Increased persistent sodium current contributes to seizure in the slamdance bang-sensitive *Drosophila* mutant

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Text pages: 37

Figures: 8

Tables: 1

Supplementary videos: 2

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Abstract

There is clinical need to extend understanding of epilepsy and to find novel approaches for treating this condition. Bang-sensitive (bs) *Drosophila* mutants, which exhibit reduced thresholds for seizure, offer an attractive possibility to combine tractable genetics, electrophysiology and high-throughput screening. However, despite these advantages, the precise electrophysiological aberrations that contribute to seizure have not been identified in any bs-mutant. Because of this, the applicability of *Drosophila* as a pre-clinical model is not yet established. In this study we show that electroshock of bs *slamdance* (*sda*) larvae is sufficient to induce extended seizure-like episodes. Whole cell voltage clamp recordings from identified motoneurons (termed aCC and RP2), show synaptic currents that are greatly increased in both amplitude and duration. Current clamp recordings indicate that these inputs produce longer-lived plateau depolarizations and increased action potential firing in these cells. An analysis of voltage-gated currents in these motoneurons, in both first and third instar larvae, revealed a consistently increased persistent Na⁺ current (I_{Nap}) and a reduced Ca²⁺ current in first instar larvae, which in older third instars appeared normal. That increased I_{Nap} may contribute to seizure-like activity is indicated by the observation that feeding *sda* larvae the anti-epileptic drug (AED) phenytoin, which we show is sufficient to reduce I_{Nap}, rescues both seizure-like episode duration and synaptic excitation of motoneurons. By contrast, feeding of either anemone toxin (rATX), a drug that preferentially increases I_{Nap}, or phenytoin to WT larvae is sufficient to induce a bs-behavioral phenotype. Finally, we show that feeding of phenytoin to gravid *sda* females is sufficient to both reduce I_{Nap}, synaptic currents and rescue the bs-phenotype in their larval
progeny indicative that heightened predisposition to seizure may arise as a consequence of abnormal embryonic neural development.

Key words: aCC, motoneuron, phenytoin, RP2, slamdance, synaptic current

Introduction

Epilepsy is a common syndrome manifesting a range of symptoms that affects ~1% of the global population. Although anti-epileptic drugs (AEDs) are available, only two-thirds of patients report relief; the remaining third are relatively insensitive to drug intervention (Schmidt 2002). Because of this, there is a need for alternative treatments based on novel targets.

A large majority of idiopathic epilepsies undoubtedly arise as a result of genetic mutations. Indeed, there is now a considerable list of single gene mutations that reduce seizure thresholds in both rats and mice (Frankel 2009). There are also, albeit fewer, rodent strains that have spontaneously developed increased prevalence of seizure (e.g. DBA and EL mice and GAERS rats (Frankel 2009)). However, the use of rodents for such studies is not without limitations. Chief amongst these, perhaps, is that rodents are labour and facility-intensive to maintain. Secondly, epilepsy is a complex trait that is significantly influenced by genetic background and it is challenging to control for this in complex animals (Schauwecker and Steward 1997).

*Drosophila melanogaster* offers tractable genetics and genotypes that readily and reliably display significantly reduced seizure thresholds. These genotypes, which were first identified by Benzer in 1971, are termed bang-sensitive (bs) mutants due to their heightened response to mechanical shock (Benzer 1971).
Subsequent studies, initiated in the Tanouye group, demonstrated that bs-mutants exhibit lower thresholds for electroshock-induced seizures than wildtype controls (Pavlidis et al. 1994). These studies utilised high frequency electroshock to the adult brain and recordings from flight muscle to report motor activity in the CNS. Such stimulation of the brain results in a discharge of excitatory potentials in flight muscle followed by an extended period of synaptic failure, before finally a recovery seizure, characterised by a volley of excitatory potentials, is observed: the whole episode lasting up to a few minutes (Zhang et al. 2002). The susceptibility to ‘seize’ is defined by the voltage-threshold required to induce the seizure-like response.

The underlying neuronal aberrations that reduce threshold for seizure induction in any bs-mutant remain unknown. Analysis of motoneuron axonal excitability indicate that threshold for excitation is significantly reduced in bs mutants which is consistent with reduced thresholds for seizure-induction (Ganetzky and Wu 1982). However, whether the central motoneurons are also hyperexcitable remains to be shown. Although more than adequate for determination of seizure-threshold, the adult preparation is not best suited to allow for a more rigorous understanding of underlying disturbances in individual neurons. By contrast, relatively sophisticated electrophysiology can be applied to central neurons in Drosophila larvae (Baines and Bate 1998; Rohrbough and Broadie 2002). In particular, two motoneurons, termed aCC and RP2, have been extensively characterized (Baines et al. 1999; Baines et al. 2001). This characterisation includes a comprehensive description of intrinsic membrane conductances and synaptic currents, the latter reflecting network properties in central motor circuitry (Baines 2004).
Seizures in bs-mutants exhibit sufficient similarities with humans to suggest that the underlying neuronal abnormalities are analogous and that the results derived have clinical relevance (Kuebler and Tanouye 2002; Reynolds et al. 2004; Tan et al. 2004). Moreover, many bs-mutants have been well characterised with respect to both gene and protein function: for example, slamdance (sda) is a mutation in the Drosophila homologue of human aminopeptidase N (Zhang et al. 2002). Aminopeptidase N (APN, also known as CD13) is a glycoprotein that is widely expressed in animal tissues, including the nervous system (Inagaki et al.). This enzyme, which catalyzes the removal of neutral and basic amino acids from the N-termini of peptide substrates, has been proposed to function in a wide spectrum of roles including acting as a receptor to mediate cell adhesion, antigen processing and in particular in cancer progression (reviewed in (Zhang et al. 2002). How this mutation is able to reduce threshold for seizure activity in Drosophila is unknown. It also remains unknown whether mutations in mammalian APN result in seizure-like activity.

