Reduced dendritic arborization and hyperexcitability of pyramidal neurons in a Scn1b-based model of Dravet syndrome

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Epileptic encephalopathies, including Dravet syndrome, are severe treatment-resistant epilepsies with developmental regression. We examined a mouse model based on a human β1 sodium channel subunit (Scn1b) mutation. Homozygous mutant mice shared phenotypic features and pharmaco-sensitivity with Dravet syndrome. Patch-clamp analysis showed that mutant subicular and layer 2/3 pyramidal neurons had increased action potential firing rates, presumably as a consequence of their increased input resistance. These changes were not seen in L5 or CA1 pyramidal neurons. This raised the concept of a regional seizure mechanism that was supported by data showing increased spontaneous synaptic activity in the subiculum but not CA1. Importantly, no changes in firing or synaptic properties of gamma-aminobutyric acidergic interneurons from mutant mice were observed, which is in contrast with Scn1a-based models of Dravet syndrome. Morphological analysis of subicular pyramidal neurons revealed reduced dendritic arborization. The antiepileptic drug retigabine, a K⁺ channel opener that reduces input resistance, dampened action potential firing and protected mutant mice from thermal seizures. These results suggest a novel mechanism of disease genesis in genetic epilepsy and demonstrate an effective mechanism-based treatment of the disease.

Keywords: epileptic encephalopathy; Dravet syndrome; sodium channel; β1 subunit; action potentials

Abbreviations: GABA = gamma-aminobutyric acid; PSC = postsynaptic current
Introduction

Epileptic encephalopathies encompass a broad range of syndromes that occur in infancy and childhood and are characterized by frequent, severe epileptic seizures, prominent interictal epileptiform discharges and progressive developmental deterioration (Berg et al., 2010). Although mutations in several genes are associated with epileptic encephalopathies (Mastrangelo and Leuzzi, 2012) the paradigmatic form of genetic epileptic encephalopathy is Dravet syndrome, which is associated with mutations in SCN1A in >70% of patients (Mullen and Scheffer, 2009; Reid et al., 2009). Dravet syndrome usually begins with prolonged seizures with fever, followed by multiple seizure types, gait abnormalities and developmental regression (Dravet et al., 2005).

Genetic mouse models based on Scn1a loss-of-function mutations develop spontaneous seizures, ataxia and early death, recapitulating the severe phenotype in humans (Yu et al., 2006; Ogiwara et al., 2007). Based on the analysis of these mouse models, a loss of sodium channel function in gamma-aminobutyric acidergic (GABAergic) neurons is thought to underlie hyperexcitability in Dravet syndrome (Yu et al., 2006; Ogiwara et al., 2007; Martin et al., 2010; Cheah et al., 2012; Dutton et al., 2012). Interestingly, although heterozygous mutations in SCN1B cause the much milder epilepsy syndrome known as genetic epilepsy with febrile seizures plus (Wallace et al., 1998), homozygous mutations can cause classical Dravet syndrome (Ogiwara et al., 2012) and an even more severe early-onset epileptic encephalopathy (Patino et al., 2009), suggesting a shared pathology with SCN1A-based disease.

Here we demonstrate that mice homozygous for the SCN1B(C121W) familial epilepsy mutation (Wallace et al., 1998) display many of the clinical and pharmaco-sensitivity features of human Dravet syndrome. In contrast with Scn1a mouse models, we identify a pyramidal neuron deficit as the likely cellular basis of hyperexcitability. Specifically, we identify a reduction in apical dendrite arborization and reduced soma area of subicular pyramids. Using an antiepileptic drug that targets the observed functional deficit, we were able to reverse the increase in neuronal excitability as well as reducing seizure susceptibility.

Materials and methods

Mice

Construction of the mouse model

SCN1B(C121W) heterozygous mice were generated by Ozgene Pty Ltd, as described previously (Wimmer et al., 2010). Briefly, homologous recombination of a targeting vector made with C57BL/6J-derived genomic DNA was achieved in C57BL/6 Thy1.1 embryonic stem cells. The C121W mutation was introduced by PCR together with an EcoRV restriction site and a PGKNeo selection cassette. Targeted clones were identified by PCR screening and confirmed with Southern blotting. For generation of the CW knock-in strain we selected a founder animal with Cre-mediated PGKvNeo cassette deletion and intact exon 3 (C121W).

Ethics and breeding

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Mice were routinely genotyped by using PCR of tail DNA at postnatal Day 7. Experiments were performed using both male and female mice of >N6 C57BL/6 generations.

