doses and exposures of
240 NBI-921352 than after a single dose ([Figure 5](#), red symbols). Animals were dosed every 12 hours,
241 morning, and evening for 13 doses. Two hours after the 13th dose, on the seventh day, efficacy
242 was tested as in acute-dosing studies. A trend toward improved efficacy was noted in all three
243 assays (see red symbols in [Figure 5](#)), but the improvement was not statistically significant when
244 comparing single-dose groups to repeated-dose groups at the same dose level in the same
245 experiment. NBI-921352 did not appreciably accumulate in the plasma or tissue and therefore
246 any trends in improved efficacy were not explained by higher drug concentrations.

247 [NBI-921352 is effective at lower brain concentrations than three Nav inhibitor ASMs](#)

248 Both the efficacy and adverse events of Nav inhibitors is driven by the drugs action in the
249 central nervous system (CNS). We found that NBI-921352 was effective in the three preclinical
250 seizure models evaluated at markedly lower brain concentrations than carbamazepine,
251 phenytoin, and lacosamide ([Figure 6](#)). The brain EC₅₀s for carbamazepine were 9.4 μM, 44 μM,
252 and 36 μM for the *Scn8a*^{N1768D/+} 6Hz model, WT mouse DC-MES, and WT rat DC-MES models,
253 respectively. The brain EC₅₀s for phenytoin were 18 μM, 13 μM, and 2.6 μM for the
254 *Scn8a*^{N1768D/+} 6Hz model, WT mouse DC-MES, and WT rat DC-MES models, respectively. The

255 brain EC₅₀s for lacosamide were 3.3 μM, 7.1 μM, and 4.3 μM for the *Scn8a*^{N1768D/+} 6Hz model,
256 WT mouse DC-MES, and WT rat DC-MES models, respectively. The lower brain concentrations
257 required for efficacy with NBI-921352 are consistent with the potent inhibition of Nav1.6
258 produced by NBI-921352 (Figure 1).

259 [NBI-921352 provided improved separation between efficacy in rats and behavioral signs](#)

260 The intent of creating a highly selective Nav1.6 antagonist was to reproduce or improve on the
261 efficacy of classic, nonselective, sodium channel inhibitor drugs while reducing or preventing
262 the adverse events caused by polypharmacy with other sodium channel and non-sodium
263 channel targets. If sparing Nav1.1 and other off-target interactions does, in fact, reduce adverse
264 events, then even higher receptor occupancy of Nav1.6 might be achievable, thereby further
265 improving efficacy.

266 To evaluate our hypothesis, we compared the window between the plasma concentrations
267 required for efficacy (plasma EC₅₀) relative to the minimal plasma concentration at which
268 treated rats showed behavioral signs of adverse effects as reported by the blinded
269 experimenter (Figure 7). We made this comparison both for NBI-921352 and for several widely
270 used Nav inhibitor ASMs: carbamazepine, phenytoin, and lacosamide.

271 NBI-921352 was well tolerated in these studies up to a plasma concentration of 71 μM.

272 Dividing this concentration by the plasma EC₅₀ of 0.15 μM in the rat DC-MES study results in a
273 behavioral signs concentration (BSC) / plasma EC₅₀ ratio of 473-fold. The same calculation was
274 repeated for the established ASMs. The minimal plasma concentrations provoking behavioral
275 signs for carbamazepine, phenytoin, and lacosamide were 110 μM, 11 μM, and 123 μM,

276 respectively. Their plasma EC₅₀s were 30 μM, 4.5 μM, and 24.4 μM, respectively. Figure 7E
277 shows BSC / Plasma EC₅₀ ratios for carbamazepine (3.7-fold), phenytoin (2.4-fold), and
278 lacosamide (5.0-fold). This data indicates that increasing Nav1.6 selectivity can improve the
279 tolerability of Nav inhibitors in rodent-seizure models.

280

281 Discussion

282 Sodium channel inhibitors have long been, and remain, a mainstay of pharmacotherapy for
283 epilepsy, as well as for pain and other neurologic, cardiac, and skeletal muscle disorders. The
284 diverse range of indications and systems affected by these drugs is a testament to their critical
285 biological role in cellular excitability. A fundamental challenge for these currently marketed
286 sodium channel drugs is that none of them are selective amongst the nine sodium channel
287 isoforms. As a result, drugs targeting the sodium channels of the brain for epilepsy can inhibit
288 both excitatory and inhibitory neurons, limiting their ability to restore balanced neuronal firing.

289 Reducing Nav1.1 current is proconvulsant due to the predominance of Nav1.1 in inhibitory
290 interneurons (Catterall et al., 2010; Claes et al., 2001; Escayg et al., 2000; Mistry et al., 2014; Yu
291 et al., 2006), while the opposite is true for Nav1.2 and Nav1.6 currents (Ben-Shalom et al., 2017;
292 Hawkins et al., 2011; Martin et al., 2007). Nav1.2 and Nav1.6 are more highly expressed in
293 excitatory neurons (Catterall et al., 2010; Du et al., 2020; Encinas et al., 2020).

294 Additionally, these nonselective agents can block the channels associated with skeletal muscle
295 (Nav1.4), cardiac tissue (Nav1.5), and peripheral neurons (Nav1.7, Nav1.8, Nav1.9). Inhibiting

296 these off-target channels can compromise muscular, cardiovascular, and sensory function.

297 These risks are highlighted by the FDA's recent drug-safety communication for the nonselective

298 Na_v inhibitor ASM lamotrigine. Lamotrigine has been linked to cardiac liabilities as a

299 consequence of $\text{Na}_v1.5$ inhibition (FDA, 2021). Likewise, Na_v inhibitors intended as local

300 anesthetics for trigeminal neuralgia or other pain syndromes and class I cardiac antiarrhythmic

301 drugs are often dose limited by CNS adverse events like dizziness, sedation, and cognitive or

302 motor impairment caused by inhibition of central nervous system Na_v channels (Caron &

303 Libersa, 1997).

304 An obvious solution to this isoform selectivity challenge is to pursue a precision-medicine

305 approach and create selective pharmacologic agents that preferentially target the sodium

306 channels specific to the desired target tissue or cell type. This selective approach has been

307 pursued for multiple channel isoforms - particularly for the peripheral Na_v s associated with pain

308 ($\text{Na}_v1.3$, $\text{Na}_v1.7$, and $\text{Na}_v1.8$). This approach has proven challenging because the nine isoforms

309 of sodium channels, $\text{Na}_v1.1$ - $\text{Na}_v1.9$, share a high degree of primary and tertiary structural

310 homology. Achieving selectivity with compounds that have tractable pharmaceutical properties

311 has been particularly difficult.

312 Previous attempts to optimize Na_v inhibitors for epilepsy have focused on either drug

313 properties or channel-state dependence. To our knowledge, this is the first description of a

314 centrally penetrant, isoform-selective Na_v inhibitor for use in CNS indications, including

315 epilepsy. NBI-921352 represents the first selective inhibitor of $\text{Na}_v1.6$ that is suitable for

316 systemic oral administration.

317 The Nav1.6 selective profile of NBI-921352 was designed to inhibit activity in excitatory neurons
318 while sparing firing in the inhibitory interneurons where Nav1.1 is preferentially expressed. We
319 found that NBI-921352 did, in fact, reduce firing in cortical excitatory pyramidal cells. In
320 contrast, inhibitory interneuron firing was not impaired and was seen to increase slightly. The
321 reason for an increase in interneuron action-potential firing is unclear. We propose that this
322 may be a consequence of network effects that arise from interactions with other neurons
323 synapsed onto the target neurons in the experiment. These data confirm that selective Nav1.6
324 inhibitors can distinguish neuronal subtypes in a way that nonselective inhibitors, like
325 carbamazepine, cannot.