In this study, we report the first detailed electrophysiological characterization of central neuron function in larvae of a defined bs-mutant. We show that sda mutant larvae exhibit prolonged bouts of seizure-like episodes induced by electroshock. Our electrophysiology shows that synaptic currents in aCC/RP2 motoneurons are increased in both amplitude and duration, an observation that may suggest heightened network excitability. We also report changes in both voltage-gated Ca\(^{2+}\) and Na\(^+\) conductances in sda aCC/RP2. In particular, we show that the voltage-gated persistent Na\(^+\) current (\(I_{\text{Nap}}\)) is increased while the transient Na\(^+\) current (\(I_{\text{Nat}}\)), which is mediated by the same ion channel (Lin et al.)
2009) is not affected. This is consistent with a change in functional properties of this ion channel to increase the apparent ratio of $I_{\text{nap}}$. We provide evidence to show that the ratio between these two components is an important determinant of seizure-like behavior. We confirm our findings in $sda$ through pharmacological treatment of wild type and, moreover, provide evidence to suggest that the aberrations observed in the $sda$ mutant may arise as a consequence of altered embryonic neural development.

Materials and Methods

*Fly stocks:* Flies were maintained on standard corn meal medium at 25°C. Embryos were collected by allowing females to lay on grape-agar (Dutscher, Essex, UK) plates supplemented with a small amount of live yeast paste at 25°C. Flies used were Canton-S (CS) wildtype (WT) and $slamdance^{iso7.8}$ ($sda$) (Zhang et al. 2002).

**Seizure induction in adult.** Adult flies (1-2 days post eclosion) were anesthetised by CO$_2$ and placed in empty plastic fly-vials (10 flies per vial). Flies were allowed to recover for at least two hours and were then subjected to 10 seconds of mechanical shock delivered via vortex (Vortex genie 2, Scientific Industries, Bohemia, FL). The time for each fly to recover, defined as standing, was scored and a mean value taken for each vial (denoted Mean Recovery Time). Any flies which did not recover were disregarded from the analysis (this number was minimal).

**Induction of seizure in third instar larvae:** Prior to stimulation, third instar larvae were washed to remove food residue and gently dried using paper tissue.
Larvae were then allowed to recover on agar plates until normal crawling behavior resumed. A stimulator was constructed comprising of two tungsten wires fixed to a non-conducting rod. The tips of the two wires (0.1mm diameter) were set ~1-2mm apart and were placed across the anterior-dorsal surface, over the approximate position of the CNS. A DC pulse (50V, 3sec), generated by a Grass S88 stimulator (Grass instruments, RI, USA), was applied. The animal responded by tonically contracting and ceasing normal, motile behavior. Time to resumption of normal behavior was timed (Mean Recovery Time).

Normal crawling was defined as a sustained, whole body length peristaltic wave that resulted in forward motion.

Third instar drug-feeding: A stock solution of phenytoin (PHY) was prepared in DMSO (4mg in 50 µl, both from Sigma, Poole, UK) and this was then added to 10ml ddH2O with added 5% dried yeast extract to produce a final phenytoin concentration of 0.4mg/ml. 2ml of this solution was then placed on to the surface of a grape-agar plate (50mm diameter) and left to dry on a warm plate (~30ºC) for 3-4 hrs. In each case control plates were made that contained only the vehicle (DMSO) which never exceed 0.02% v/v and yeast extract. Anemone toxin (rATXII) was diluted with ddH2O into stock aliquots of 10µM and further diluted to the working concentration as needed; 150µl of the final working concentration was coated onto a well of a 24-well plate (Costar 3524, Corning Incorporated NY) that had previously had each well one third filled with grape-agar. The rATXII coated plate was then left to dry on a warm plate for 1-2 hours. In both cases, larvae were allowed to feed on these plates for 24hrs prior to testing.
Adult drug feeding: Mated adult females were fed on phenytoin (0.4mg/ml) for 2 days by adding flies to food vials containing drug. The flies were then transferred to non-drug containing vials and allowed to lay for a further 2 days before being removed. These drug-free vials were left at 25°C and wall-climbing third instar larvae used for analysis. To determine levels of phenytoin ingestion by female flies and transfer to oocyte and third instar, the added phenytoin was spiked with $^{14}$C-labelled phenytoin (0.074MBq per vial, PerkinElmer, Cambridge, UK) and counts normalised to mg protein (Biorad, Hemel Hempstead, UK).

