Selective Na$_{1.1}$ activation rescues Dravet syndrome mice from seizures and premature death

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Dravet syndrome is a catastrophic, pharmacoresistant epileptic encephalopathy. Disease onset occurs in the first year of life, followed by developmental delay with cognitive and behavioral dysfunction and substantially elevated risk of premature death. The majority of affected individuals harbor a loss-of-function mutation in one allele of SCN1A, which encodes the voltage-gated sodium channel Na$_{1.1}$. Brain Na$_{1.1}$ is primarily localized to fast-spiking inhibitory interneurons; thus the mechanism of epileptogenesis in Dravet syndrome is hypothesized to be reduced inhibitory neurotransmission leading to brain hyperexcitability. We show that selective activation of Na$_{1.1}$ by venom peptide Hm1a restores the function of inhibitory interneurons from Dravet syndrome mice without affecting the firing of excitatory neurons. Intracerebroventricular infusion of Hm1a rescues Dravet syndrome mice from seizures and premature death. This precision medicine approach, which specifically targets the molecular deficit in Dravet syndrome, presents an opportunity for treatment of this intractable epilepsy.

Dravet syndrome is a catastrophic pediatric epilepsy characterized by childhood onset of intractable polymorphic seizures accompanied by cognitive impairment, psychomotor regression, autistic traits, and ataxia (1–4). Clinically it presents as one of the most pharmacoresistant epilepsy syndromes (5). Further, the incidence of sudden unexpected death in epilepsy is 30-fold higher in Dravet syndrome than in other childhood-onset epilepsies, accounting for up to 50–60% of mortality (4, 6). Therefore there is an urgent need to develop new therapeutic strategies to treat this devastating disease.

About 80% of Dravet syndrome cases result from de novo heterozygous loss-of-function mutations in SCN1A, which encodes the voltage-gated sodium channel Na$_{1.1}$ (7, 8). In the brain, Na$_{1.1}$ is expressed predominantly in the axon initial segment of fast-spiking parvalbumin-positive (PV+) GABAergic inhibitory interneurons (9). Selective reduction of Na$_{1.1}$ expression in PV+ interneurons is sufficient to recapitulate many of the clinical symptoms of Dravet syndrome (9–13). A potential clinical correlate of this mechanism is that sodium-channel blockers, which will normally work in an antiepileptic fashion, can make symptoms worse in some Dravet syndrome patients (10, 14). This might be explained by decreased inhibitory neuron firing, which in these patients has a devastating impact that cannot be compensated by sodium-channel inhibition on excitatory neurons (10, 14). In contrast, for Dravet syndrome patients, compounds that enhance GABAergic neurotransmission are more effective anticonvulsants (8, 15).

Precision therapies that rescue Na$_{1.1}$ haploinsufficiency should therefore be clinically effective and reduce seizures and attendant comorbidities. Hm1a is a spider venom peptide that selectively potentiates Na$_{1.1}$ channels (16). It does so by inhibiting gating movement of the domain IV (DIV) voltage sensor of Na$_{1.1}$, which hinders both fast and slow inactivation of the channel and leads to a persistent current at depolarized potentials (16, 17). Hm1a is therefore an obvious candidate for a precision therapy that targets WT channels in patients with Dravet syndrome.

The ability of Hm1a to rescue Na$_{1.1}$ haploinsufficiency was evaluated in a mouse model of Dravet syndrome in which the mouse carries one mutated and one unaffected allele (9). Hm1a selectivity was confirmed in a heterologous expression system with preferential action on hNa$_{1.1}$ channels over other Na$_{1.2}$ channels.

Significance

Spider venom is a rich source of peptides, many targeting ion channels. We assessed a venom peptide, Hm1a, as a potential targeted therapy for Dravet syndrome, the genetic epilepsy linked to a mutation in the gene encoding the sodium channel alpha subunit Na$_{1.1}$. Cell-based assays showed Hm1a was selective for Na$_{1.1}$ over other sodium and potassium channels. Utilizing a mouse model of Dravet syndrome, Hm1a restored inhibitory neuron function and significantly reduced seizures and mortality in heterozygote mice. Evidence from the structure of Hm1a and modeling suggest Hm1a interacts with Na$_{1.1}$ inactivation domains, providing a structural correlate of the functional mechanisms. This proof-of-concept study provides a promising strategy for future drug development in genetic epilepsy and other neurogenetic disorders.


Conflict of interest: G.F.K. and S.P. are listed as inventors on a patent application on Hm1a (WO/2017/096431).

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channels and was without significant action on voltage-gated potassium (Kv) channels. Hm1a rescued action potential (AP) firing in GABAergic interneurons from Dravet syndrome mice and spared excitatory neuron firing. Hm1a treatment in Dravet syndrome mice showed remarkable efficacy with a significant reduction in seizure counts and mortality. This study demonstrates that a precision-medicine strategy that targets fundamental disease mechanisms can achieve efficacy in an otherwise refractory disease.