326 *SCN8A*-RES patients most often carry de novo genetic variants. While some variants are known
327 to be recurrent, many variants are represented by a single patient (Meisler, 2019). We
328 therefore wanted to assure that NBI-921352 inhibition was not limited to wild-type Nav1.6
329 channels. We tested 9 distinct, patient-identified variants and found that 8 of them were
330 inhibited by NBI-921352 at very similar concentrations as wild-type channels (Figure 2). One
331 variant, R1617Q, was found to be 6.8-fold less sensitive to inhibition than the wild-type
332 channel. R1617Q has been identified in multiple *SCN8A*-DEE patients and is in the domain IV
333 voltage-sensor domain (VSD4). NBI-921352 is an aryl sulfonamide with structural similarity to
334 the Nav1.7 targeted aryl sulfonamides where the binding site has been identified as the Nav1.7
335 VSD4 (Ahuja et al., 2015; McCormack et al., 2013). It is likely that the R1617Q directly or
336 allosterically impairs the tight association of NBI-921252 with Nav1.6 due to its proximity to the
337 binding site. Despite this, NBI-921352 remains markedly more potent than existing Nav inhibitor
338 drugs on the N1617Q variant Nav1.6 channel. This would suggest that while NBI-921352 may be

339 an effective treatment for *SCN8A*-DEE patients carrying R1617Q variants, higher plasma levels
340 of the compound could be required for efficacy in those patients.

341 Many *SCN8A*-RES associated variants produce their GoF effects by disrupting or destabilizing
342 the inactivation-gating machinery of Nav1.6 channels. This can lead to pathological persistent or
343 resurgent currents that contribute to neuronal hyperexcitability (Pan and Cummins, 2020).

344 Most known small molecule inhibitors of Nav channels, except some marine toxins like
345 tetrodotoxin, bind preferentially to inactivated or open gating states of the channels and
346 stabilize the channels in inactivated, non-conductive conformations. This state dependence is
347 manifested as a protocol dependence of the apparent drug potency. State dependence
348 inhibition has been described in many ways. Use-dependent, frequency-dependent, resurgent
349 current -selective, and persistent current-selective inhibition are all consequences of a
350 preference for binding to inactivated and/or open channels. Stabilizing inactivated states of the
351 channel reduces persistent and resurgent currents, and this feature has been suggested to
352 contribute to the efficacy of many Nav targeted ASMs including phenytoin, carbamazepine,
353 oxcarbazepine, lacosamide, cannabidiol, and lamotrigine (Wengert and Patel, 2021). NBI-
354 921352 is also highly state dependent, with a >750-fold preference for open and inactivated
355 channels vs. rested, closed-state channels (sometimes referred to as *peak current*). Forcing all
356 Nav1.6 channels into the closed state by applying voltages more hyperpolarized than
357 physiological (-140 mV) results in very weak inhibition of the channels (Figure 3). Biasing the
358 channels toward activated states (open or inactivated states) by holding the membrane
359 potential more positive in a protocol designed to monitor inactivated state, resurgent current,
360 or persistent current protocol yields potent inhibition. In physiologic conditions, channels are

361 distributed among closed, open, and inactivated states, thus allowing equilibration of potent
362 inhibition of the channel by NBI-921352.

363 Increasing the selectivity of a Na_v inhibitor provides the expectation of an improved safety
364 profile by reducing adverse events caused by off-target activity. An inherent risk of this
365 approach is the potential loss of efficacy that could come from reduced polypharmacy. We have
366 developed a potent, highly selective $Na_v1.6$ inhibitor in NBI-921352. Our studies with NBI-
367 921352 indicate that a $Na_v1.6$ specific compound can retain a robust ability to prevent seizures
368 in rodent models at modest plasma and brain concentrations, consistent with the important
369 role of $Na_v1.6$ in seizure pathways. Our data also suggests that this selectivity profile does
370 improve the tolerability of NBI-921352 relative to commonly employed nonselective sodium
371 channel ASMs in rodents. Whether these results will translate to humans is not yet established,
372 but Phase I clinical trials have shown that NBI-921352 was well tolerated at plasma
373 concentrations higher than were required for efficacy in the preclinical rodent studies described
374 here. NBI-921352 is currently being developed for both *SCN8A*-DEE epilepsy and adult focal-
375 onset seizures by Neurocrine Biosciences (Neurocrine, 2019). Phase II clinical trials will soon
376 evaluate the efficacy of NBI-921352 in patients (Neurocrine, 2021). These clinical trials will
377 provide the first evidence for whether the robust efficacy and tolerability demonstrated in
378 rodents translates to human epilepsy patients.

379

380 Materials and Methods

381 Electrophysiological determination of potency and selectivity

382 Cell lines

383 Electrophysiology experiments were performed with HEK293 cells either stably transfected or
384 transiently transfected. The stable cell lines were transfected with an expression vector containing the
385 full-length cDNA coding for specific human and mouse sodium channel α -subunit, grown in culture
386 media containing 10% fetal bovine serum, and 0.5 mg/mL Geneticin (G418) at 37°C with 5% CO₂. The
387 Nav_v1.x stable cell lines and accessory constructs used correspond to the following GenBank accession
388 numbers: Human Nav_v1.1 (NM_006920); mouse Nav_v1.1 (NM_018733.2); human Nav_v1.2 (NM_021007);
389 mouse Nav_v1.2 (NP_001092768.1); human Nav_v1.5 (NM_198056); human Nav_v1.6 (NM_014191); mouse
390 Nav_v1.6 (NM_001077499); human Nav_v1.7 (NM_002977); human Nav_v1.4 (NM_000334); human Nav_v1.3
391 (NM_0069220). The human Nav_v β 1 subunit (NM_199037) was co-expressed in all cell lines. Human and
392 mouse Nav_v1.6 channels were also coexpressed with human FHF2B (NM_033642) to increase functional
393 expression. Human Nav_v1.2 channels were also coexpressed with Contactin 1 (NM_001843) to increase
394 functional expression.

395 For studies of mutant channels, cDNA plasmids in pcDNA™4/TO Mammalian Expression Vector were
396 transiently transfected into Expi293F™ cells stably expressing human FHF2b and human SCN1B subunit
397 (polyclonal) background using ExpiFectamine™ 293 Transfection Kits (Gibco, Thermo Fisher Scientific CAT
398 #: A14524). Induction was achieved using Tetracycline (Sigma Aldrich). Transfected cells were used in
399 automated patch-clamp experiments 24 hours postinduction.

400 Na_v channel automated planar patch-clamp assay

401 NBI-921352 requires several seconds to equilibrate with activated channels, and this property of the
402 compound must be taken into consideration in the design of state-dependent assay voltage-clamp
403 protocols.