Electrophysiology: Recordings were performed in both young first instar larvae (1-4 hours post hatching) and wall climbing third instars at room temperature (22–24°C). First instars were dissected with sharpened tungsten wires as described previously (Baines and Bate 1998). Third instar CNS was dissected free with fine forceps and fixed to Sylgard (Dow Corning, MI, USA) coated cover slips with cyanoacrylate glue (Vetbond, WPI, Stevenage, UK). Rupture of the neurolemma surrounding the CNS was as described (Baines and Bate 1998) but with the larger third instar CNS requiring a slightly larger bore micropipette.

Whole-cell voltage and current clamp recordings were achieved using thick-walled borosilicate glass electrodes (GC100F-10; Harvard Apparatus, Edenbridge, UK), fire polished to resistances of between 15 - 20 MΩ (firsts) and 10 - 15 MΩ (third instar). aCC and RP2 motoneurons were initially identified based on both size and dorsal position in the ventral nerve cord. Unequivocal identification was determined after recording by labelling with 0.1% Alexa Fluor 488 hydrazide, sodium salt (Molecular Probes, Eugene, OR), which was
included in the patch saline. Recordings were made using a Multiclamp 700B amplifier controlled by pClamp (v10.2, Molecular Devices, Sunnyvale, CA). Recordings were sampled at 20KHz and filtered online at 10KHz. Capacitance was measured by integrating the area under the capacity transient resulting from a step protocol from -60mV to -90mV.

Synaptic currents showing multiple peaks were quantified as a single event; the amplitude was taken from the largest peak, which invariably was the first one. Such multiple events are rare in both wildtype and sda (see Fig 2). A more detailed explanation of how we measure both amplitude and frequency is provided in Stacey et al (Stacey et al.). To measure synaptic current amplitude, the change from pre-firing baseline to peak current amplitude was determined using MiniAnalysis (Synaptosoft, Decatur, GA). Duration of each synaptic current was defined as time from current initiation until return to baseline (± 3 pA), determined in Clampfit v10.2. For all histograms shown, synaptic current parameters were determined for each cell recorded and an average value calculated. This was repeated for multiple cells and a cumulative average ± SEM was calculated and is shown. For cumulative probability plots (Fig. 2F-G) all synaptic current amplitudes or durations obtained from multiple cells are shown.

Because we observe a change to the persistent $I_{Na}$ and not to the transient $I_{Nat}$, we have determined, and report, the ratio of these two conductances, which are mediated by the same ion channel. Normalising our data to a ratio additionally compensates for the fact that the magnitude of both components show variability between genotypes (and to a lesser extent within genotypes).
Electrophysiology Solutions: External saline for dissection and normal whole-cell recording of synaptic currents consisted of (in mM): 135 NaCl, 5 KCl, 4 MgCl₂, 2 CaCl₂, 5N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), and 36 sucrose. Potassium conductance was isolated using a saline that consisted of (in mM): 135 NaCl, 5 KCl, 4 MgCl₂, 2 CaCl₂, 5 TES, 36 sucrose and 2μM TTX. Calcium conductance saline consisted of (in mM): 50 NaCl, 6 KCl, 50 BaCl₂, 50 tetraethylammonium chloride (TEA), 10 4-aminopyridine, 10 MgCl₂, 10 HEPES, and 10 glucose. Sodium conductance saline consisted of (in mM): 100 NaCl, 6 KCl, 50 TEA, 10 4-aminopyridine, 10 MgCl₂, 10 HEPES, and 10 sucrose. All solutions were pH 7.4.

Internal saline consisted of (in mM): 140 KCH₃SO₃, 5 KCl, 2 MgCl₂, 11 EGTA, and 20 HEPES, pH 7.4. When recording either calcium or sodium conductances, CsCH₃SO₃ was substituted for KCH₃SO₃, and CsCl₂ was substituted for KCl. TTX and rATXII were obtained from Alomone Labs (Jerusalem, Israel), KCl, KOH, NaOH, NaCl, sucrose and glucose from Fisher scientific (Loughborough, UK), HEPES, MgCl₂, CaCl₂ from BDH (Poole, UK), all remaining chemicals and drugs were obtained from Sigma (Poole, UK).

Statistics: All values are shown as means ± SEM. Significance was determined using a non-paired t-test. Results were deemed significant at *P<0.05 or **P<0.01.
Results

Although increased susceptibility to seizure in bs-mutants, including sda, has been previously characterised in adult flies (Zhang et al. 2002), precise underlying electrophysiological defects in central neurons have not been described. This is primarily because the adult CNS has not, until recently, been considered suitable for electrophysiological recordings from identified neurons. By contrast, whole-cell patch clamp recordings from larval central neurons are now routine and, as such, there exists a considerable body of background literature describing, for example, the voltage- and ligand-gated conductances present in larval motoneurons (Baines 2004; Baines et al. 2001; Choi et al. 2004; Pym et al. 2006; Rohrbough and Broadie 2002). To exploit this, we developed an electroshock assay to test for increased susceptibility for seizure-like phenotype in third instar bs-mutants.