**Results**

**Hm1a Delays Na\textsubscript{v}1.1 Inactivation.** Hm1a was previously shown to be highly selective for Na\textsubscript{v}1.1 over Na\textsubscript{v}1.2–Na\textsubscript{v}1.8 when the α subunits alone were expressed in Xenopus oocytes (16). However, since the pharmacology of venom-derived sodium-channel toxins can be influenced by auxiliary subunits (18) and post-translational channel modifications (19), we employed automated planar patch-clamp electrophysiology to reexamine Hm1a selectivity using mammalian cells expressing human (h) Na\textsubscript{v}1.1–Na\textsubscript{v}1.8 in the presence of the human sodium channel β1 subunit. At concentrations \(\geq 5\) nM Hm1a enhanced hNa\textsubscript{v}1.1 peak current amplitude and potently delayed channel inactivation in a dose-dependent manner, leading to a large sustained current (Fig. 1A). A similar action was seen on hNa\textsubscript{v}1.3 channels (Fig. 1B), although with lower efficacy (a measurement of the magnitude of change in total charge versus peptide concentration) and lower sensitivity (calculated as a change in response versus concentration, compared with hNa\textsubscript{v}1.1) (Fig. 1I and J). Application of up to 50 nM Hm1a had no effect on other hNa\textsubscript{V} subtypes (Fig. 1C–H and SI Appendix, Fig. S1).

We next examined the effect of Hm1a on biophysical properties of hNa\textsubscript{v}1.1. Current family traces illustrate potent inhibition of hNa\textsubscript{v}1.1 inactivation by 5 nM Hm1a (Fig. 1A). Hm1a had no effect on the voltage dependence of steady-state activation (Fig. 2B) and at 50 nM induced a significant depolarizing shift in the voltage dependence of inactivation (Fig. 2B). With increasing concentration of Hm1a, the slope factor of the steady-state inactivation curve increased (Fig. 2B). The effect of Hm1a on hNa\textsubscript{v}1.1 inactivation was quantified by fitting a double exponential curve. The time constant of fast inactivation was significantly increased at 50 nM (Fig. 2C). A significant slowing of inactivation was also seen at the lower concentration of 5 nM (Fig. 2D), resulting in the appearance of a persistent current across the entire duration of the voltage step (Fig. 2E). Hm1a did not have any impact on recovery from fast inactivation (Fig. 2F).

**Hm1a Does Not Modulate K\textsubscript{Ca} Channels.** Hm1a was previously shown to have no effect on Kv1.1–Kv1.6 and Kv3.4, but it was reported to be a weak inhibitor of Kv2.2, Kv4.1, and Kv4.3 (IC\textsubscript{50} \sim 300 nM) (20). The lack of data on calcium-activated potassium (K\textsubscript{Ca}) channels and biologically important Kv channels such as Kv11.1 (hERG) motivated the extension of the selectivity profile of Hm1a (20). At a concentration of 300 nM (i.e., 25-fold higher than the EC\textsubscript{50} on Na\textsubscript{v}1.1), Hm1a had no significant effect on Kv1.7, Kv10.1 (hEAG), Kv11.1 (hERG), K\textsubscript{Ca}1.1, K\textsubscript{Ca}2.2, or K\textsubscript{Ca}3.1 (SI Appendix, Figs. S2 and S3).

**Hm1a Is a Knottin Peptide with Moderate in Vivo Stability.** We produced recombinant Hm1a and determined its structure using 2D homonuclear NMR techniques. The final ensemble of NMR-derived structures is high resolution based on measures of precision (backbone rmsd of 0.18 ± 0.04 Å) and stereochemical quality (SI Appendix, Table S1) (21). The structure of Hm1a (Fig. 3A and SI Appendix, Fig. S4) reveals a classical knotton fold in which a central β-hairpin is stabilized by three disulfide bonds that form an inhibitor cystine knot (ICK) motif (Fig. 3B) (22). The Cys2–Cys16 and Cys9–Cys21 disulfide bonds and the intervening sections of the polypeptide backbone form a 14-residue ring that is pierced by the Cys15–Cys28 disulfide bond to form the cystine knot (Fig. 3C).