404 Data was collected using the Qube 384 (Sophion) automated voltage-clamp platform using single hole
405 plates. To measure inactivated state inhibition, the membrane potential was maintained at a voltage
406 where inactivation is complete. For each Na_v channel subtype, the V_h used to quantify compound
407 inhibition were as follows: $\text{Na}_v1.6$ (-45 mV), $\text{Na}_v1.1$ (-45 mV), $\text{Na}_v1.2$ (-45 mV), $\text{Na}_v1.3$ (-45 mV), $\text{Na}_v1.5$ (-
408 60 mV), $\text{Na}_v1.7$ (-60 mV), $\text{Na}_v1.4$ (-45 mV). The mutant channel $\text{hNa}_v1.6^{\text{N1768D}}$ was found to have
409 accelerated run-down compared with wild-type $\text{hNa}_v1.6$, so the holding potential was adjusted to -60
410 mV to provide sufficient signal window. The voltage was briefly repolarized to a negative voltage (-150
411 mV) for 20 milliseconds for ($\text{Na}_v1.5$, $\text{Na}_v1.7$, $\text{Na}_v1.3$, $\text{Na}_v1.4$) or for 60 milliseconds (for $\text{Na}_v1.1$, $\text{Na}_v1.2$,
412 and $\text{Na}_v1.6$) to allow recovery from fast inactivation, followed by a test pulse to -20 or 0 mV for 10
413 milliseconds to quantify the compound inhibition. The repolarization step allows compound-free
414 channels to recover from fast inactivation, but compound-bound channels remain inhibited during the
415 subsequent test step. For rested state “Peak” current V_h was set to -120 mV. Appropriate filters for
416 minimum seal resistance were applied (typically >500 M Ω membrane resistance), and series resistance
417 was compensated at 100%. The pulse protocols were run at 1 Hz for $\text{hNa}_v1.7$, $\text{hNa}_v1.5$, $\text{hNa}_v1.3$, and
418 $\text{hNa}_v1.4$ or 0.04 Hz for $\text{Na}_v1.6$, $\text{Na}_v1.1$ and $\text{Na}_v1.2$.

419 To construct concentration response curves, baseline currents were established after 20 minutes in
420 vehicle (0.5% DMSO). Full inhibition response amplitudes were determined by adding tetrodotoxin (TTX,
421 300 nM) or tetracaine for $\text{Na}_v1.5$ (10 μM) to each well at the end of the experiment. Compounds were
422 then exposed at a single concentration for 20 minutes. One-sixth of every experimental plate was

423 dedicated to vehicle-only wells that enabled correction for nonspecific drift (i.e., rundown) of the signal
424 in each experiment. For all channel subtypes, inhibition by the compound reached steady state within
425 20 minutes of incubation. The current inhibition values ($I_{(CPD)}$) were normalized to both the vehicle
426 ($I_{(control)}$) and the full response defined by supramaximal TTX ($I_{(TTX)}$) or tetracaine (for Nav1.5) addition
427 responses according to Equation 1:

428 *Equation 1*

$$429 \quad I_{norm(CPD)} = (I_{CPD} - I_{control}) / (I_{TTX} - I_{control}).$$

430 This normalized inhibition was then further normalized to the span of the assay to account for the run-
431 down seen in cells exposed to vehicle alone for 20 minutes as follows:

432 *Equation 2*

$$433 \quad I_{norm, span} = (I_{norm(CPD)} - I_{norm(VEH)}) / (1 - I_{norm(VEH)}),$$

434 where:

435 $I_{norm, span}$ = the current response normalized to within the span of the assay.

436 $I_{norm(CPD)}$ = the normalized response in the presence of compound.

437 $I_{norm(VEH)}$ = the normalized response in the absence of compound.

438 This normalization ensures that the data ranges were between 0 and 1, and there is no rundown in the
439 plots. The normalized data from all cell recordings at a concentration were grouped together and
440 plotted with GraphPad Prism 8, and IC_{50} values were calculated for grouped data using the following
441 version of the Hill equation:

442 *Equation 3*

$$443 \quad Y = RD + (1 - RD) \times [CPD] / (IC_{50} + [CPD]),$$

444 where:

445 Y = the fraction of sodium current blocked in the presence of the compound.

446 $[CPD]$ = the concentration of compound.

447 IC_{50} = the IC_{50} concentration.

448 RD = the “rundown” of sodium current in vehicle alone, which is equal to 0 in this case, as the inhibition
449 has already been normalized to the span.

450 The Hill slope was fixed to 1.

451 The 95% CI for the IC_{50} from the fitted curve to the mean data were reported unless otherwise noted.

452 To evaluate inhibition of hNav1.6(N1768D) resurgent currents, synthetic Nav β 4 peptide
453 (KKLITFILKKTREKKKECLV) was added to the intracellular recording solution at 200 μ M and a dedicated
454 protocol to elicit resurgent currents was employed (Barbosa et al., 2015). Cells were voltage clamped at
455 (V_h = -80 mV) and subjected to a strong depolarization (+60 mV) for 20 milliseconds. Following the
456 strong depolarization, cells were partially repolarized to the voltage where resurgent current was
457 maximal (-20 mV) for 50 milliseconds, and resurgent current amplitude was measured. This resurgent
458 current-specific waveform was repeated at 5 Hz for 100 s in vehicle, followed by 100 s in test compound,
459 then 300 nM TTX. Fractional inhibition was calculated using the same normalization procedure as
460 above.

461 Experiments were all performed at $27^\circ\text{C} \pm 2^\circ\text{C}$.

462 Automated patch-clamp recording solutions

463 The recording solutions for Nav1.1, Nav1.2, Nav1.3, Nav1.4 and Nav1.6 cell line studies contained:

464 Intracellular solution (ICS): 5 mM NaCl, 10 mM CsCl, 120 mM CsF, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM

465 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer), 10 mM EGTA (ethylene glycol
466 tetraacetic acid); adjusted to pH 7.2 with CsOH. Extracellular solution (ECS): 140 mM NaCl, 5 mM KCl,
467 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES; adjusted to pH 7.4 with NaOH. Solutions with a reversed Na⁺
468 gradient were used for Na_v1.5 and Na_v1.7 studies since they improved technical success. ICS: 120 mM
469 NaF, 10 mM CsCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM EGTA; adjusted to pH 7.2 with
470 CsOH. ECS: 1 mM NaCl, 139 mM CholineCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES; adjusted
471 to pH 7.4 with NaOH. Osmolarity in all ICS and ECS solutions was adjusted with glucose to 300 mOsm/kg
472 and 310 mOsm/kg, respectively.

473 [Current-clamp recording of cortical pyramidal neurons and inhibitory interneurons](#)

474 [Slice preparation](#)

475 Parasagittal cortical brain slices were prepared from >P21 mice using standard procedures (adapted
476 from Tai et al., PNAS 2014). Briefly, the mouse was deeply anaesthetized with isoflurane and
477 decapitated. The brain was removed and placed into chilled artificial cerebrospinal fluid (aCSF) solution
478 containing (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 2 MgCl₂, 10 d-glucose, pH 7.3,
479 osmolarity adjusted to ~306 mOsm using sucrose. All solutions were saturated with 95% O₂ and 5% CO₂
480 constantly perfused with 95% O₂/5% CO₂. Slices with a thickness of 400 μm were prepared using a
481 vibratome (Ted Pella, Inc.). Following sectioning, the slices were placed in a holding chamber and
482 incubated in a water bath at 34°C for 15 minutes. The brain slices were removed from the water bath
483 and held at room temperature for 60 minutes prior to recording.

484 [Brain slice electrophysiology assay](#)

485 All experiments involving rodent subjects were performed in accordance with the guidelines of the
486 Canadian Council on Animal Care (CCAC). Following a 60-minute incubation at room temperature, a
487 brain slice was selected and placed on the stage of an upright microscope (SliceScope Pro 2000,

488 Scientifica). The slice was constantly perfused with room temperature aCSF, containing 0.1% DMSO as a
489 vehicle control, and oxygenated with 95% O₂/5% CO₂. The slice was visualized using brightfield
490 microscopy, and a healthy neuron was selected from neocortical layer 5. Whole-cell configuration was
491 achieved with a pipette (bath resistance 4 – 6 MΩ) containing internal solution. Stimulation was applied
492 in current-clamp mode, and consisted of a series of 1000 ms square pulses, beginning at -20 pA and
493 increasing by +20 pA increments (3000 ms between pulses).