*sda larvae exhibit prolonged seizures following electroshock*

It has previously been shown that adult bs-mutants, including sda, exhibit an initial seizure-like phenotype followed by a period of paralysis and then a short recovery seizure in response to a mechanical stimulus (Zhang et al. 2002). The total duration of this seizure-like episode can be measured by the Mean Recovery Time: defined as the time taken for all flies to be standing following the stimulus. We have repeated this determination for sda adults which shows a significantly longer Mean Recovery Time compared to wildtype flies (Fig. 1A). Increased Mean Recovery Time in adults is correlated with decreased seizure threshold (i.e. increased seizure sensitivity) and, as such, is routinely used to determine predisposition to ‘seize’ in flies (Song and Tanouye 2008).
Whilst bs-behavior is well documented in adults, how the larval stages of \textit{sda}, or other bs-mutants, respond to similar extreme stimulation is unknown. Because larvae are not suited to mechanical shock testing, we instead applied a brief electric shock to the dorsal cuticle. Such stimulation of wildtype third instar results in a full-body paralysis (seizure/paralysis) that lasts on average 24.9 ± 3.4 sec (n = 40) before full recovery is observed (Fig. 1B). Full recovery was scored as sustained peristaltic contractions, travelling the whole body length of the larva that resulted in sustained forward motion. Partial recovery was frequently observed in body segments that had not been directly stimulated (often posterior to the site of stimulation). This partial recovery would often take the form of the larva attempting, but failing, to resume full body motile behavior. On occasion, a failure of body lifting during the peristaltic wave was also observed. This behavior in combination with myogenic mouth hook flexing would cause the larva to move forward (via the mouth hooks) and then backward (via failed body lifting during the peristaltic wave), producing a rocking motion. Both of these behaviors were discounted in terms of ‘full’ recovery. In contrast to wildtype, identical electroshock of \textit{sda} larvae resulted in a significantly increased Mean Recovery Time (164.9 ± 20.0 sec, n = 40, Fig. 1B) indicative of a heightened predisposition to seizure-like behavior. Videos showing a typical wildtype and \textit{sda} response to electroshock are available in supplementary data. Identical stimulation was sufficient to increase Mean Recovery Time in another bs-mutant, \textit{easily-shocked}, indicative that this method may have general applicability (Marley and Baines, unpublished data).
Synaptic excitation of motoneurons is increased in sda mutants:

Our electroshock data indicate that sda larvae, similar to adults, display increased duration of seizure-like activity in response to extreme stimulation. To determine a possible mechanistic basis for this we focused our attention on larval motoneurons. These cells, which lie within the CNS, project axons to the body wall muscles that are responsible for the seizure-like activity we observe following electroshock.

Larval motoneurons receive cholinergic excitatory synaptic drive from presynaptic interneurons (Baines 2003). Voltage clamp recordings (Vh -60 mV) are sufficient to reveal these currents; termed spontaneous rhythmic currents (SRCs) (Rohrbough and Broadie 2002) which occur at a robust frequency, amplitude and duration. To allow direct comparisons to be made we restricted our recordings to two motoneurons (termed aCC and RP2) that receive identical synaptic drive (Baines et al. 2001). The mean cell capacitance of both wildtype and sda motoneurons (a measure of cell size and potentially therefore cell development) was not statistically different (12.1 ± 0.7 vs. 11.9 ± 0.8 pF, WT vs. sda, P > 0.05). Voltage clamp recordings of SRCs in third instar larvae show a significant increase in both amplitude (55.6 ± 3.2 vs. 68.9 ± 5.4 pA/pF) and duration in sda (1.79 ± 0.3 vs. 2.7 ± 0.2 secs, Fig 2A-D, F-G). Analysis of mini’s (i.e. synaptic currents that remain in the presence of TTX) show no significant difference in amplitude (7.5 ± 1.6 vs. 6.2 ± 0.7 pA/pF, WT vs. sda, respectively, P < 0.05). This suggests that the increased amplitude and duration seen in SRCs is due to increased transmitter release from presynaptic interneurons. By contrast, frequency of SRCs was significantly reduced in sda (14.4 ± 2.1 vs. 6.0 ± 1.4 per min, Fig 2E). Analysis of larval crawling shows that third instar sda
larvae are significantly slower than their wildtype counterparts, reflective of the reduced frequency of motor excitation (3.2 ± 0.1 vs. 6.2 ± 0.2 cm per min peak speed, P < 0.01, W. Ockert and Baines, unpublished data). Unfortunately, our attempts to combine simultaneous electroshock with patch recording from motoneurons, in an attempt to determine what happens to network activity during a seizure-like episode has proven not possible (the shock causes loss of seal resistance on our patch recording). We conclude from these data that network activity in the motor pattern generator, that underlies movement in Drosophila larvae, is constitutively increased in strength but reduced in frequency in the sda mutation.