One face of the Hm1a structure is comprised of a set of contiguous hydrophobic residues (Tyr4, Leu5, Phe6, Tyr7, Trp30, and Phe34) (Fig. 3D, *Left*) that are conserved in the paralogous toxin Hm1b, which has similar pharmacology (16). This face of the peptide likely mediates the interaction between Hm1a and Na\textsubscript{v}1.1, since all the residues that differ between Hm1a and Hm1b form a spine on the opposite face of the molecule (Fig. 3D, *Right*). We previously showed that both the S1–S2 and S3–S4 extracellular loops of the domain IV voltage sensor of Na\textsubscript{v}1.1 are important for recognition of Hm1a (16), and hence we propose that Hm1a engages the channel by lodging into the water-filled cavity between the S1–S2 and S3–S4 helical bundles and acts as a wedge that impedes outward movement of
Stability of the peptide was analyzed in an isolated cerebrospinal fluid (CSF) preparation (Fig. 3F). The knotted architecture of ICK peptides typically provides them with very high levels of stability in serum and other biological fluids (23), but their stability in CSF is mostly unknown. We found that Hm1α is more stable than human atrial natriuretic peptide (hANP) in human CSF but is significantly less stable than ziconotide, a venom-derived ICK peptide drug that is administered intrathecally for treatment of intractable chronic pain (24).

Hm1α Rescues Interneuron Function from Dravet Syndrome Mice. Mice carrying an Scn1a variant identified in human Dravet syndrome (R1407X), bred into a mixed C57BL/6J/129S1/SvImJ background, developed spontaneous seizures and early mortality (9, 25). Whole-cell recordings from interneurons in the CA1 stratum radiatum of these Dravet syndrome mice were made to assess the effect of Hm1α on AP firing activity. Collapse of AP firing at current injections >300 pA was a robust cellular phenotype in Dravet syndrome mice interneurons (Fig. 4 A–D), confirming previous findings (9, 26). Remarkably, 10 nM Hm1α rescued the AP firing deficit with average AP count significantly greater than control for current injections >300 pA (Fig. 4 A and B).

Detailed analysis of nine sequential APs at a current step that elicited collapse was also made (Fig. 4 C–E). Interestingly, Hm1α caused a significant change in the amplitude of the first few APs, but increased the amplitude of subsequent APs (Fig. 4 C and D). The waveform of the first AP elicited at rheobase was unchanged in the presence of the peptide (Fig. 4E). Hm1α induced a small increase in rheobase current (baseline, 59 ± 10 vs. Hm1α, 81 ± 13 pA, P < 0.01, n = 10; paired t test) as well as a depolarizing shift in threshold voltage (44 ± 2 vs. 36 ± 4 mV, P < 0.02, n = 10; paired t test) and resting membrane potential (77 ± 1 vs. 73 ± 1 mV, P < 0.02, n = 10; paired t test), but had no impact on input resistance (550 ± 73 vs. 460 ± 60 MΩ; P = 0.11, n = 10; paired t test).

Hm1α Does Not Alter Firing of GABAergic Interneurons from WT Mice or Excitatory Neurons from Dravet Syndrome Mice. Hm1α had minimal impact on the firing properties of noncollapsing WT CA1 GABAergic interneurons (Fig. 5 A–C, n = 6). There were no significant differences in the amplitude, rise time, width, and threshold of APs at rheobase (Fig. 5C). Further, Hm1α had no effect on rheobase (111 ± 18 vs. 123 ± 18 pA, P = 0.5, n = 6; paired t test) and caused a nonsignificant right shift in the input current vs action potential output (i–o) relationship at intermediate current injections (Fig. 5B). Hm1α also had no impact on resting membrane potential (Fig. 5C) or input resistance (270 ± 40 vs. 330 ± 40 MΩ; P = 0.76, n = 6; paired t test).

Hm1α (10 nM) had no impact on the firing properties of CA1 pyramidal neurons recorded from Dravet syndrome mice (Fig. 5 D–F), consistent with reports that Na−v.1.1 expression in brain is primarily localized to GABAergic interneurons (9, 26). Hm1α caused a small depolarizing shift in resting membrane potential (Fig. 5F), but had no impact on input resistance (450 ± 70 vs. 420 ± 60 MΩ, P = 0.8, n = 6; paired t test), rheobase (98 ± 15 vs. 101 ± 16 pA, P = 0.8, n = 6; paired t test), or the amplitude, rise time, width, and threshold of APs measured at rheobase (Fig. 5F).

Acute Hm1α Infusion Reduces Whole-Brain Hyperexcitability in Dravet Syndrome Mice. To overcome poor CNS availability of systemically delivered peptides, we used intracerebroventricular (ICV) delivery for in vivo tests of Hm1α efficacy. After postnatal day (P)19, electrocorticography (ECoG) recordings from Dravet syndrome mice revealed high-amplitude interictal epileptiform discharges or “spikes” (Fig. 6A and SI Appendix, Fig. S5), which were used as a biomarker for assessing Hm1α efficacy.

the DIV voltage sensor during membrane depolarization. Hm1α most likely engages the channel with its hydrophobic face pointing into the DIV cavity, making contacts with both the S1–S2 and S3–S4 helices, while the poorly conserved and largely polar opposing face points toward the extracellular space (Fig. 3E).
Spike analysis was conducted before, during, and after acute ICV infusion of 5 μM Hm1α, dissolved in saline and fatty-acid free BSA (fBSA) vehicle, delivered over a period of 1 h. In contrast to the significant spike activity observed before Hm1α infusion, very few high-amplitude spike events were detected in ECoG recordings 1 h after Hm1α administration (Fig. 6). Power spectrum analysis for a 30-min epoch showed peak activity at 0.5–2 Hz before Hm1α delivery, and this was significantly reduced by Hm1α infusion (Fig. 6B and SI Appendix, Table S2). Average spike frequency in Dravet syndrome mice was significantly reduced by Hm1α infusion (Fig. 6C). There was no difference in spike frequency in Dravet syndrome mice measured before and after vehicle infusion (27.5 spikes/h [95% CI 0.07–54.9] preinfusion versus 24.5 spikes/h [95% CI 0.910–49.9] postinfusion; P = 0.19; n = 4).