Morphology

Tissue preparation and staining for gross morphology

Gross morphology of the brains of mice aged between postnatal Days 14–16 were assessed using Nissl stained sections. Mice were lightly anaesthetized using isoflurane inhalation, followed by deep anaesthesia with Nembutal™ (80 µ100 g body weight intraperitoneally). Animals were transcardially perfused with 0.1 M phosphate buffer (pH 7.4) followed by fixative (4% formaldehyde in 0.1M phosphate buffer). Brains were removed from the skull and post-fixed overnight at room temperature. Tissue was washed in 0.1 M phosphate buffer three times and prepared for frozen sectioning by incubating in sucrose solutions (20% then 30%) in 0.1 M phosphate buffer at 4°C. The brain was then embedded in a mould containing Tissue Tek® O.C.T. compound and rapidly frozen by immersion in iso-pentane in a liquid nitrogen bath to prevent freezing artefacts. Frozen coronal sections (30µm) were cut on a cryostat (Leica, CM3000), collected on 1% gelatine-coated slides and stored at −20°C until stained. A minimum of three animals per genotype were analysed. Representative Nissl stained sections for each genotype were compared including the cortex, hippocampus, thalamus and cerebellum. Briefly described, sections were air-dried, dehydrated and defatted, then rehydrated and stained with 0.1% Cresyl violet in 0.1 M acetate buffer and differentiated in 70% ethanol. Sections were then dehydrated through a graded alcohol series and histolene, mounted with DPX and cover-slipped. Nissl-stained sections were viewed using a Nikon i80 brightfield microscope and images were captured using a ×4 objective and MacroFire® camera (Microbrightfield Systems, Inc.). Images were exported into Adobe Photoshop CS (Adobe Systems) for processing.

Single neuron morphology

For neuron analysis, slices used during electrophysiological recordings were post-fixed in 4% paraformaldehyde overnight. Endogenous peroxides in the slices were then suppressed by incubating in hydrogen peroxide, membranes permeabilized in 1% Triton™ X-100 before incubation with the Vectastain® Elite ABC Kit PK-6100 (Vector Laboratories). Neuron tracings were performed using a ×60 oil-immersion objective (Olympus), using the NeuroLucida and NeuroLucida Explorer software package (MBF Biosciences). Dendrite length and volume analysis, along with the Sholl analysis, was done with the in-built analysis suite available in NeuroLucida Explorer 10.

Labelling of biocytin-filled neurons

Slices were post-fixed in 4% paraformaldehyde overnight as above, and stored at 4°C in phosphate buffer (pH 7.4) until ready for processing. Slices were then incubated in blocking solution for 30 min and then incubated with rabbit anti-parvalbumin (1:5000; Swant) overnight at 4°C. After primary incubation, slices were washed in phosphate buffer several times and then incubated in secondaries, donkey anti-rabbit Alexa Fluor® 488 and streptavidin Alexa Fluor® 594 diluted in blocking solution (1:200) for 3 h at room temperature. Slices were then washed in phosphate buffer several times and mounted onto glass slides using ProLong® Gold (Invitrogen) medium and cover-slipped.
**Behavioural testing**

**Electrocorticograms**

Mice were anaesthetized with 1–3% isoflurane and four silver electrodes implanted in each quadrant of the skull with a ground electrode placed just posterior to the olfactory bulb as previously described (Tan et al., 2008). Signals were low pass filtered at 200 Hz and high pass filtered at 0.1 Hz and sampled at 1 kHz using Powerlab 16/30 data acquisition system (ADInstruments).

**Seizure testing**

To determine spontaneous seizure frequency mice were placed in a glass chamber and video recorded at room temperature. Each mouse was recorded for a period of 2 h, which was the limit set by our animal ethics committee for a pre-weaned animal. Seizure classification was based on the revised Racine scale (Luttjohann et al., 2009).

Thermogenic seizure testing was done by placing postnatal Day 14–17 mice in a thick-walled metal container heated from below. The air temperature within the chamber was continuously recorded and heat adjusted until a constant 42–42°C was achieved. Mice were monitored from direct contact with metal by a thick layer of paper. The time to first tonic-clonic seizure (class 6 on the modified Racine Scale) was recorded as previously described (Reid et al., 2013). Antiepileptic drugs were all injected intraperitoneally before subjecting mice to thermal stress. Diazepam (Sigma-Aldrich) was injected 30 min before testing and lamotrigine (Tocris Biosciences), stiripentol (Sigma-Aldrich) and retigabine (ChemPacific) were injected 60 min before testing. Lamotrigine, stiripentol and retigabine were suspended in 1% Tween 80 in saline. Diazepam stock (20 mg/ml) dissolved in dimethyl sulphoxide (DMSO) was diluted in saline for injection. Vehicle controls had no impact on seizure susceptibility (DMSO 25%, n = 3; Tween 1%, n = 3; saline controls, n = 5). All vehicle controls and saline-treated animals were combined for the control situation. Mice were sacrificed immediately after the first observed seizure to comply with our ethics approval.

**Gait analysis**

Gait was assessed using DigigaitTM (Mouse Specifics Inc.) when mice were 17 days old. The Digigait™ system uses video capture of the paws of the mice during treadmill locomotion. Analysis software was used to determine when individual paws were in contact with the treadmill and to calculate standard gait parameters. Treadmill speed was set to 4 cm/s.

**Electrophysiology**

Mice (post-natal Day 14–16) were anaesthetized using isoflurane and sacrificed 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 with artificial CSF solution at 34°C, unless stated, consisting of (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 Na₂HPO₄, 1 MgCl₂, 2 CaCl₂ and 10 glucose, aerated with 95% O₂ and 5% CO₂ to a final pH of 7.4. Whole cell patch clamp recordings were made using a MultiClamp 700 A amplifier and pClamp acquisition software (Molecular Devices) from neurons visually identified using infrared differential interference contrast imaging (BX51, Olympus). Electrodes were pulled using a Sutter P-2000 puller (Sutter Instruments) from borosilicate micropipettes (World Precision Instruments) with an initial resistance of ~2–3 MΩ.