To determine how the increased magnitude SRCs in motoneurons influenced action potential firing, we repeated our recordings in current clamp, where membrane voltage is free to change. These recordings show that presynaptic excitatory drive produces sustained depolarisations in aCC/RP2 that fire multiple action potentials (Fig 3A) (Baines et al. 2001). The sustained depolarisations result from synaptic excitation of the motoneuron due to release of ACh from presynaptic pre-motor interneurons. The spikes that ride the depolarisation are action potentials fired by the postsynaptic motoneuron during this excitation (Baines et al. 1999). These spikes originate in the dendritic regions that are some distance away from the cell body, from where our recordings are made. This limits our ability to fully interpret the changes to spiking in these neurons due to changes in synaptic excitation that we observe using voltage clamp. Nevertheless, the depolarisations observed in sda are slightly larger in amplitude and considerably longer in duration and result in increased action potential firing (Fig 3B). This observation is consistent with the
increased amplitude and duration we observe in the synaptic drive to these motoneurons and indicates that they are exposed to significantly increased synaptic excitation in the sda mutant (Fig. 2).

Motoneurons in sda mutants show altered membrane conductances

The increased SRC amplitude and duration we record, in voltage clamp, in motoneurons in sda are indicative of increased transmitter release by presynaptic interneurons. Our current clamp recordings also show larger and longer sustained depolarizations, that are the result of the enhanced synaptic excitation. We also considered, however, the possibility that motoneurons are also intrinsically more excitable in sda. Consistent with this, a previous study reports that motor axon excitability in the bs-mutant, bang-senseless is increased (Ganetzky and Wu 1982).

We again used voltage clamp to determine whether changes in membrane conductances had also occurred in sda aCC/RP2 motoneurons (peak membrane conductances in these two neurons are near identical and data were pooled (Baines et al. 2001)). Initially, we isolated and recorded voltage-gated Na⁺, K⁺ and Ca²⁺ currents in first instars because they provide better space clamp (first instar sda show the same changes in SRC amplitude, duration and frequency as seen in third instars, data not shown). Values for each current component were: WT controls $I_{kf} 84.1 \pm 5.9$; $I_{ks} 64.0 \pm 5.5$; $I_{Ca} -21.2 \pm 2.7$; $I_{Nat} -24.1 \pm 2.7$; $I_{Nap} -4.6 \pm 0.4$. Sda $I_{kf} 77.4 \pm 14.2$; $I_{ks} 66.6 \pm 12.0$; $I_{Ca} -13.9 \pm 1.8$; $I_{Nat} -25.5 \pm 2.1$; $I_{Nap} -7.3 \pm 1.0$ pA/pF. Figure 4 shows normalised values for each current investigated, the comparative wildtype value being set at 100%. Any current value below 100% is reduced, and values above 100% are increased. A
particularly striking observation was that the persistent Na⁺ current (I_{Nap}) is significantly increased in sda (159%) whilst there is no change in the transient I_{Nat} (Fig 4C). This is notable because both current components are mediated by the same ion channel (paralytic, see Discussion). We also observed a significant reduction in I_{Ca} in sda (Figs 4A, D) but no change to I_{K} (Figs 4A, B).

To confirm that these changes are maintained in later third instar larvae (which we use for electroshock testing) we repeated our voltage clamp determination of both I_{Na} and I_{Ca} in aCC/RP2 at this later developmental stage. Despite the poorer space-clamp, we found that I_{Nap} is again significantly elevated in sda (14.2 ± 1.2 vs. 7.5 ± 0.8 pA/pF, P ≤ 0.01) whilst I_{Nat} remains unchanged from WT controls (see Fig. 5A,B). Significantly, however, I_{Ca} in sda was not different from WT in third instar larvae (-50.9 ± 7.8 vs. -48 ± 8.2 pA/pF, data not shown).

Whilst the reason that I_{Ca} is only reduced in first instar sda mutants remains unclear, it may be because of differential expression of Ca channels between young and older aCC/RP2 motoneurons. In embryos, the predominant I_{Ca} is sensitive to amiloride indicative of it being similar to vertebrate T-type channels (Baines and Bate 1998). However, by third instar an L-type I_{Ca} encoded by Dmca1D predominates (Worrell and Levine 2008). Even though the influence of sda on I_{Ca} remains to be fully explained, the consistent increase in I_{Nap} is indicative that this current underlies heightened seizure activity rather than the reduction in I_{Ca} which we observed only in first instar larvae.

To show experimentally how these changes might affect neuronal excitability, we used current clamp to inject depolarizing current and counted action potentials fired (i.e. input-output determination). We performed these experiments in both third (Fig. 4E) and first instars (data not shown) and
observed the same result. Injection of constant current into sda aCC/RP2 neurons result, on average, in fewer action potentials being fired compared to wildtype controls. Thus, we conclude that aCC/RP2 motoneurons, in sda, have an intrinsically reduced membrane excitability. However, whether this reduction is due to the increase in $I_{Na_p}$ in these neurons remains to be determined (see discussion). Regardless of precise mechanism, the reduction in intrinsic excitability in sda motoneurons (as evidenced from controlled injections of constant current, Fig 4E) is clearly insufficient to fully compensate for the heightened excitation that results from the increased endogenous synaptic drive we observe in this mutation (c.f. Fig 3).

Reduction of $I_{Na_p}$ rescues bang-sensitivity.