We observed a spike-free period of ~1 h following cessation of Hm1α infusion (SI Appendix, Fig. S6), which likely correlates with a gradual clearance and/or metabolism of peptide within the CSF. The in vitro half-life for Hm1α in CSF (~1.7 h) (Fig. 3F) closely mirrored the spike-free period following cessation of Hm1α infusion.

**Long-Term Hm1α Infusion Reduces Seizures and Mortality in Dravet Syndrome Mice.** Previous studies have shown that survival of Dravet syndrome mice is reduced from P17 (9). Our cohort of Dravet syndrome mice out-crossed with C57BL/6J (N2, second backcross generation) also had dramatically decreased survival compared with WT littermates (Fig. 7A). The mortality of heterozygous Dravet syndrome mice was highest between P18 and P26. Video and visual observation showed that mortality was always correlated with a preceding spontaneous seizure event. We used this high seizure and mortality epoch to assess the impact of long-term Hm1α infusion. Video monitoring was performed from P18 for the duration of the experiment. Mice that displayed a minimum of two seizures qualified as subjects for long-term efficacy trials and were randomly assigned to either the Hm1α-treated or the vehicle-treated groups. At initiation of the trial, average seizure frequencies were identical for the treatment and control groups (Fig. 7B).

During continuous Hm1α infusion, seizure frequency was significantly reduced or completely abolished in six of nine mice (67%) after 3 d. In contrast, seizure frequency increased dramatically in the control group (Fig. 7B). In the first 24-h period after control infusion was initiated, 62% of mice had died (Fig. 7C), and by day 3 all control-treated mice were dead. Hm1α infusion caused no obvious adverse events; Hm1α-treated mice displayed normal home cage activity, including eating, drinking, and grooming behavior [see Movies S1 and S2 for examples of...
four Dravet syndrome mice before (Movie S1) and during (Movie S2) Hm1a infusion.

Discussion

The aim of this study was to assess the utility of the venom peptide Hm1a as a targeted treatment for Dravet syndrome caused by loss of Na\textsubscript{v}1.1 function. We demonstrated that Hm1a enhances Na\textsubscript{v}1.1 currents, rescues an interneuron AP firing deficit, and reduces seizure susceptibility and mortality in a mouse model of Dravet syndrome. Our results provide strong proof of concept that therapy based on directly targeting a molecular deficit in intractable epilepsy is feasible, providing a promising strategy for future drug development in genetic epilepsy and other neurogenetic disorders.

The primary effect of Hm1a on hNa\textsubscript{v}1.1 was to delay fast inactivation, which led to a substantial persistent current. Hm1a had a similar impact on hNa\textsubscript{v}1.3 channels, although with lower efficacy and sensitivity, and had no effect on other Na\textsubscript{v} channels. Our results differ subtly from those previously reported, in which Hm1a had little effect on Na\textsubscript{v}1.3 but altered peak current and inactivation in Na\textsubscript{v}1.1 channels (16). These earlier data were obtained using Xenopus oocytes without coexpression of auxiliary \(\beta1\) subunits, while data from this study were obtained using mammalian cells expressing this subunit.

The lack of efficacy at Na\textsubscript{v}1.3 demonstrated here is an important point, as drugs that potentiate Na\textsubscript{v}1.2 would likely be proepileptic and certainly would be contraindicated in Dravet syndrome. The weak activity of Hm1a at Na\textsubscript{v}1.3 is likely to have minimal effect on neuronal function. In both rodent and human brain, there is a continuous postnatal increase in Na\textsubscript{v}1.1 levels, whereas Na\textsubscript{v}1.3 expression is highest just before birth, with a rapid postnatal decline (27, 28), with no compensatory up-regulation of expression occurring in the Dravet syndrome mice (9). Essentially, the mean age at seizure onset in Dravet syndrome patients (5.7 mo) closely approximates the point at which the increasing level of Na\textsubscript{v}1.1 exceeds the diminishing level of Na\textsubscript{v}1.3 in normal human brain development (27).