494 Once the recordings in vehicle were completed, and while still holding the patch on the same neuron,
495 the bath solution was changed from 0.1% DMSO in aCSF to 0.25 μM NBI-921352 or 100 μM
496 Carbamazepine in aCSF. The slice was incubated in circulating compound for 10 minutes before
497 repeating the series of square pulse stimulations. Working stock solutions were prepared in DMSO at a
498 concentration of 20 mM.

499 All data analysis was done offline using ClampFit 10.7 (Molecular Devices). Data are presented as a mean
500 ± SEM. For each sweep, the number of evoked APs was counted, and plotted as a function of current
501 injection (beginning with -20 pA). These generated “input/output” (or “F/I”) curves demonstrating the
502 relationship between stimulus and average AP frequency. Statistical significance was assessed using
503 paired, two-way, student’s t-test applied at each current injection level with significance considered
504 P<0.05.

505 [Formulation and oral dosing of NBI-921352](#)

506 [Vehicle preparation](#)

507 The vehicle for oral dosing solutions was 0.5% methyl cellulose and 0.2% Tween-80 in deionized (DI)
508 water. DI water (0.8 L) was heated up to 70°C to 80°C. Five grams of methyl cellulose was slowly added
509 to heated DI water. The mixture was stirred until it formed a homogeneous milky suspension. The
510 suspension was moved to a cold room and stirred overnight to get a clear solution. Two milliliters of

511 Tween-80 was added to the clear solution and diluted up to 1 L with DI water. The vehicle solution was
512 stored at 2°C to 8°C.

513 [Drug formulation](#)

514 NBI-921352 was weighed into vials. An appropriate amount of vehicle was added to the NBI-921352
515 powder then mixed on a T18 ULTRA TURRAX homogenizer (IKA, Wilmington, NC) to create a uniform
516 suspension at the desired concentration. The vials were then wrapped in aluminum foil to protect them
517 from light and placed on a stir plate until the time of dosing. Carbamazepine and lacosamide were
518 formulated in the same manner. Phenytoin was formulated in 0.9% physiological saline.

519 [Dosing](#)

520 NBI-921352, carbamazepine, and lacosamide were administered orally using a stainless-steel gavage
521 needle at a dose volume of 10 ml/kg. Phenytoin was formulated in physiologic saline and was
522 administered intraperitoneally (i.p.) using a 25-gauge needle at a dose volume of 10 mL/kg. All
523 compounds were administered 2 hours prior to electrical seizure induction for all seizure models
524 employed in this study.

525 [Bioanalytical assessment of plasma and brain concentrations](#)

526 [Sample collection](#): Approximately 0.5 mL of blood was collected from each mouse at the end of
527 the assay via cardiac puncture under deep anesthesia. The blood samples were collected in a
528 syringe and transferred to tubes containing EDTA. Blood was stored at 4°C until centrifuged
529 within 30 minutes of collection. Plasma was harvested and placed on dry ice and stored in a
530 freezer set to maintain a temperature of -70°C to -80°C until analysis. Brains were harvested
531 immediately after blood collection and placed on dry ice prior to storage in a freezer set to
532 maintain a temperature of -70°C to -80°C until analysis.

533 **Plasma samples:** Extraction of plasma samples was carried out by protein precipitation using
534 acetonitrile. Plasma samples (50 μ L) were mixed with 50 μ L of internal standard (IS) solution in
535 water followed by addition of 10 μ L of concentrated ortho-phosphoric acid and 200 μ L of
536 acetonitrile. Samples were vortexed for 30 seconds, centrifuged at 13,000 rpm for 20 minutes,
537 decanted in to a 96-well plate, and further centrifuged at 4,000 rpm for 20 minutes. The
538 samples were analyzed by UHPLC-ESI-MS/MS as described below.

539 **Brain samples:** Prior to extraction, pre-weighed whole brains were homogenized in 1:1
540 acetonitrile/water (v/v) (4 mL per mouse brain) using an IKA T18 ULTRA-TURRAX Homogenizer
541 at the setting of 4 for approximately 2 min. The homogenate was centrifuged at 13,000 rpm for
542 20 min and 50 μ L of the supernatant were treated exactly as described above for plasma
543 samples. 50 μ L of the brain homogenate were then treated exactly as the plasma samples
544 described above.

545 **Standards and quality control (QC) samples:** K₂EDTA Blank mouse plasma purchased from Valley
546 Biomedical, California, USA was used to prepare standards and QC samples for plasma
547 quantitation and as surrogates for brain homogenate quantitation. Calibration samples ranged
548 from 2.34 ng/mL to 4,800 ng/mL. QC samples concentration included 14 ng/mL (QC-L), 255
549 ng/mL (QC-M) and 3,600 ng/mL (QC-H). Standards and QC samples were processed the same
550 way as the sample extracts described above.

551 **Analytical methods and statistics for plasma and tissue samples:**

552 Samples were analyzed by UHPLC-ESI MS/MS using a TQ-5500 Sciex triple quadrupole mass
553 spectrometer equipped with a Shimadzu Nexera UHPLC pump and auto-sampler system using an ACE
554 C18 PFP, 2.50 x 50 mm, 1.7 μ particle size column and gradient elution consisting of solvent A (0.1%

555 formic acid in water) and solvent B (0.1% formic acid in acetonitrile) starting at 20% B from 0 min to 0.4
556 min and then increased to 100% B from 0.4 min to 0.6 min. At 2.0 min, the mobile phase composition
557 was switched back to 60% B for 1 min. The flow rate used throughout the experiment was 0.4 min/mL.
558 The analyte, NBI-921352, and the IS were detected by electrospray in the positive ion mode using the
559 following transitions: m/z 460/91 for NBI-921352 and m/z 503/341m/z for the IS. The UHPLC-ESI
560 MS/MS system was controlled by Analyst 1.6.

561 Sample concentrations were determined using a linear calibration function, weighted 1/X, generated by
562 the regression of analyte to IS peak area ratios in the standard samples to their respective
563 concentrations. Acceptance criteria for the analytical run required that the back calculated values of the
564 standards and the QC samples fell within $\pm 20\%$ of their nominal values, except for the lowest standard
565 or lower limit of quantitation (LLOQ), for which the acceptance criterion was $\pm 25\%$. At least 6 out of 12
566 standard points had to show back-calculated values within $\pm 20\%$ of their nominal concentrations for the
567 calibration to be accepted. At least three QC samples, one at each level, had to show back-calculated
568 values within $\pm 20\%$ of their nominal concentrations for the whole sample batch to be valid.

569 [Animals](#)

570 After delivery, animals were allowed sufficient time to acclimate prior to testing (~1 week). All animals
571 were housed in plastic cages in rooms with controlled humidity, ventilation, and lighting (12 hr/12 hr
572 light–dark cycle). All animal procedures were performed using protocols approved by Xenon Animal
573 Care Committee and the Canadian Council on Animal Care.