Our electrophysiology indicates that motoneurons in sda are exposed to increased synaptic excitatory drive. Drosophila motoneurons, similar to mammalian neurons, are able to compensate for increased synaptic excitation by reducing intrinsic membrane excitability (Baines 2003; Mee et al. 2004). In the case of Drosophila, increased synaptic excitation of motoneurons is countered, at least in part, by a homeostatic reduction in expression of voltage-gated Na$^+$ (VgNa$^+$) channels which manifests as a significantly reduced $I_{Na}$ in voltage clamp recordings (Mee et al. 2004; Muraro et al. 2008). Thus, from the outset we predicted that in sda, both the transient ($I_{Na_t}$) and persistent ($I_{Na_p}$) components of $I_{Na}$ would be reduced. Our analysis of intrinsic conductances in aCC/RP2, however, shows that this prediction does not hold true: $I_{Na_t}$ is unchanged and $I_{Na_p}$ increased.
It is significant for the behavioral phenotype associated with sda that an increased $I_{\text{Nap}}$ is already implicated in human seizure and, moreover, is the target of certain antiepileptic drugs, including phenytoin (Lampl et al. 1998; Segal and Douglas 1997). In order to test whether phenytoin affects this, and other, currents in *Drosophila*, we repeated our voltage clamp analysis of conductances in third instar aCC/RP2 with the added presence of phenytoin (30μM) in the bath saline. We found that this amount of phenytoin was sufficient to inhibit $I_{\text{Nap}}$ by ~43% ($7.0 \pm 1.0$ vs. $4.0 \pm 0.5$ pA/pF, control vs. phenytoin, n = 5, $P \leq 0.05$). By contrast, this amount of phenytoin did not significantly reduce $I_{\text{Nat}}$ nor $I_{\text{Ca}}$ ($I_{\text{Nat}}$: $34 \pm 5.7$ vs. $30 \pm 5.5$; $I_{\text{Ca}}$: $48 \pm 8.2$ vs. $61 \pm 4.7$ pA/pF, WT vs. sda, respectively, $P > 0.05$).

Having established that acute exposure to phenytoin is sufficient to selectively block $I_{\text{Nap}}$, we tested whether this AED might influence this current in intact larvae, and through doing so reduce seizure severity in sda. To do so we fed phenytoin to third instar larvae (0.4mg/ml for 24hrs) and initially used voltage-clamp to determine efficacy of action. Figure 5A shows that feeding phenytoin to sda again has no effect on the aCC/RP2 transient Na$^+$ current ($I_{\text{Nat}}$). As expected, however, phenytoin ingestion resulted in a significant decrease in $I_{\text{Nap}}$ in sda larvae (Fig. 5B). Analysis of $I_{\text{Ca}}$ showed no effect for phenytoin-feeding on this conductance ($50.9 \pm 7.8$ vs. $48.4 \pm 2.6$ pA/pF, vehicle control vs. sda, respectively, $P > 0.05$). We also noted that feeding phenytoin to wildtype third instar larvae paradoxically resulted in a significant increase in $I_{\text{Nap}}$ (with no significant change to $I_{\text{Nat}}$) (Fig 4B). We next calculated the $I_{\text{Nap}} / I_{\text{Nat}}$ ratio, with and without phenytoin, which is shown in Fig. 5C. We did this to normalise our data because the absolute value of both $I_{\text{Nat}}$ and $I_{\text{Nap}}$ varies from cell to cell.
After exposure to phenytoin, this ratio in sda is reduced from 45% to 21% which is approximately equivalent to wildtype. Treatment of WT wildtype with phenytoin increased this ratio to 50.7%. To determine whether the electroshock-induced seizure episode induced in sda larvae could also be ameliorated by treatment with phenytoin, we measured Mean Recovery Times in identically-treated third instars. Feeding phenytoin to sda larvae significantly reduced Mean Recovery Time (Fig. 5D). Thus, treatment of sda with phenytoin not only reduces INap to near control, non-bs, levels but also reduces the duration of the seizure episode induced by electroshock. We also measured the Mean Recovery Time of WT fed phenytoin, which increases INap (see above). WT fed with phenytoin exhibit a significantly longer Mean Recovery Time to electroshock indicative that phenytoin, when fed to wildtype, acts as a proconvulsive. The same phenomenon has also been reported in rat (Callaghan and Schwark 1980; Rundfeldt et al. 1990) Taken together, our data are consistent with INap being an important contributor to seizure-like behavior in this Drosophila bs-mutant.

Phenytoin rescues synaptic currents.

We show above that feeding of phenytoin ameliorated seizure-like behavior in sda larvae. In order to test whether feeding of phenytoin suppresses Mean Recovery Time by reducing network activity, SRMs of drug fed sda third instar larvae were recorded. Third instar larvae were allowed to feed on phenytoin (0.4mg/ml for 24 hrs) and then SRMs were recorded from aCC/RP2. Figure 6A shows the effects on SRC amplitude: significant reductions were seen in both WT and sda when fed phenytoin. Similar marked reductions were seen in synaptic current duration, (Fig 6B), whilst no significant changes were observed
for frequency (Fig 6C). We conclude that feeding phenytoin to sda reduced SRC amplitude and duration to below that seen in WT controls. This correlates well with the marked reduction we observe in Mean Recovery Time to electroshock in this genotype (Fig. 5D). Moreover, the fact that feeding phenytoin to wildtype larvae, which is proconvulsive, increases $I_{\text{NaP}}$ but also reduces SRC amplitude and duration is consistent with the former ($I_{\text{NaP}}$) contributing to seizure and not increased synaptic excitation.