Recently identified is the role that Na\textsubscript{v}1.1 also plays in transcription of mechanical pain via primary sensory afferents in the peripheral nervous system (16, 17). Na\textsubscript{v}1.1 activators therefore may enhance the sensation of pain if given systemically. It is important to note that Dravet patients are haploinsufficient for Na\textsubscript{v}1.1 and therefore may be less sensitive to this effect. Furthermore, Hm1a delivered directly within the CNS is unlikely to result in elevated pain sensitivity, and this is consistent with mice showing no overt adverse effects to ICV infusion of Hm1a.

Fig. 4. Hm1a reverses AP firing collapse in Dravet syndrome mice CA1 GABAergic neurons. (A, Upper) Raw traces recorded from single CA1 GABAergic neuron at progressively more depolarizing current injections. (A, Lower) Raw traces from the same neuron following the addition of Hm1a (10 nM). (Scale bars: 200 ms horizontal; 20 mV vertical.) (B) Summary of \(i-o\) data. Control (black) and Hm1a-treated (pink) show the recovery of AP collapse in the presence of the peptide (AP counts at \(>300 \text{pA}; P < 0.02, n = 10\), paired t test). (C) Raw traces (control, black trace; Hm1a, pink trace) of the first and ninth individual APs from a train of APs at the point of collapse. (Scale bars: 5 ms horizontal; 20 mV vertical.) (D) Summary of peak AP amplitude (control, black; Hm1a, pink) of sequential APs in a train. The amplitude of the fourth to ninth APs is significantly larger in Hm1a-treated cells than untreated, control cells. \(P < 0.04\), paired t test. (E) Morphology of the first AP at rheobase (R\textsubscript{b}) and the first AP (C\textsuperscript{in}) and ninth AP (C\textsuperscript{out}) at a current injection causing collapse. Control, black bars; Hm1a, pink bars; ***P < 0.005, paired t test.

Fig. 5. Hm1a has no impact on WT GABAergic or pyramidal CA1 neurons of Dravet mice. (A, Upper) Raw traces (black) recorded from single WT CA1 GABAergic neuron at progressively more depolarizing current injections. (Lower) Raw traces (pink) from the same neuron following the addition of Hm1a (10 nM). (Scale bars: 200 ms; 20 mV vertical.) (B) Summary of \(i-o\) data. Control (black) and Hm1a (pink) data (n = 6) indicate a small right shift in the relationship at intermediate current injections that normalized at higher currents in the presence of the Hm1a. (C) No significant change in AP amplitude, rise, width, or threshold was detected at rheobase or resting membrane potential in WT GABAergic neurons shown without (black bars) and with (pink bars) Hm1a. (D, Upper) Raw traces (black) recorded from a single mutant CA1 pyramidal neuron at progressively more depolarizing current injections. (Lower) Raw traces (pink) from the same neuron following the addition of Hm1a (10 nM). (Scale bars: 200 ms horizontal; 20 mV vertical.) (E) Summary of \(i-o\) data. Control (black) and Hm1a (pink) data (n = 6). (F) No significant change in AP amplitude, rise, width, or threshold was detected at rheobase with only a small depolarizing shift in resting membrane potential (\(P < 0.04\), n = 6, paired t test) in mutant CA1 pyramidal neurons shown without (black bars) and with (pink bars) Hm1a.
Interneurons are key regulators of cortical excitability, and their dysfunction is believed to underlie pathology in Dravet syndrome (9, 26). Hm1a was able to rescue AP collapse in fast-spiking PV⁺ inhibitory interneurons from Dravet syndrome mice, ostensibly through its ability to enhance Nav1.1 function. The peptide had no effect on the firing properties of excitatory pyramidal neurons in Dravet syndrome mice. This strongly implies that Hm1a specifically targets the emerging cellular deficit in Dravet syndrome mice. This may also explain the remarkable efficacy of Hm1a in reducing both seizure susceptibility and mortality without apparent adverse effects.

Clinical development of peptide CNS therapeutics in general is difficult. One of the major challenges is that peptides such as Hm1a are not blood–brain barrier permeant; delivery therefore needs to occur via injection into the intrathecal or intracerebroventricular spaces. A number of parameters, including the dynamics of fluid flow from the CSF to the interstitial fluid and half-life, will be critical in determining the final distribution of a peptide within the brain. Hm1a will likely need to be delivered using microinfusion pumps to overcome brain-accessibility problems. Microinfusion of peptides has already been used clinically for intrathecal delivery of ziconotide, a synthetic equivalent of a naturally occurring conopeptide, which is used as an effective, Food and Drug Administration-approved treatment for severe chronic pain (29).