**Action potential and passive properties**

Electrodes were filled with intracellular solution consisting of (in mM): 125 Kglu, 4 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 4 ATP-Mg and 0.3 GTP-Na, and 8 biocytin hydrochloride adjusted to a final pH of 7.3 with KOH. D-Mannitol was used to adjust osmolarity to 300 mOsm. Bridge balance was applied to all current clamp recordings. Voltage recordings were filtered at 30 kHz and sampled at 100 kHz. The bulk of action potential characterization was performed on these data but for measuring action potential threshold and display, data were further filtered at 3 kHz. A holding current was injected into neurons if required setting their holding potential to approximately −80 mV. A current injection/ action potential frequency relationship was established by injecting a 400-ms square pulse of progressively depolarizing currents. An automated action potential detection algorithm in pClamp9 (Molecular Devices) was used to detect action potential with visual confirmation. Area under the curve of the i-f curves was calculated (Axograph X) to allow comparison between genotypes. Action potential threshold voltage was defined as the voltage at which velocity reached 10 mV/ms. For the average action potential waveform analysis, action potentials were assigned to the threshold (10 mV/ms). Amplitude was measured from threshold to peak. Rise-time was determined as the time between 10% to 90% of the action potential amplitude. In a subset of subicular neurons the first and second (d²V/dt²) derivative was calculated numerically as previously described (Wimmer et al., 2010). Membrane input resistance and capacitance were measured using the membrane test analysis tool in PClamps. These values were then averaged. Resting membrane potential was measured as the potential when no current was injected. Liquid junction potential correction was made using the JPCalc program in Clampex 10.0 and for the solutions used above was estimated to be −14.8 mV at 34°C.

**Synaptic properties**

Electrodes were filled with an intracellular solution consisting of (in mM): 105 CsCl, 35 CsOH, 10 HEPES, 10 EGTA, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 14 D-mannitol and adjusted to a final pH of 7.3 with KOH. Recordings were filtered at 30 kHz and sampled at 100 kHz. Spontaneous inhibitory postsynaptic currents (PSCs) were measured in voltage clamp at −70 mV with the glutamatergic receptor blockers, D-APV (20 μM) and CNQX (25 μM) in the bath. Currents were recorded ≥5 min after achieving whole cell recording. For analysis, current traces were filtered at 3 kHz using a software Bessel filter (Axograph X). Spontaneous inhibitory PSCs were identified using event detection in Axograph X. The detection threshold of spontaneous inhibitory PSCs was set at 2.5 times the standard deviation of the noise. Each automatically identified event was manually confirmed. For each cell, at least 250 events were averaged to achieve an estimate of the spontaneous inhibitory PSC characteristics. Inter-event intervals were calculated between successive events and averaged. Rise time was defined as the time in between 10% to 90% of the peak amplitude. Spontaneous events in the absence of glutamatergic blockers (spontaneous PSCs) were also recorded as a marker of local network activity in both subiculum and CA1 pyramidal neurons. An identical protocol as described above was used to analyse these data.

**Statistical data analysis**

All group data are expressed as mean ± standard error of the mean (SEM) and compared using unpaired Student’s t-tests (Graphpad Prism, Graphpad Software), unless stated. When variance did not have a normal distribution the non-parametric Mann-Whitney test was used as indicated. Pearson’s chi-squared test (χ²) was used to compare the proportion of bursting cells. Comparison between
Kaplan-Meier curves was made using a Log-rank test (Graphpad Prism). Cumulative probability curves constructed from synaptic events were analysed using the Kolmogorov-Smirnov test (Graphpad Prism). \( P < 0.05 \) was taken as statistical significance. For cellular analysis \( n \) represents the number of neurons recorded. Cells were selected from a minimum of four mice with an average of 7.8 ± 0.7 mice per group. All drugs and salts were obtained from Sigma.

**Results**

**Complex neurological phenotype of homozygous SCN1B (C121W) mice**

At post-natal Day 14, gross brain morphology of SCN1B(C121W) homozygous mice seemed similar to controls (Fig. 1A). However, homozygous mice had an obvious tremor with continuous jerking movements and, at rest, assumed a hunched posture (Fig. 1B). Homozygous mice post-natal weights were significantly lower at post-natal Day 7 (wild-type: 4.7 ± 0.1 g, \( n = 26 \); homozygous: 3.3 ± 0.1 g, \( n = 30 \); \( P = 0.0001 \)), and despite gaining weight during the first few weeks of life they were always smaller than their wild-type littermates (post-natal Day 18, wild-type: 6.6 ± 0.2 g, \( n = 26 \); homozygous: 4.7 ± 0.1 g, \( n = 29 \); \( P = 0.0001 \)). Homozygous mice also exhibited increased mortality, with few surviving beyond post-natal Day 25 (Fig. 1C; \( P = 0.001 \), log-rank test).

Homozygous SCN1B(C121W) mice had spontaneous seizures at room temperature ranging from relatively mild seizures with facial twitching and rhythmic chewing [Class 1 and 2 based on the modified Racine scale (Luttjohann et al., 2009)] to more major seizures that included rearing and falling (Class 4) and sometimes exacerbating seizures in patients (Guerrini et al., 1998). Assessment of the properties of these two drugs in the SCN1B(C121W) homozygous mouse reflected this efficacy profile: stiripentol was effective whereas lamotrigine was not (Fig. 2B).