574 [Scn8a^{N1768D}/+ mice:](#)

575 Xenon Pharmaceuticals Inc. licensed the mouse with the missense mutation p.Asn1768Asp (N1768D) in
576 the neuronal sodium channel Nav1.6, characterized and developed by Dr. M Meisler (University Of
577 Michigan, MI, USA). The *Scn8a*^{N1768D} knock-in allele was generated by TALEN targeting of (C57BL/6JXSJL)

578 F2 eggs at the University of Michigan Transgenic Animal Model Core. The line was propagated by
579 backcrossing N1768D/+ heterozygotes to C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor,
580 ME). Male N1768D/+ heterozygotes on a C57BL/6J background were subsequently backcrossed to
581 C3HeB/FeJ female mice. All the experiments were performed using animals following at least 7 such
582 backcrosses. Experiments were performed using (B6 × C3He) F7 (F7. N1768D/+) offspring aged 35-42
583 days.

584 WT mice:

585 Adult male CF-1 WT albino mice 26-35 g were obtained from Charles River, Senneville, Quebec, Canada.
586 All the assays were carried out in mice 9 weeks or older.

587 Sprague-Dawley rats:

588 Adult male Sprague-Dawley albino rats weighing 150-200 g were obtained from Envigo, Livermore, CA,
589 USA. All the assays were carried out in rats 5 weeks or older.

590 The modified 6 Hz psychomotor seizure assay

591 The modified 6 Hz seizure assay in *Scn8a*^{N1768D/+} heterozygous mice was adapted from the
592 traditional 6 Hz assay psychomotor seizure assay to provide a measure of in vivo on target
593 (Nav1.6 mediated) efficacy (Barton et al., 2001). The modified assay used a low frequency (6 Hz)
594 but long-duration stimulation (3 seconds) to induce seizures. We identified 12 mA and a 0.3
595 millisecond pulse interval as a suitable current for testing in *Scn8a*^{N1768D/+} mice, since it
596 differentiated mutant and wild-type (WT) mice. An electroshock (6 Hz, 12 mA) was delivered for
597 3 seconds (at 0.3 millisecond pulse interval) by corneal electrodes (Electro Convulsive Therapy
598 Unit 57800 from Ugo Basile). Immediately prior to the electroshock, the animals' eyes were
599 anesthetized with a drop of Alcaine (0.5% proparacaine hydrochloride). Upon corneal

600 stimulation, WT mice experienced mild seizure behaviors such as facial clonus, forelimb clonus,
601 Straub tail, rearing, and falling, but did not experience a generalized tonic-clonic seizure (GTC)
602 with hindlimb extension. *Scn8a*^{N1768D/+} animals, however, in addition to mild seizure behaviors,
603 experienced a GTC with hindlimb extension. The modified assay showed a clear differentiation
604 of seizure behavior between WT and *Scn8a*^{N1768D/+} mice. *Scn8a*^{N1768D/+} mice exhibited GTC with
605 hindlimb extension but not WT mice.

606 For the single-dose and repeated-dose efficacy experiments, *Scn8a*^{N1768D/+} animals were dosed
607 PO with vehicle or NBI-921352 two hours before the administration of the electric stimulation.
608 An animal was considered protected in the assay upon prevention of GTC with hindlimb
609 extension and was then scored “0”. An animal displaying GTC with hindlimb extension was
610 considered not protected and is then scored “1”. The experimenter scoring the seizure behavior
611 was blinded to the treatment.

612 DC-Maximal electroshock seizure assay in rodents

613 The maximal electroshock seizure (MES) assay has been extensively used in the search for
614 anticonvulsant substances (Loscher et al., 1991; Piredda et al., 1985; White et al., 1995). The
615 MES assay is sensitive to nonselective NaV inhibitors. It is considered a model for generalized
616 tonic-clonic (GTC) seizures and provides an assessment of seizure spread. Briefly, an
617 electroshock of direct current (DC) was delivered by corneal electrodes (Electro Convulsive
618 Therapy Unit 57800 from Ugo Basile). The parameters of stimulation were different between
619 mice and rats. In CF1 mice, a direct current of 50 mA (60 Hz) was delivered for 0.2 seconds
620 (pulse width of 0.5 ms), whereas in Sprague Dawley (SD) rats, a direct current of 150 mA (60 Hz)
621 was delivered for 0.3 seconds (pulse width of 0.5 ms). Immediately prior to the electroshock,

622 the animals' eyes were anesthetized with a drop of Alcaine (0.5% proparacaine hydrochloride).
623 Upon corneal stimulation, naïve animals experienced a generalized tonic-clonic seizure (GTC)
624 with hindlimb extension.

625 For the efficacy experiments, single dose and repeated dose, animals were dosed PO with
626 vehicle or NBI-921352 two hours before the administration of the electric stimulation. An
627 animal was considered protected in the assay in the absence of a GTC with hindlimb extension
628 and is then scored "0". An animal displaying GTC with hindlimb extension was considered not
629 protected and is then scored "1". The experimenter scoring the seizure behavior was blinded to
630 the treatment.

631 [Blinding of in vivo efficacy experiments](#)

632 On each testing day, individual treatment groups were assigned a random label (e.g., A, B, C,
633 etc.) by the technical staff administering the compound. To ensure blinding, the technical staff
634 member performing drug administration differed from the person performing the test.
635 Therefore, the experimenter conducting testing was blinded to treatment group (e.g., drug or
636 vehicle treatment, dose, and time point).

637 [Randomization of in vivo efficacy experiments](#)

638 Randomization of animals into various treatment groups occurred on a per-animal (e.g., rather
639 than a per-cage) basis. Therefore, each animal was randomly assigned to a treatment group,
640 and all animals tested in each experiment had an equal chance of assignment to any treatment
641 group. Prior to each study, a randomization sequence was obtained
642 (www.graphpad.com/quickcalcs).

643

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651

652 Competing Interests

653 All authors are, or were previously, employees of Xenon Pharmaceuticals Inc. They receive or
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655 Xenon Pharmaceuticals Inc.

656

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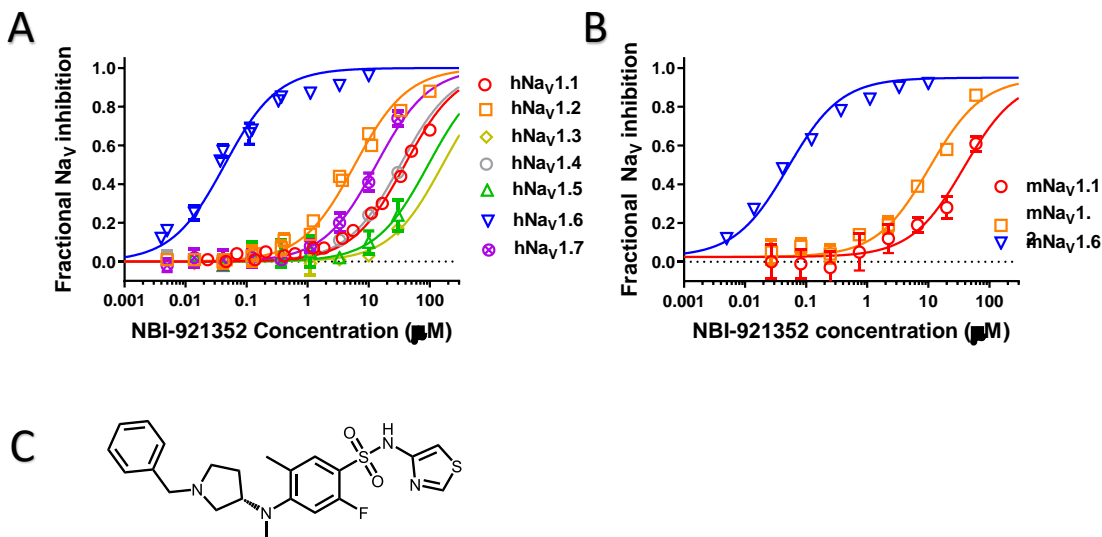
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811

812

813 **Figure 1** Potency and isoform selectivity of NBI-921352 for human and mouse Na_V
814 channels.