In humans, neonatal/infantile seizure onset in Dravet syndrome often marks disease progression and subsequent comorbidities, including cognitive and behavioral deficits (30). Additional studies will determine if Hm1a therapy can prevent such comorbid states. Furthermore, the molecular and cellular selectivity of Hm1a suggest that it might have a broader application beyond Dravet syndrome. Other SCN1A-associated epilepsies (31), including intractable temporal lobe epilepsy for which partial loss of PV⁺ interneurons appears to be a pathological hallmark (32). Nav1.1 agonists such as Hm1a might also be useful for the treatment of schizophrenia, in which there appears to be both loss and reduced connectivity of PV⁺ interneurons (33), and Alzheimer’s disease in which reduced levels of Nav1.1 in PV⁺ interneurons have been linked to network hyperactivity and cognitive dysfunction (34).

Our data indicate that selective activation of Nav1.1 restores normal network excitability in a genetic model of Dravet syndrome. This precision medicine approach has the potential both to control seizures and to ameliorate Dravet syndrome comorbidities, and it should be applicable to other genetic epilepsies.

Materials and Methods

Tissue Cell Culture and Stable Transfection. HEK293T cells stably transfected with hSCN1A, hSCN2A, hSCN3A, hSCN5A, hSCN9A, and hSCN10A were maintained in DMEM Nutrient Mixture F-12 (Invitrogen) supplemented with 10% (vol/vol) FBS (Invitrogen), 0.9% penicillin/streptomycin (P/S) solution, and 100 μg/mL hygromycin or 100 μg/mL neomycin. CHO cells stably transfected with hSCN4A and hSCN8A were maintained in Ham’s F-12 Nutrient Mixture (Invitrogen) supplemented with 10% (vol/vol) FBS, 0.9% P/S solution, and 100 μg/mL hygromycin. Cells were grown to ∼70% confluency in T25 flasks (BD Biosciences).

Planar Patch-Clamp Electrophysiology. Patch-clamp recordings were made using a Patchliner (Nanion Technologies) in the whole-cell configuration. Before recordings, cells were detached from culture flasks with Accutase Cell Detachment Solution (Innovative Cell Technologies Inc.) and resuspended at a density of 1 × 10⁶ cells/mL in 50% serum-free medium and 50% external recording solution (vol/vol). The external recording solution comprised (in mM) 140 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 5 ω-glucose, 10 Hepes (pH 7.4) with NaOH, 298 mOsm. The internal recording solution comprised (in mM) 50 CsCl, 60 CsF, 10 NaCl, 20 EGTA, 10 Hepes (pH 7.2) with CsOH, 285 mOsm. High-fidelity seals were established through brief exposure to a high-calium-containing solution (in mM) 80 NaCl, 35 CaCl₂, 10 MgCl₂, 3 KCl, 10 Hepes (pH 7.4) with HCl, 298 mOsm. Solutions were filtered using a 0.2-μm membrane filter (Minisart; Sartorius Stedim Biotech). Cells were kept in suspension by automatic pipetting. Hm1a was dissolved in H₂O with 0.1% fatty acid-free BSA (fafsBSA) (Sigma-Aldrich). Medium single-hole planar NPC-16 chips with an average resistance of ∼2.5 MΩ were used. Chip and whole-cell capacitance were fully compensated, and 500 series resistance compensation was applied. Recordings were acquired at 50 kHz with the low-pass filter set to 10 kHz in PATCHMASTER (HEKA Instruments) and performed at 27 °C. When data needed further filtering for the analysis of sustained current, recordings were additionally filtered...
at 500 Hz offline using a Gaussian filter algorithm. Offline analysis was performed using Microsoft Excel, MATLAB R2015a (MathWorks), Igor Pro 6.37 (WaveMetrics Inc.), and GraphPad Prism 7 (Molecular Devices). Biophysical analysis was performed using tailored MATLAB scripts. Currents were normalized after leak subtraction. The area under the curve (AUC) was used to measure the efficacy and sensitivity of Hm1a for Na\textsubscript{1.1} and Na\textsubscript{1.3}. Efficacy was calculated as a mean percentage change (%) using the area of the negative peaks under the raw current traces with the baseline set at zero. Efficacy describes the magnitude of the change in total charge, a combination of delayed rectification and inactivation and increased current amplitude. Sensitivity was calculated from the mean normalized AUC as a change in response versus concentration, essentially producing a concentration-response curve.