**Increased excitability of bursting subicular pyramidal neurons in homozygous SCN1B(C121W) mice is due to increased input resistance and decreased capacitance**

Hippocampal subicular pyramidal neurons contribute to seizure genesis (Huberfeld et al., 2011). As typical for subicular pyramidal neurons, current clamp recordings revealed two main populations based on propensity for burst firing (Menendez de la Prida et al., 2003). In homozygous mutants the proportion of burst firing neurons was 81% compared with 56% in controls (wild-type, \( n = 23 \); homozygous, \( n = 17 \); \( P = 0.03 \), \( \chi^2 \)). Non-bursting neurons were not investigated further. The properties of action potentials during both the bursting and tonic firing phases were analysed (Fig. 3A and Table 1). For tonic firing the input current versus action potential frequency \((i-f)\) relationships for neurons from homozygous mutants were left-shifted, consistent with increased excitability (Fig. 3B; \( P < 0.05 \) for all current injections \( \geq 170 \mu A \)).

Action potential firing thresholds from neurons of homozygous mutants and controls did not differ (Fig. 3C; see Table 1 for description of other single action potential properties) suggesting that increased excitability in the mutants may have resulted from changes in neuronal input resistance or capacitance. Analysis of both resting membrane input resistance \((R_{m})\) and capacitance revealed changes consistent with reduced neuronal size: input resistance in neurons from mutants was \( \sim 50\% \) higher than controls (Fig. 3D; \( P < 0.05 \)) and cell capacitance was 25% lower (wild-type, \( 140 ± 4 \mu F \), \( n = 15 \) versus homozygous, \( 106 ± 10 \mu F \), \( n = 10 \); \( P < 0.002 \)).

A more depolarized resting membrane potential was also observed in subicular neurons from mutants (Table 1).

**Homozygous SCN1B(C121W) mice share the pharmaco-sensitivity profile as Dravet syndrome**

We next tested if thermogenic seizures were responsive to anti-epileptic drugs. Diazepam significantly increased the time to first seizure in a dose-dependent manner confirming that this model reflects clinical efficacy of benzodiazepines in febrile seizures (Chiron and Dulac, 2011) (Fig. 2A). Patients with Dravet syndrome respond to certain antiepileptic drugs, with stiripentol controlling (Chiron et al., 2000; Kassai et al., 2008) and lamotrigine sometimes exacerbating seizures in patients (Guerrini et al., 1998). Assessment of the properties of these two drugs in the SCN1B(C121W) homozygous mouse reflected this efficacy profile: stiripentol was effective whereas lamotrigine was not (Fig. 2B).
SCN1B(C121W) phenotypes differ, with no clear gene-dosing effect on axon initial segment or neuronal passive properties.

**Reduced neuronal size and dendritic arborization in subicular neurons**

Subicular neurons that were biocytin filled during whole-cell recordings were reconstructed and analysed. We show that neurons from homozygous mutants were smaller overall and had less dendritic arborization than wild-types (Fig. 4A). Both soma area (Fig. 4B; $P < 0.05$) and apical dendrite volume (Fig. 4C; $P < 0.01$) were significantly smaller, consistent with the reduced membrane capacitance and increased resistance of these neurons. In addition, Sholl analysis, a common method used to quantify morphological characteristics of neurons, showed a significant reduction in the extent of tertiary branching (Fig. 4D; $P < 0.05$) and length of the distal apical dendrites (Fig. 4E). Taken together, these results suggest that the primary cause of enhanced neuronal excitability in the homozygous mutants is the altered passive properties of the neurons as a result of reduced neuron size.
Layer 2/3 pyramidal neurons show a similar increase in excitability

To see if the changes observed in subicular neurons were evident in pyramidal neurons from another brain region we recorded from L2/3 pyramidal neurons in the somatosensory cortex. An increase in excitability was observed in L2/3 pyramidal neurons from mutants with a left-shifted \( i-f \) curve and a \( R_m \) increase similar to those seen in subicular pyramids (Fig. 5). Single action potential and resting membrane properties are described for L2/3 pyramids in Table 1.

CA1 and L5 pyramidal neurons from homozygous mutant mice do not display any change in excitability

To test if other excitatory neuron classes display similar increases in excitability, we recorded from both L5 and CA1 pyramids. There were no significant differences between mutant and wild-type \( i-f \) curves, \( R_m \) or action potential firing thresholds (Supplementary Figs 2 and 3) for either neuron class. Similarly, analysis of action potential morphology demonstrated no significant differences between the genotypes (Table 1).