815
816 Concentration-response curves were generated by automated patch-clamp electrophysiology using the
817 Sophion Qube. Concentration-response curves were generated for human (A) or mouse (B) Na_V channel
818 isoforms heterologously expressed in HEK293 cells. The analysis included only those cells that met pre-
819 specified acceptance criteria for seal quality, current amplitude, and series resistance. Normalized data
820 from all cell recordings at a concentration were grouped together and plotted with GraphPad Prism 8.
821 Details regarding the number of cells analyzed for each Na_V channel and concentration can be found in
822 the source data sheet. Error bars indicating the standard error of the mean fraction were plotted for all
823 points, but, in some cases, they were smaller than the data point symbols and, therefore, not visible.
824 The chemical structure of NBI-921352 is shown (C).

825

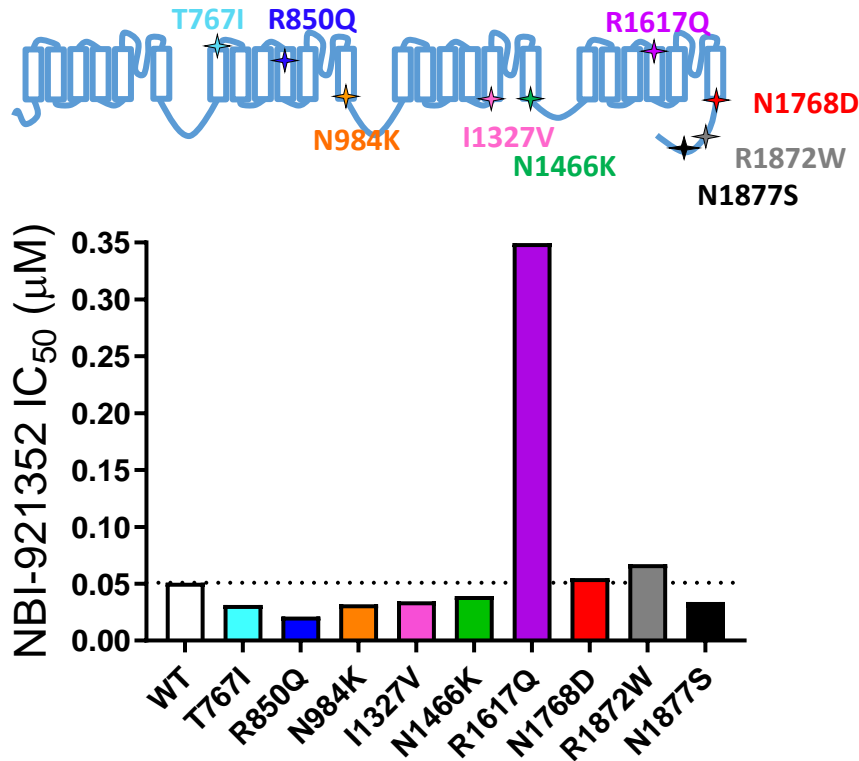
826 **Table 1 Potency and isoform selectivity of NBI-921352 for human and mouse Nav**
 827 **channels.**

	Nav1.6	Nav1.1	Nav1.2	Nav1.3	Nav1.4	Nav1.5	Nav1.7
Human IC₅₀ (μM)	0.051	39	6.9	>30	>30	>30	14
Human Selectivity hNav1.X / hNav1.6	1	756	134	>583	>583	>583	276
Mouse IC₅₀ (μM)	0.058	41	11				
Mouse Selectivity mNav1.X / mNav1.6	1	709	191				

828 IC₅₀s corresponding to the concentration-response curves in Figure 1 were calculated in GraphPad Prism
 829 8 using the Hill equation described in the Materials and Methods. Note that IC₅₀s for the neuronal
 830 sodium channels, Nav 1.1, Nav 1.2, and Nav1.6, have been more accurately defined than those for non-
 831 neuronal sodium channels.

832

833 **Figure 2 Comparison of NBI-921352 potency on human wild-type Nav1.6 and patient-**
834 **identified variants of Nav1.6.**



835 IC₅₀s were calculated in GraphPad Prism 8 using the Hill equation described in the Materials and
836 Methods. All constructs were transiently transfected into Expi293F™ cells and evaluated by automated
837 patch-clamp electrophysiology using the Sophion Qube. The voltage-clamp methods and data analysis
838 were identical to those used for evaluation of the wild-type channels in Figure 1. Details regarding the
839 number of cells analyzed for each Nav channel and concentration can be found in the source data sheet.
840 The dotted line indicates the IC₅₀ for wild-type Nav1.6 from [Figure 1](#).

841

842

843 **Table 2 Comparison of NBI-921352 potency on human wild-type Nav_v1.6 and patient-**

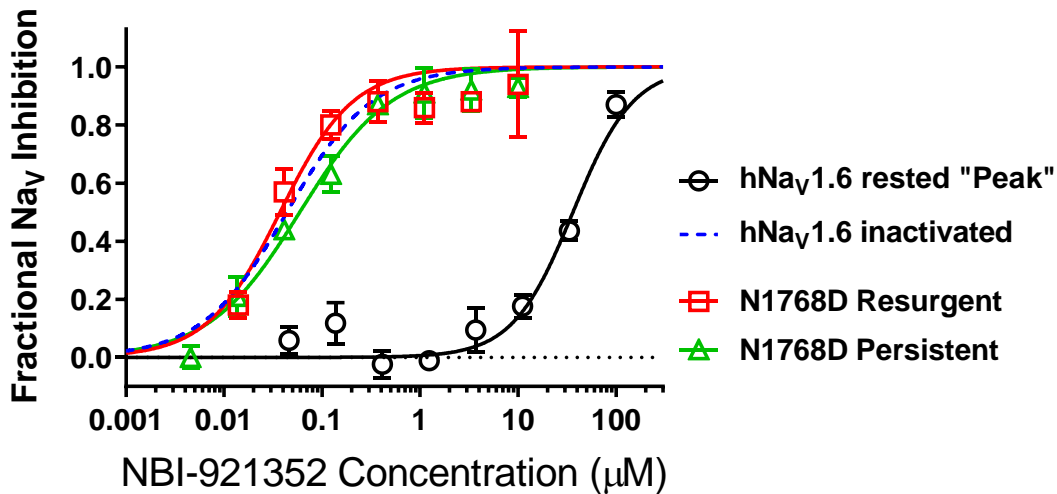
844 **identified gain-of-function variants of Nav_v1.6.**

	WT	T767I	R850Q	N984K	I1327V	N1466K	R1617Q	N1768D	R1872W	N1877S
hNav_v1.6 IC₅₀ (μM)	0.051	0.031	0.021	0.032	0.035	0.039	0.349	0.054	0.067	0.034
Fold change WT / Variant	-	0.6	0.4	0.6	0.7	0.8	6.8	1.1	1.3	0.7