Increasing $I_{\text{NaP}}$ generates a bs phenotype in wildtype larvae

To provide additional support for our hypothesis that seizure-like severity in sda is primarily influenced by the magnitude of $I_{\text{NaP}}$ we artificially increased this current component in WT third instar motoneurons and tested the effect this had on Mean Recovery Time to electroshock. To increase $I_{\text{NaP}}$, we fed third instars rATXII, a toxin known to selectively potentiate this current component in neurons (Hartung and Rathmayer 1985; Mantegazza et al. 1998) including Drosophila motoneurons (Mee et al. 2004). By contrast, acute (added to recording saline) or chronic (24hr feeding) exposure to ATX has no effect on $I_{\text{Ca}}$ as measured in third instar larvae (48 ± 8.2 vs. 46.3 ± 16.2pF/pF and 42.8±14.4 WT vs. ATX acute and ATX chronic, respectively, $P > 0.05$). Serial dilutions of rATXII were added to grape agar and WT third instars allowed to feed freely for 24 hours before electrophysiological determination of $I_{\text{NaP}} / I_{\text{Nat}}$ ratio or Mean Recovery Time to electroshock. Again we determined the $I_{\text{NaP}} / I_{\text{Nat}}$ ratio to normalise our data to compensate for any changes in $I_{\text{Nat}}$ that may also result from ingestion of this toxin (although this was minimal).
Our results show that there is a strong correlation between $I_{\text{Nap}} / I_{\text{Nat}}$ ratio and Mean Recovery Time (Fig 7). For example, feeding rATXII at 40nM and above results in a significant increase in $I_{\text{Nap}}$ ($I_{\text{Nat}}$ was unchanged and therefore the $I_{\text{Nap}} / I_{\text{Nat}}$ ratio increases) and also a correspondingly significant increase in Mean Recovery Time. Larvae fed rATXII below this dose (i.e. 10nM) are borderline statistically different from controls with respect to both ratio and Mean Recovery Time ($P = 0.05$). This dose-response indicates that the threshold for a seizure-like phenotype in WT larvae lies close to a $I_{\text{Nap}} / I_{\text{Nat}}$ ratio of ~35%. Above this level, Mean Recovery Time is significantly increased. This correlation provides strong support for a causal relationship between an elevated $I_{\text{Nap}}$ and an increased seizure-like phenotype. Thus, taken together our data indicates that an elevated $I_{\text{Nap}}$ contributes to increased seizure-like activity in sda. This bs-mutant may offer, therefore, an attractive model for understanding the role of this current in the aetiology of those human epilepsies with which it has been implicated.

Does a predisposition for seizure-like behavior arise from altered neural development?

Recent studies, in both rats and flies, provide support for the possibility that at least some forms of epilepsy are due to aberrant early neural development (Blumenfeld et al. 2008; Hekmat-Scafe et al. 2005). This possibility is significant because it offers a potential to cure these epilepsies through early drug intervention. To determine whether the seizure-like phenotype in sda might also arise as a consequence of altered embryonic neural development we attempted to influence this early stage. To do so we allowed gravid sda females to feed continually on food containing phenytoin (0.4mg/ml made up in DMSO). We
collected the eggs laid by these females on phenytoin-free agar plates and
allowed development to third instar to occur in the absence of drug. Our
rationale for doing this was to effect transfer of phenytoin to the developing
embryo only (see below). Remarkably, when tested for response to
electroshock at third instar, the offspring of such phenytoin-fed sda females
show a complete rescue of Mean Recovery Time relative to sda vehicle
(DMSO)-fed controls (Table 1). Because these third instar larvae were grown in
conditions that did not include drug for the duration of larval development, we
tentatively conclude that early treatment is sufficient to prevent the bs-
phenotype characteristic of this mutant. By contrast, feeding phenytoin to gravid
wildtype females resulted in offspring that showed greatly increased Mean
Recovery Times (Table 1) which recapitulates the effect of feeding wildtype
larvae this drug for 24hrs (c.f. Fig. 5). Although we have yet to fully understand
this effect, this observation is consistent with disturbed patterns of activity
during neurogenesis increasing the likelihood of seizures.

We consider it highly unlikely that the phenytoin fed to gravid females would
persist in appreciable amounts in larvae during development. However, to
measure how much might persist and, moreover, how much might be
transferred from adult female to oocyte we repeated the feeding regime with the
addition of radiolabelled $^{14}$C- phenytoin to the phenytoin used to feed the female
flies. We find that approximately 32% of the amount of drug ingested by adult
females is transferred to oocytes (based on a per mg protein basis). This figure
drops to just 6% by third instar (178, 58 and 11 cpm above background per mg
protein for adult, embryo and third instar, respectively). This level is equivalent
to feeding third instar larvae phenytoin at 0.02mg/ml (i.e. 6% of 0.4mg); a
concentration which is insufficient to rescue Mean Recovery Time to
electroshock when fed directly to sda third instar (data not shown). Thus, we
conclude that the amount of phenytoin present in third instar, the stage at which
our Mean Recovery Time determination is undertaken, is negligible and cannot
account for the rescue observed.