Hippocampal inhibitory neurons of homozygous mutants are identical to wild-type

Inhibitory interneuron dysfunction is a feature of Scn1a models of Dravet syndrome and the prevailing view is that this form of epilepsy is due to an ‘interneuronopathy’ (Yu et al., 2006; Ogiwara et al., 2007). This deficit was robustly seen in a heterogeneous population of Scn1a-deleted hippocampal interneurons (Yu et al., 2006). To test whether interneuron dysfunction also occurs in our Scn1b homozygous model, CA1 stratum radiatum inhibitory neurons were analysed. Only regular firing inhibitory neurons were included in the analysis. The \( i-f \) relationship of homohygos mutant inhibitory neurons could not be distinguished from wild-type (Fig. 6A and B). Moreover, input resistance and capacitance was also similar to control values (Fig. 6C and D). Single action potential properties are described in Table 1. Some studies have specifically identified parvalbumin-positive inhibitory neuron dysfunction, as the likely cellular substrate in their NaV1.1 heterozygous null mouse model (Ogiwara et al., 2007; Dutton et al., 2012). This broad subclass accounts for ~25% of interneurons in CA1 (Kosaka et al., 1987). We used immunohistochemistry to identify parvalbumin-positive inhibitory neurons that were then correlated to their functional properties. We localized examples of parvalbumin-positive interneurons using post-experimental immunohistochemistry and showed that firing properties in homozygous mutants and controls were the same (Supplementary Fig. 4). This demonstrates that the parvalbumin positive interneurons recorded in this study and implicated in NaV1.1 Dravet syndrome (Yu et al., 2006; Ogiwara et al., 2007) are not altered in the Scn1b(C121W) mouse model. Initial data implicating dysfunction of GABAergic neurons as the pathological bases of Dravet syndrome demonstrated action potential collapse in dissociated hippocampal neurons at room temperature (Yu et al., 2006). To test if higher recording temperature used in the experiments above was masking a similar functional deficit in the Scn1b homozygous model we next recorded a separate cohort of hippocampal interneurons at room temperature. Consistent with the properties observed at the higher recording temperature, the \( i-f \) relationships of homozygous mutant inhibitory neurons recorded at room temperature were indistinguishable from wild-type neurons (Supplementary Fig. 5). In summary, no observable changes in GABAergic neuron firing properties were observed in the Scn1b-based model of Dravet. These results are in stark contrast to those seen in NaV1.1 heterozygous null models where a fundamental aspect of the cellular phenotype is marked collapse of inhibitory neuron action potential firing (Yu et al., 2006; Ogiwara et al., 2007).

Next we investigated GABAergic synaptic transmission by measuring spontaneous inhibitory PSCs from subicular pyramids. There was
no difference in the average amplitude or inter-event interval (Fig. 6E) of spontaneous inhibitory PSCs recorded from mutant and wild-type neurons. This indicates that there is no overt GABAergic synaptic dysfunction within this region. There were also no changes seen in rise-time (wild-type, $1.3 \pm 0.1$ ms, $n = 6$ versus homozygous, $1.4 \pm 0.1$ ms, $n = 6$; $P = 0.37$) and half-width ($2.4 \pm 0.15$ ms versus $2.6 \pm 0.3$ ms; $P = 0.37$) of spontaneous currents.

**Spontaneous synaptic activity is increased in the SCN1B(C121W) mouse subiculum**

To get a measure of local network activity within the subiculum we recorded spontaneous PSCs from subicular pyramids in the absence of glutamatergic receptor blockers. A mix of excitatory and inhibitory events contributes to synaptic currents under these recording conditions (Fig. 7A). A reduction in inter-event interval (Fig. 7B and C) was observed in mutant pyramids when compared with wild-types suggesting that network excitability was increased within the subiculum. No change in amplitude (Fig. 7B and C), rise-time (wild-type, $1.6 \pm 0.2$ ms, $n = 6$ versus homozygous, $1.5 \pm 0.1$ ms, $n = 7$; $P = 0.37$) or half-width (wild-type, $2.4 \pm 0.3$ ms versus homozygous, $2.2 \pm 0.3$ ms; $P = 0.60$) was observed between the genotypes. We next investigated spontaneous synaptic events recorded from CA1 pyramids under identical conditions (Fig. 7D). In this case there was no significant difference in the average inter-event interval (Fig. 7E and F) or amplitude (Fig. 7E and F) between the genotypes consistent with the absence of altered CA1 pyramid excitability in the neurons from mutant mice. As might be expected, there was also no difference in the rise-time (wild-type, $1.7 \pm 0.4$ ms, $n = 4$ versus homozygous, $1.7 \pm 0.2$ ms, $n = 4$; $P = 0.98$) and half-width ($2.8 \pm 0.11$ ms versus $3.1 \pm 0.19$ ms, $P = 0.2$) of spontaneous PSC recorded from CA1 pyramids.
Retigabine restores action potential firing and reduces hyperthermic seizure susceptibility