**Pulse Protocols.** Electrophysiological protocols were as previously described (35). Briefly, the voltage dependence of activation was studied by measuring normalized peak currents during 100-ms depolarizations from −120 mV to +30 mV in 5-mV increments. The resulting current-voltage curve was fit to the equation \( I = (1 + \exp(-x(V - V_{1/2})))(g(V - V_i)) \), where \( I \) is the current amplitude, \( x \) is the apparent gating charge, \( V \) is the test potential, \( V_{1/2} \) is the half-maximal voltage, \( g \) is a factor related to the maximum number of open channels, and \( V_i \) is the reversal potential. Conductance \( (G) \) was determined using \( G = \exp((-V - V_{1/2}))(I(V - V_i)) \). Calculated conductance values were then fit with the Boltzmann equation \( G = 1/(1 + \exp((V - V_{1/2}))(I(V - V_i))) \), where \( a \) is the slope of the half-maximum, \( V \) is the potential of the given pulse, and \( V_{1/2} \) is the potential for the half-maximal activation. To study steady-state fast inactivation, cells were held at conditioning prepulse potentials ranging from −120 mV to +30 mV in 5-mV increments from a holding potential of −120 mV and a test pulse at 5 mV for 20 ms. Peak currents \( (I) \) during the subsequent test pulses were normalized to the peak current during the first test pulse \( (I_{max}) \) and were plotted against the potential of the conditioning pulse and fitted with the Boltzmann equation \( y = 1/(1 + \exp(-(V - V_{1/2}))(I(V - V_i))) \). Inactivation \( I_{max} \) was fit using a double exponential function \( I_{max} = I_{0} + A1 \exp(-(t-t_{0})(1)) + A2 \exp(-(t-t_{0})(2)) \), where \( I_0 \) is the non-inactivating component, \( I_{max} \) is the peak current, \( t \) is time, and \( A1 \) and \( A2 \) are the components for the time constants \( t_{1} \) and \( t_{2} \), respectively. Time constants were plotted against voltage, and the data were fitted with a Boltzmann equation \( V = V_{1/2} + (V_{max} - V_{1/2}) \exp(-(t-t_{0})(1)) \), where \( V_{max} \) is the maximum current, \( t_{0} \) is the starting point of the curve, \( K \) is the decay factor, \( plateau \) is the value to which the curve decays, and \( x \) is time. To examine the effects of Hm1a on hNa\textsubscript{1.1} from 0 to 39 ms in 3-ms increments. To test channel availability, the voltage was stepped to 0 mV for 30 ms. Peak current \( (I) \) was plotted as fractional recovery against the recovery period by normalizing to the maximum current \( (I_{max}) \) during the conditioning potentials. Recovery currents were plotted against voltage, and data were fitted with the equation \( I_{max} = 1 - \exp(-\alpha x + \beta) \), where \( I_{max} \) is maximum current, \( \alpha \) is the recovery rate constant, and \( \beta \) is time. Sensitivity to Hm1a was assessed during the critical epileptogenic period. (A) Survival curves for standard-housed heterozygous Dravet syndrome mice (black trace; \( n = 45 \)) compared with WT littermates (green trace; \( n = 20 \)) revealed significantly increased mortality in Dravet syndrome mice \( (P < 0.0001; \text{Mantel-Cox test}) \). The highest mortality of Dravet syndrome mice occurred between P18 and P26; thus long-term Hm1a efficacy was assessed during the critical epileptogenic period. (B) Mean seizure count for Dravet syndrome mice randomly assigned to either vehicle treatment (control, black circles) \( (3.63 \text{ CI } 95\% \ 1.90-5.35; \ n = 8) \) or Hm1a treatment (pink circles) \( (3.50 \text{ CI } 95\% \ 2.06-4.94; \ n = 10) \) was significantly different before ICV infusion of Hm1a (day 0). At day 1 post-infusion, the mean seizure count for vehicle-treated control mice \( (12.00 \text{ CI } 95\% \ 3.82-20.18) \) was significantly higher than for Hm1a-treated mice \( (3.70 \text{ CI } 95\% \ 0.005-7.40; \ n = 0.003) \). The majority of Dravet syndrome mice were seizure free after 3 d of continuous Hm1a infusion. (C) Survival curves for Dravet syndrome mice treated with vehicle (black trace) or Hm1a (pink trace). Ninety percent of Hm1a-treated mice were alive at day 3, whereas no vehicle-treated mice survived to day 3. Data are mean ± SEM.

**Hm1a Structure.** Hm1a \( (300 \mu \text{L} \text{ of a 1- mM solution in } 95\% \text{ H}_2\text{O}/5\% \text{ D}_2\text{O, pH } 4) \) was placed in a susceptibility-matched Shigemi microcell, and NMR spectra were acquired at 900 MHz on a Bruker AVANCE spectrometer.
equipped with a cryogenic probe. 2D 1H–1H total correlation spectroscopy (TOCSY) (mixing time (τ) = 80 ms), 1H–1H NOESY (τ = 250 ms), 1H–13C heteronuclear multiple quantum coherence (HMQC) and 1H–13C heteronuclear single quantum coherence (HSQC) experiments were acquired with WATERGATE water suppression used in homonuclear NMR experiments. Sequence-specific resonance assignments were made using CCPNMR (36); chemical shifts have been deposited in the Biological Magnetic Resonance Data Bank (accession no. 25770). Backbone dihedral-angle restraints for structure calculations were obtained from TALOS+ chemical shift analysis (37). NOESY cross-peaks were manually picked and integrated; then CYANA 3.0 (38) was used to automatically assign NOESY peaks and calculate a structural ensemble. Using a tolerance of 0.02 ppm and 0.025 ppm in the direct and indirect dimensions, respectively, CYANA assigned 94.3% of 1,314 NOESY cross-peaks. CYANA was used to calculate 300 structures from random starting conformations; then the 30 best-ranked structures were evaluated by MolProbity (39) to generate an ensemble consisting of the 20 conformers with highest stereochemical quality. Atomic coordinates are available from the Protein Data Bank (ID code 2N6O).