845 IC₅₀s corresponding to Figure 2 are shown in the table and were calculated as indicated for Table 1.

846

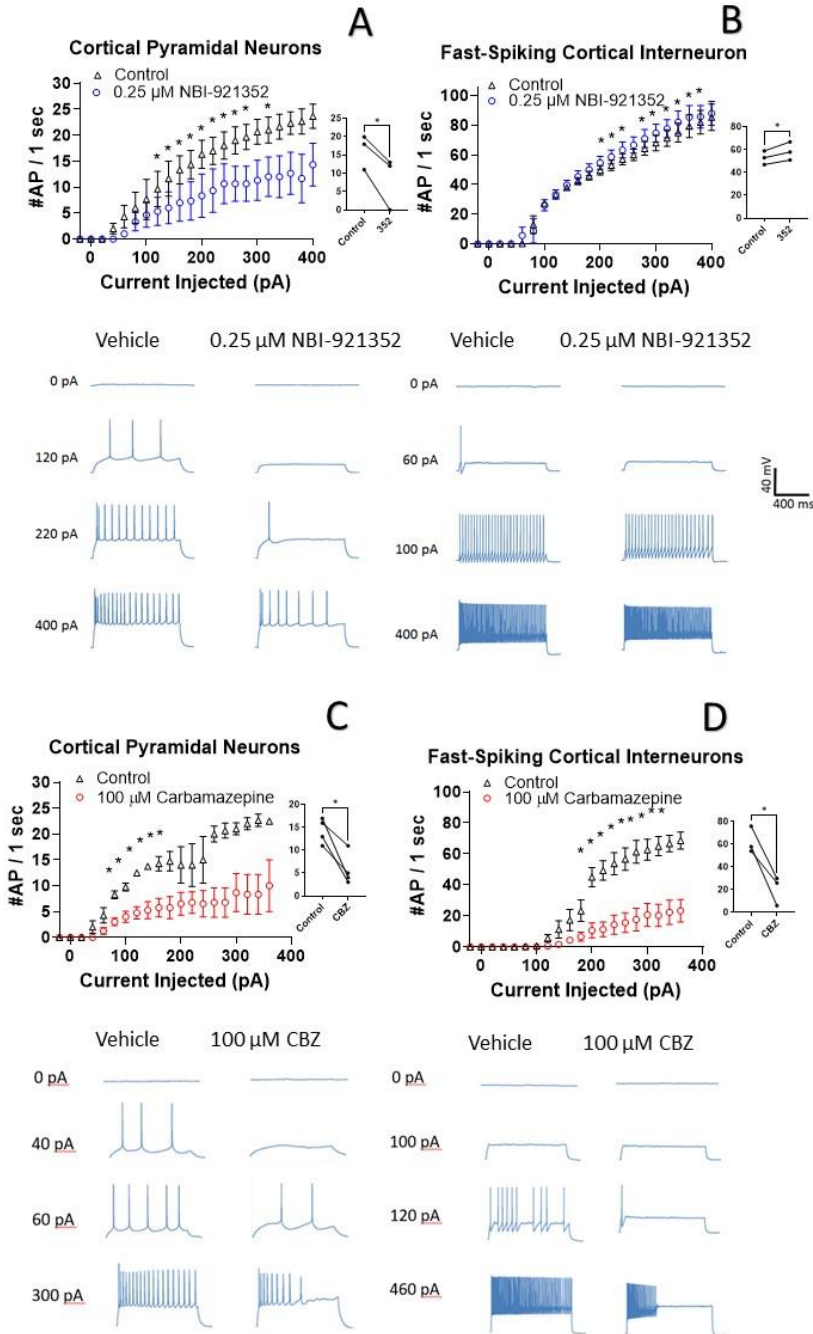
847 **Figure 3 NBI-921352 is a state-dependent inhibitor of Na_v1.6 and preferentially inhibited**
848 **activated (open or inactivated) channels.**



849

850 Concentration-response curves were generated for human WT and N1788D channel isoforms
851 heterologously expressed in HEK293 cells. The analysis included only those cells that met pre-specified
852 acceptance criteria for seal quality, current amplitude, and series resistance. Normalized data from all
853 cell recordings at a concentration were grouped together and plotted with GraphPad Prism 8. Details
854 regarding the number of cells analyzed for each Na_v channel isoform and concentration can be found in
855 the source data sheet. Error bars indicating the standard error of the mean fraction were plotted for all
856 points. The blue dotted line indicates the concentration-response curve for wild-type Na_v1.6 from
857 [Figure 1](#). When Na_v1.6 channels were equilibrated with NBI-921352 at voltages that allow full
858 equilibration with inactivated states (-45 mV), the compound provided potent inhibition, as seen in
859 [Figure 1](#). Measuring persistent or resurgent sodium current after equilibration of cells with NBI-921352
860 resulted in similar potency (see Materials and Methods and text). Forcing channels to the rested, closed
861 state by hyperpolarizing to -120 mV resulted in very weak inhibition. Current evoked from very negative
862 potentials is sometimes referred to as "peak current".

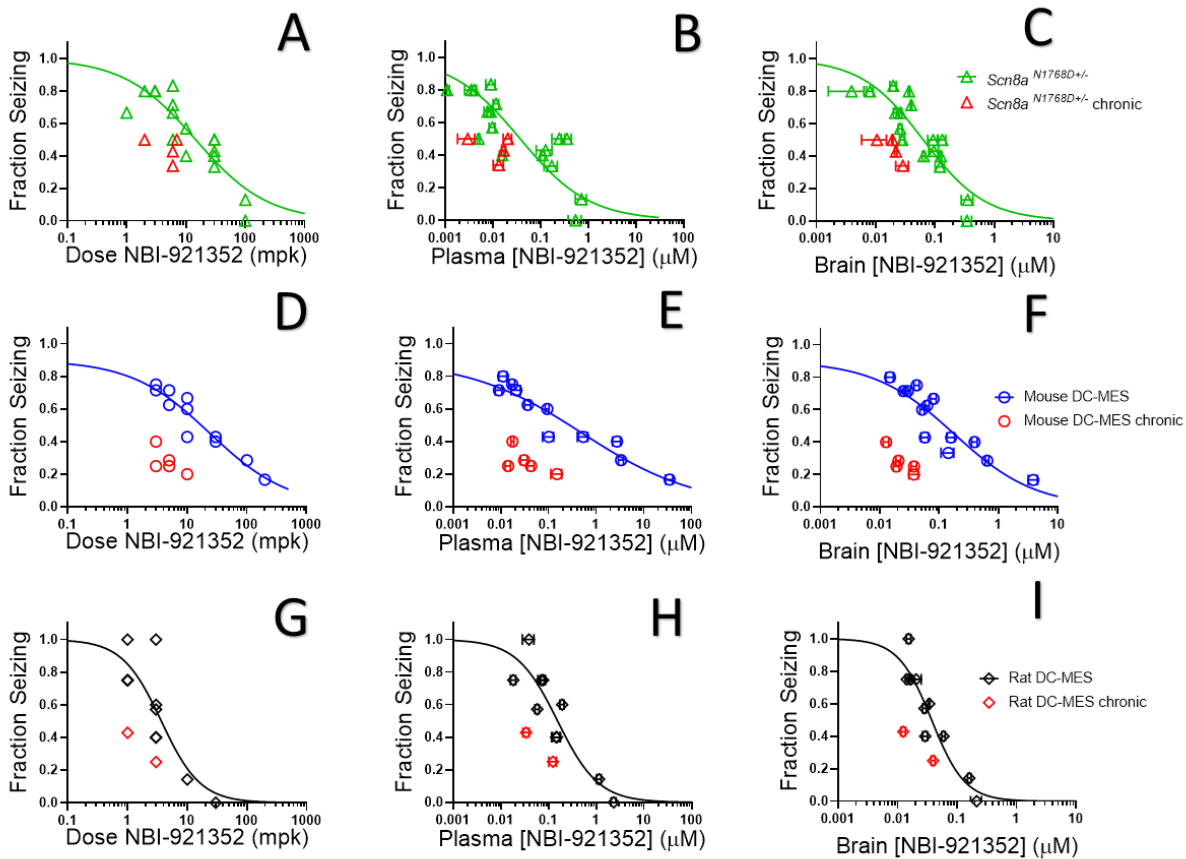
863 **Figure 4 NBI-921352 inhibits firing in pyramidal neurons but spares fast-spiking**
 864 **interneurons.**