Retigabine is a recently approved anti-epileptic drug (Large et al., 2012) that is a KCNQ/Kv7 voltage-gated potassium channel opener (Gunthorpe et al., 2012). Opening of these channels by retigabine reduces neuronal input resistance (Otto et al., 2002), which should reverse the increased resistance seen in pyramidal neurons of homozygous SCN1B(C121W) mice in a disease mechanism-based manner. To test this idea we applied retigabine (25 μM) while recording from mutant subicular pyramidal neurons and demonstrated a significant reduction in Rm and action potential firing (i-f) essentially reversing the observed functional deficit (Fig. 8A–C). Retigabine also restored network function in neurons from mutant mice towards control levels with an increase in the inter-event interval (control, 0.22 ± 0.04 s versus retigabine, 0.36 ± 0.07 s, n = 5, P = 0.01, paired t-test) with no changes in other synaptic parameters (P > 0.5). Moreover, retigabine, at doses that do not alter body temperature (Kristensen et al., 2011), had an in vivo impact on the homozygous mice, reducing thermogenic seizure susceptibility towards wild-type levels (Fig. 8D).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Action potential characteristics and resting membrane potential of patched neurons</th>
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<tbody>
<tr>
<td>Subicular bursting pyramids</td>
<td>WT (n = 15)</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>110.99 ± 2.89</td>
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<tr>
<td>Half-width (ms)</td>
<td>1.35 ± 0.09</td>
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<tr>
<td>10V s⁻¹ threshold (mV)</td>
<td>−50.91 ± 2.66</td>
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<tr>
<td>Tonic firing action potentials</td>
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<tr>
<td>Amplitude (mV)</td>
<td>86.26 ± 2.73</td>
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<tr>
<td>Half-width (ms)</td>
<td>1.42 ± 0.10</td>
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<tr>
<td>Rise (ms)</td>
<td>0.40 ± 0.03</td>
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<td>10V s⁻¹ threshold (mV)</td>
<td>−54.28 ± 1.11</td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>−71.69 ± 1.47</td>
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<tr>
<td>Cortical layer 2/3 pyramids</td>
<td>WT (n = 20)</td>
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<tr>
<td>Amplitude (mV)</td>
<td>91.55 ± 3.01</td>
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<tr>
<td>Half-width (ms)</td>
<td>1.32 ± 0.07</td>
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<tr>
<td>Rise (ms)</td>
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<tr>
<td>10V s⁻¹ threshold (mV)</td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>−74.87 ± 2.32</td>
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<tr>
<td>CA1 interneurons</td>
<td>WT (n = 7)</td>
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<td>34°C</td>
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<tr>
<td>Amplitude (mV)</td>
<td>78.61 ± 4.14</td>
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<tr>
<td>Half-width (ms)</td>
<td>0.818 ± 0.074</td>
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<tr>
<td>Rise (ms)</td>
<td>0.337 ± 0.021</td>
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<tr>
<td>10V s⁻¹ threshold (mV)</td>
<td>−52.06 ± 1.5</td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>−68.36 ± 4.96</td>
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<td>22°C</td>
<td>WT (n = 9)</td>
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<td>Amplitude (mV)</td>
<td>72.59 ± 5.11</td>
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<tr>
<td>Half-width (ms)</td>
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<td>Rise (ms)</td>
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<td>10V s⁻¹ threshold (mV)</td>
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<td>Resting membrane potential (mV)</td>
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<tr>
<td>Cortical layer 5 pyramids</td>
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<td>Half-width (ms)</td>
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<td>Rise (ms)</td>
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<td>10V s⁻¹ threshold (mV)</td>
<td>−51.44 ± 2.03</td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
<td>−75.98 ± 1.88</td>
</tr>
<tr>
<td>CA1 pyramids</td>
<td>WT (n = 10)</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>92.87 ± 2.29</td>
</tr>
<tr>
<td>Half-width (ms)</td>
<td>1.52 ± 0.078</td>
</tr>
<tr>
<td>Rise (ms)</td>
<td>0.293 ± 0.011</td>
</tr>
<tr>
<td>10V s⁻¹ threshold (mV)</td>
<td>−51.49 ± 1.96</td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
<td>−72.35 ± 2.01</td>
</tr>
</tbody>
</table>

WT = wild-type; Hom = homozygous.
**Discussion**

Homozygous SCN1B(C121W) mice recapitulate many of the classical phenotypic features of Dravet syndrome (Yu et al., 2006; Ogiwara et al., 2007; Dravet et al., 2012). We observed a structural change in subicular pyramidal neurons consistent with the enhanced excitatory neuronal phenotype that represents a novel disease mechanism in Dravet syndrome. Retigabine, a K⁺ channel opener, reversed the excitatory electrophysiological phenotype and was effective at low doses in protecting SCN1B(C121W) mice against thermogenic seizures. These data illustrate that the ‘electrophysiological consequences’ of altered neuronal morphology can be reversed. More importantly these observations highlight the potential use of understanding disease mechanisms in genetic mouse models of epilepsy syndromes, which in turn could direct disease mechanism-based therapies.
Heterozygous SCN1B variants associated with generalized epilepsy with febrile seizures plus occur within or near the Ig loop of the protein as do the homozygous R125C and I106F mutations observed in the published patient with typical Dravet syndrome and the more severe epileptic encephalopathy case, respectively (Patino et al., 2009; Ogiwara et al., 2012). The C121W mutation falls between these two described variants in the homozygous human cases. Although no human case of Dravet syndrome with a homozygous C121W mutation has yet been identified, the proximity to the R125C residue and shared behavioural phenotype with the Scn1a models and patients provide evidence that the homozygous SCN1B(C121W) mutant mice may represent a valid experimental model of Dravet syndrome. The lack of an impact of the mutation in L5 pyramidal neurons in the homozygous mice suggest that they are different to the homozygous b1 knock-out mice, which displayed increased excitability in these neurons (Marionneau et al., 2012).