Hm1a Stability. CSF was obtained from patients undergoing lumbar puncture at St. Vincent’s Hospital, Sydney (Ethics Approval SVH15/024) and informed consent was obtained from all participants. Hm1a was added to human CSF (final concentration 0.5 μM); then the sample was incubated at 37 °C for 30 min (Human Research Ethics Approval UO 20160085). Samples were collected at various time points; then the reaction was quenched and proteins were precipitated by the addition of 5% tri-fluoroacetic acid. Samples were centrifuged at 25,000 × g for 10 min at 4 °C; then 20 μL of the supernatant was transferred to a new tube containing 5% formic acid (FA), and the sample was centrifuged again at 25,000 × g for 10 min. The supernatant (10 μL) was then processed a TripleTOF 6500 mass spectrometer (AB SCIEX) with a cycle time of 0.2751 s. Peak areas were measured for triple-, quadruple-, and quintuple-charge states and were analyzed using PeakView and MultiQuant (Sciex) software. hNAP (1 μM; GenScript) and ziconotide (ω-conotoxin MVIIA, 1 μM; Alomone Labs) served as controls.

Mice. Mice were housed in a temperature-controlled environment maintained at 23 °C with a 12 h light/dark cycle. Scn1a (R1407X) Dravet syndrome mice were maintained on the 129S1/SvImJ background and were crossed to the C57BL/6j (N2) background for experiments. At P7, mice were genotyped using the PCR protocol described previously (9). Mice for electrophysiology experiments were used at P14–P16. Mice aged P18–P26 were used for in vivo animal experiments.

Brain Slice Electrophysiology. Heterozygote Dravet syndrome mice and WT littermates (P14–P16) were anesthetized using isoflurane and were killed by decapitation. Brain slices (300 μm thick) were cut using a vibratome in the sagittal plane. Slices were kept at room temperature until recording. The slices were transferred to a recording chamber constantly perfused at 34 °C and equilibrated to a solution consisting of (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 1 MgCl2, 2 CaCl2, and 10 glucose, aerated with 95% O2 and 5% CO2, to a final pH of 7.4. Hm1a (10 nM) was dissolved in 25% ethanol and 75% human CSF (final concentration 0.5 M); then the sample was incubated at 37 °C for 30 min (Human Research Ethics Approval UO 20160085). Samples were collected at various time points; then the reaction was quenched and proteins were precipitated by the addition of 5% tri-fluoroacetic acid. Samples were centrifuged at 25,000 × g for 10 min at 4 °C; then 20 μL of the supernatant was transferred to a new tube containing 5% formic acid (FA), and the sample was centrifuged again at 25,000 × g for 10 min. The supernatant (10 μL) was then processed a TripleTOF 6500 mass spectrometer (AB SCIEX) with a cycle time of 0.2751 s. Peak areas were measured for triple-, quadruple-, and quintuple-charge states and were analyzed using PeakView and MultiQuant (Sciex) software. hNAP (1 μM; GenScript) and ziconotide (ω-conotoxin MVIIA, 1 μM; Alomone Labs) served as controls.

To determine the ability of Hm1a to modulate seizures in the long term, animals were video-monitored for seizure events at least 24 h after ICV guide cannula surgery. Animals with at least two seizure events were assigned to either the Hm1a-treated or vehicle-treated control group; solutions [either vehicle (saline/hafBSA) or Hm1a (0.5 μM in vehicle)] were delivered continuously for up to 5 d via ICV with a flow rate 0.2 μL/min using a Hamilton syringe and drive. Seizure frequency and survival were measured using continuous video recordings of ICV-tethered animals from P19. Video recording was done using a Vivotek video server (VS8102) connected to an infrared day and night digital color camera (EV02; Pacific Communications). Two independent observers conducted long-term infusion experiments and analyzed digital video images offline. During experiments mice were housed individually with a 12 h light/dark cycle and ad libitum access to water and food.

Statistical Analyses. All statistical analyses were performed using GraphPad Prism 7 (Molecular Devices) software, with a P value <0.05 considered statistically significant. Data values are expressed as mean ± SEM. Comparisons between datasets were made using a paired or unpaired Student’s t test; survival curves were created using the Kaplan–Meier method and evaluated using a Mantel–Cox test. One-way ANOVA with Bonferroni correction was applied to take into account multiple comparisons in Figs. 1 and 2.

Study Approval. All animal experiments were approved by the Animal Ethics Committee, Florey Institute of Neuroscience and Mental Health (approval nos. 13-097 and 14-046) and were conducted in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

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