866 Current input versus action-potential output evaluations in wild-type mouse brain slices treated with
867 vehicle or 0.25 μ M NBI-921352 (A & B), or 100 μ M carbamazepine (C & D) was plotted. In cortical
868 pyramidal neurons, both NBI-921352 (A) and carbamazepine (C) reduced AP spiking. In fast-spiking
869 cortical interneurons, NBI-921352 increased firing frequency slightly (B), while carbamazepine markedly
870 reduced firing (D). The main upper panels compare average AP count of 3-4 neurons in each condition
871 +/- the standard error of the mean. The inserts in the upper panels show the results for each tested
872 neuron at approximately 3X the cell rheobase. *Indicates a $p < 0.05$ relative to the control condition
873 using a paired, two-way, student's t-test. The lower panels show recordings for individual representative
874 neurons for each condition. No inhibitors of synaptic inputs were used for these experiments.

875

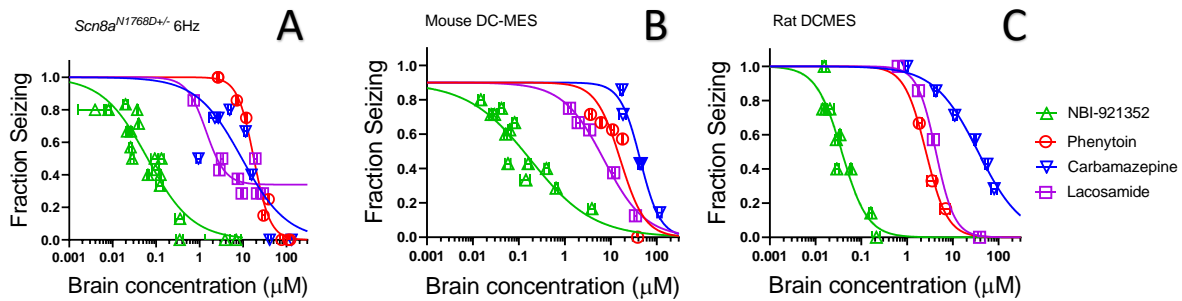
876 **Figure 5 NBI-921352 inhibited electrically induced seizures in rodents.**



877
878 Dose versus efficacy, plasma concentration versus efficacy, and brain concentration versus efficacy
879 relations are shown for *Scn8a*^{N1768D+/-} mice in the modified 6Hz psychomotor seizure assay in A, B, and C,
880 respectively (green open triangles). Dose versus efficacy, plasma concentration versus efficacy, and
881 brain concentration versus efficacy relations are shown for wild-type mice in the DC-MES assay in D, E,
882 and F, respectively (blue open circles). Dose versus efficacy, plasma concentration versus efficacy, and
883 brain concentration versus efficacy relations are shown for wild-type rats in the DC-MES assay in G, H,
884 and I, respectively (black open diamonds). Each point represents the fraction of animals exhibiting a GTC
885 with hindlimb extension after stimulus from a dosing group of 4-8 animals (See source data sheet for all
886 additional information). Horizontal error bars show the standard error of the mean plasma (B, E, H) or

887 brain (C, F, I) concentrations measured from the animals in that dosing group immediately after assay.
888 Where error bars are not visible, they are smaller than the symbols. No error bars are shown for the dose
889 levels (A, D, G), since those were dictated by the experimenter.
890

891 **Figure 6 NBI-921352 is more potent than 3 commonly prescribed Nav inhibitor ASMs.**



892

893 Brain concentration versus fraction of animals exhibiting is plotted for NBI-921352 versus that for

894 phenytoin, carbamazepine, and lacosamide in the *Scn8a*^{N1768D +/-} modified 6Hz model (A), the wild-type

895 mouse DC-MES model (B), and the wild-type rat DC-MES model (C). Data from animals at a given dose

896 were grouped together and plotted with GraphPad Prism 8. Details regarding the number of animals

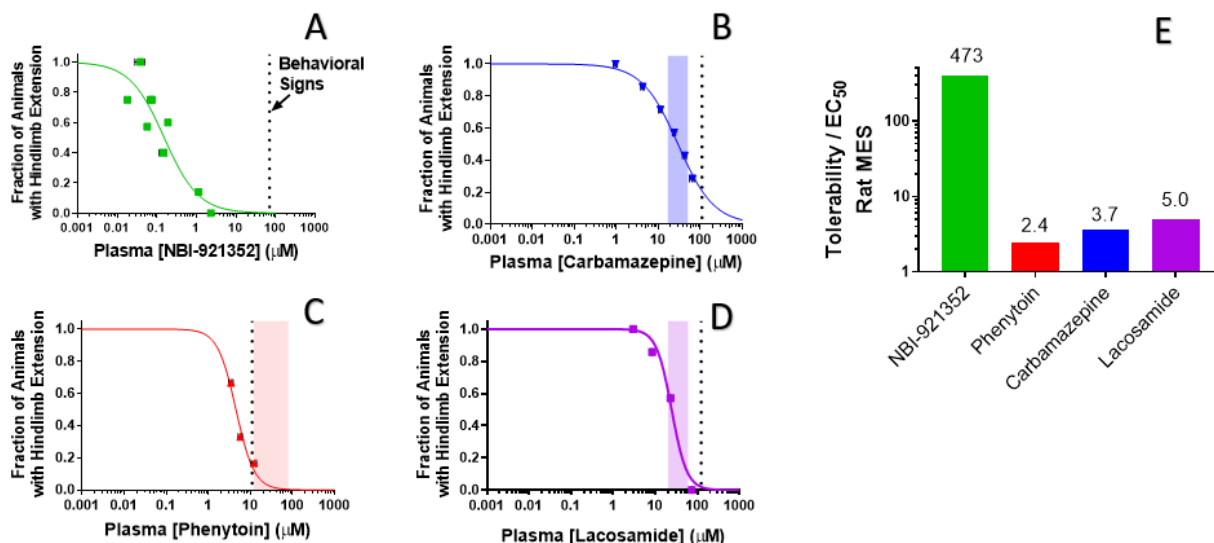
897 analyzed per model and per dose group can be found in the source data sheet. Error bars indicating the

898 standard error of the mean concentration were plotted for all points on the concentration-response

899 curves.

900

901 **Figure 7 Rat efficacy compared to acute tolerability for NBI-921352 relative to Nav_v inhibitor**
902 **ASMs.**



903
904 Plasma concentration versus efficacy data is shown for the rat DC-MES assay for NBI-921352 (A),
905 carbamazepine (B), phenytoin (C), and lacosamide (D). The vertical dotted lines indicate the lowest
906 plasma concentration at which a rat was observed to exhibit atypical behavioral signs indicative of an
907 adverse reaction to drug in the assay format. Animals exhibiting such signs were excluded from efficacy
908 evaluation. The shaded bars in B, C, and D indicate the approximate human plasma concentrations
909 observed in clinical practice. Data from animals at a given dose were grouped together and plotted with
910 GraphPad Prism 8. Details regarding the number of animals analyzed per model and per dose group can
911 be found in the source data sheet. Error bars indicating the standard error of the mean concentration
912 were plotted for all points on the concentration-response curves. Panel E shows the ratio of the (rat
913 plasma EC₅₀ / the plasma concentration where behavioral signs were noted for each compound).