A prominent excitable neuronal phenotype in SCN1B(C121W) homozygous mutants was increased action potential firing of subicular and layer 2/3 pyramidal neurons. It was surprising that threshold of action potential firing was not altered given the role of b1 in modulating the biophysical properties of sodium channel gating (Kazen-Gillespie et al., 2000) and its location at the axon initial segment (Brackenbury et al., 2010; Wimmer et al., 2010). However, it is consistent with observations in the null model in which the effect of b1 on sodium channels is subtle with gating properties and sodium channel density unchanged in pyramidal and bipolar neurons (Chen et al., 2004; Patino et al., 2009; Brackenbury et al., 2010), possibly reflecting compensatory mechanisms. An increase in neuronal input resistance is likely to underlie the excitable phenotype observed in mutant neurons. Recent evidence shows that b1 acts as an auxiliary subunit to the Kv4.2 potassium channel increasing channel density (Marionneau et al., 2012). However, because the Kv4.2 channel rapidly inactivates after opening, its contribution to passive leak, as observed in the current study, would be expected to be minimal.

Morphological analysis revealed that mutant subicular neurons had a smaller soma and less dendritic arborization consistent with reduced membrane capacitance and higher input resistance. Aberrant dendritic excitability and morphology may be a common pathophysiological mechanism in epilepsy and other neurological diseases (Nestor and Hoffman, 2012). More recently, in a cortical stroke model of epilepsy, Paz et al. (2013) demonstrate increased thalamocortical neuron excitability due to an
increase in input resistance caused by a reduction in neuron size. Similarly, we propose that the reduction in dendritic arborization is a key contributor to excitability in the genetic Scn1b mouse model. The sodium channel β1 subunit is a member of the Ig superfamily of cell adhesion molecules and is implicated in many ‘non-conducting’ roles within the brain. Interactions with protein phosphatases, kinases and several other cell adhesion molecules and extracellular matrix proteins have been reported (reviewed...
in Brackenbury and Isom, 2011) some of which will likely be impacted by the C121W mutation and potentially contribute to the phenotype. One non-conducting function possibly relevant is the role of $\beta_1$ in controlling neurite outgrowth (Davis et al., 2004; Brackenbury et al., 2008, 2013; Patino et al., 2011). Interestingly, the $\beta_1$ null model (Brackenbury et al., 2013) and although not specifically investigated here could also be part of the underlying pathology of the homozygous SCN1B(C121W) mice.

In addition to the reduction in rheobase, subicular pyramids from mutant mice were depolarized relative to controls and also displayed more bursting behaviour that may be important for the initiation and amplification of epileptiform activity (Traub and Wong, 1982). This is in contrast with CA1 pyramids for which no hyperexcitable phenotype was observed. CA1 pyramidal neurons provide a major input to the subiculum but are unlikely to

Figure 7 Synaptic activity is increased in the SCN1B(C121W) mouse subiculum but not CA1. (A) Raw traces of spontaneous PSCs (sPSC) recorded in subicular pyramids from wild-type (WT) and homozygous (Hom) mice in the absence of glutamateric blockers. (B) A significant increase in the average spontaneous PSC event frequency was observed for mutant mice (wild-type, $n = 6$; homozygous, $n = 7$, $P = 0.04$, unpaired $t$-test). There was no difference in the average event amplitude (wild-type, $n = 4$; homozygous, $n = 4$, $P = 0.3$, unpaired $t$-test). (C) Cumulative probability curves also identify an increase in frequency of spontaneous PSC ($P = 0.01$, Kolmogorov-Smirnov test) but not amplitude. (D) Raw traces of spontaneous PSC recorded in CA1 pyramids from wild-type and homozygous mice. (E) No difference in the average event frequency wild-type, $n = 4$; homozygous, $n = 4$, $P = 0.22$, unpaired $t$-test) or amplitude was observed for mutant (wild-type, $n = 4$; homozygous, $n = 4$, $P = 0.87$, unpaired $t$-test). (F) Similarly, the cumulative probability curves showed no significant changes in spontaneous PSC characteristics between mutant and wild-type CA1 pyramidal neurons (Kolmogorov-Smirnov test).
directly contribute to the increased excitability. However, input into the subiculum is complex with projections arising from several other brain regions (Van Groen and Lopes da Silva, 1986), which may independently provide increased synaptic drive. Alternatively, subicular pyramids can fire bursts in response to local synaptic excitation with different responsiveness that is determined by intrinsic membrane properties (Menendez de la Prida, 2003). So even in the context of normal input the reduced dendritic arborization of subicular neurons observed here will increase excitatory output. Another possibility is local subiculum excitability being the primary driver of seizure generation. Interestingly, pre-ictal local population bursts in the subiculum are a feature of an epileptic state with the recurrence of these discharges proposed to lead to seizures (Huberfeld et al., 2011). An increase in spontaneous synaptic activity in subicular pyramids, but not CA1 pyramids, is consistent with an increase in local hyperexcitability. Further investigation is required to determine the full consequence of a reduced dendritic arborization of subicular neurons on local and long-range network function. However, taken together our data argue strongly that this change is a key driver of pathogenesis in the SCN1B(C121W) mouse.

At post-natal Day 16, when the electrophysiology experiments were conducted, mice were having spontaneous seizures. The cellular dysfunction noted in our model may, therefore, be due to the consequence of seizures rather than a direct impact of the mutation on function. However, recent data suggest that cellular phenotypes that can be attributed to SCN1B's cell adhesion role occur before seizures in the Scn1b-null mouse model (Brackenbury et al., 2013). Irrespective of this, the changes in morphology and consequent passive properties seen in the homozygous SCN1B(C121W) mice are likely to contribute to ongoing seizure activity and hence be a valid model of Dravet syndrome.

Benzodiazepines are used to treat Dravet syndrome (Chiron and Dulac, 2011) and in the present study diazepam was effective at reducing thermogenic seizures in the Scn1b mouse model (Cao et al., 2012). Stiripentol was also effective at protecting against thermogenic seizures, consistent with the effect observed in the Scn1a mouse model (Cao et al., 2012) and also clinically in patients with Dravet syndrome (Chiron et al., 2000; Kassai et al., 2008). Stiripentol has multiple actions including enhancement of GABA_A receptors (Fisher, 2011). It is particularly

**Figure 8** Retigabine was effective at reversing the neuronal deficit and protected homozygous SCN1B(C121W) mice from thermal seizures. (A) Representative traces of action potential firing of a single homozygous neuron before and after retigabine (Hom RTG). (B) Action potential frequency before and after retigabine for the example illustrated in A. (C) Summary data of neuron membrane resistance for homozygous neurons before and after retigabine compared to wild-type (WT) neurons alone (homozygous versus homozygous retigabine, n = 9; *P < 0.05, t-test). (D) Kaplan-Meier curves showing dose-dependent protection from thermal-induced tonic-clonic seizures of homozygous mice following intraperitoneal injection of two doses of retigabine, 5 mg/kg and 10 mg/kg compared with untreated homozygous mice (RTG 5, n = 4; RTG 10, n = 5; P < 0.05, Log-rank test compared to injection controls, n = 11). Non-injected wild-type Kaplan-Meier curve is provided as a reference point (n = 13).
effective at GABA<sub>A</sub> receptors containing α3 subunits, which are at their highest expression level in immature brains (Fisher, 2011). Interestingly, lamotrigine was not effective at reducing thermogenic seizures, as expected from human data that even shows exacerbation of seizures in some Dravet cases (Guerrini et al., 1998). One suggestion is that the further loss of sodium channel function in GABAergic neurons (Yu et al., 2006) may underlie lamotrigine's exacerbation of seizures, although there is no direct evidence for this premise. Our data suggest that the mechanism underlying the lack of efficacy of lamotrigine is more complex as we do not see similar dysfunction in GABAergic neurons. Other modes of action of lamotrigine have been identified, such as effects on I<sub>N</sub> and Ca currents (Stefani et al., 1996; Poolos et al., 2002). Also, a recent suggestion that the drug increases the surface expression of both wild-type and Dravet mutant Na<sub>V</sub>1.1 channels, which may be part of the explanation underlying exacerbation in some patients (Thompson et al., 2012). Further work comparing and contrasting the Scn1a and Scn1b models will help identify the precise mechanism through which exacerbation occurs.

The effectiveness of retigabine in reversing both the cellular and behavioural deficits (at low doses) suggests that morphologically-mediated changes are an important mechanism of excitability and hence a valid therapeutic target in SCN1B-based Dravet syndrome. Although we cannot rule out an anti-seizure effect of retigabine through an alternative mechanism, the exquisite sensitivity of this drug on the thermogenic seizures in our model is consistent with the idea of efficacy relating to pathological mechanism. Additional studies comparing a variety of syndrome-specific models, each with their unique disease mechanism, are needed to develop a complete understanding of the mechanistic bases of the epileptic encephalopathies and to devise precision medicine-based therapeutic strategies.

Genetic heterogeneity has been a surprising finding in apparently homogeneous epilepsy syndromes, especially the epileptic encephalopathies (Mastrangelo and Leuzzi, 2012; Allen et al., 2013; Carvill et al., 2013). The phenotype in our homozygous SCN1B(C121W) mouse closely matches those described in Scn1a mice models of Dravet syndrome, including seizures, ataxia and premature death (Yu et al., 2006; Kalume et al., 2007; Ogiwara et al., 2007; Oakley et al., 2009; Martin et al., 2010). The primary cellular deficit in the Scn1a mice models occurs in GABAergic inhibitory neurons with loss of function of the Na<sub>v</sub>1.1 channel causing disinhibition that is proposed to underlie increased network excitability (Yu et al., 2006; Ogiwara et al., 2007; Martin et al., 2010; Cheah et al., 2012; Dutton et al., 2012). In stark contrast, we observed unique changes in firing properties of two different pyramidal neuron types, but saw no change in the action potential firing properties of GABAergic neurons. Further, we did not observe any overt deficit in GABA<sub>A</sub> receptor-mediated transmission. Therefore, despite Na<sub>v</sub>1.1 and SCN1B(C121W) mice models and patients having a convergent behavioural phenotype of Dravet syndrome, the underlying pathology can be driven by either interneuron or pyramidal neuron deficits. Our data provide evidence that common neuronal network dysfunction can arise from distinct cellular mechanisms, which may have implications for mechanism-based therapeutics. The current study suggests a novel pathogenic mechanism in genetic epilepsy and provides an important proof-of-concept for disease-based therapy.

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**Supplementary material**

Supplementary material is available at Brain online.

**References**