Because feeding phenytoin to gravid sda females effectively rescued Mean
Recovery Times in their progeny, we next asked what effect this treatment had
on both \( I_{\text{nap}} / I_{\text{nat}} \) ratio and SRC kinetics. Determination of the \( I_{\text{nap}} / I_{\text{nat}} \) ratio, in
aCC/RP2, shows a significant reduction relative to untreated sda third instar
(28% vs. 46%, respectively, Fig. 8A). Actual \( I_{\text{na}} \) current amplitudes recorded:
WT \( I_{\text{nat}} = -35.3 \pm 4.7 \); \( I_{\text{nap}} = -6.9 \pm 0.9 \); untreated sda \( I_{\text{nat}} = -31.8 \pm 5.1 \); \( I_{\text{nap}} = -14.6 \pm 
2.9 \); treated sda \( I_{\text{nat}} = -45.8 \pm 4.1 \); \( I_{\text{nap}} = -12.7 \pm 1.1 \) pA/pF. Analysis of SRCs show
that sda larvae from phenytoin-fed females are significantly (\( P \leq 0.05 \)) reduced
in both amplitude and duration, but show an unchanged frequency compared to
controls (progeny of sda females fed vehicle alone, which is itself proconvulsive
- see Table 1). Actual values are: vehicle-treated sda controls: 113.2 \pm 14.5,
2.05 \pm 0.38, 5.7 \pm 1.1; phenytoin-treated sda: 59.3 \pm 17, 0.76 \pm 0.08, 5.2 \pm 2.02
(amplitude, duration and frequency; pA/pF, secs and per min, respectively, \( n = 
4 \)).

We conclude that feeding of gravid sda females with phenytoin is sufficient to
suppress synaptic excitation of aCC/RP2 and to also reduce the \( I_{\text{nap}} / I_{\text{nat}} \) ratio
in their progeny to below the bs-threshold (~35%) and, by doing so, is able to
rescue Mean Recovery Time to electroshock. However, whilst the \( I_{\text{nap}} / I_{\text{nat}} \) ratio
was reduced in almost all cells examined (12/15 cells), closer examination of
individual cells recorded revealed an intriguing phenomenon. In 7/15 cells recorded, the ratio was reduced primarily due to a reduction in $I_{\text{Nap}}$. In almost all of the other cells, the ratio was decreased primarily by an increase in $I_{\text{Nat}}$ (Fig. 8B). Although we cannot yet explain this difference, the current component that changed seemingly correlates to which motoneuron was recorded: thus, reduction in $I_{\text{Nap}}$ was mostly observed in aCC, whilst an increase in $I_{\text{Nat}}$ was associated in RP2 (Fig. 8B). Regardless of the precise mechanism, this regulation is strongly indicative of an intrinsic capability of neurons to ‘determine’ the ratio of $I_{\text{Nap}} / I_{\text{Nat}}$ and effect change where this ratio lies outside of pre-determined physiological limits.

Discussion

Experimental validity of using *Drosophila* for human epilepsy research is incomplete. This is primarily because we do not have a good understanding of the specific neurological defects that underlie heightened seizure-like episodes in the many bs-mutants that are increasingly being used for such research. To address this shortfall, we have exploited a larval preparation that allows unprecedented access to characterise seizure-behavior and neuronal function in these mutant backgrounds. Our demonstration that $I_{\text{Nap}}$ is elevated in *sda* mutants is significant because this current is already implicated in human epilepsy and has long been utilised as a target for drug-intervention (George 2005; Rhodes et al. 2004). Our finding serves to validate the use of, at least this, bs-mutant as an appropriate model to further our understanding of human epilepsy and, excitingly, for the possible development of novel treatments.
Although extensively characterised in terms of behavior and genetics, the underlying causes of reduced seizure thresholds and prolonged Mean Recovery Times in bs-mutants is unknown (Song and Tanouye 2008). This is arguably because the adult fly has until recently been considered intractable for detailed electrophysiology of central neurons. By comparison, application of whole cell recording techniques has been possible in both the embryo and larvae of *Drosophila* for more than a decade (Baines and Bate 1998). This possibility offered us the opportunity to characterise, in detail, neuronal abnormalities present in bs-larvae. However, it was first essential that we convincingly show that bs-larvae share the bs-phenotype of their adult counterparts. To do so we used electroshock which clearly demonstrates that *sda* exhibit seizure-like episodes that last significantly longer than WT. It should be noted, however, that these episodes can be also induced in WT larvae, much as they can in wildtype adults, but that their severity is much reduced. In bs-adult flies, seizure-like activity can be elicited at lower stimulus intensity than in WT adults (Kuebler and Tanouye 2000).

Our analysis of *sda* shows clear alterations in membrane conductances in, and increased synaptic excitation of, motoneurons. It is tempting to speculate that these events are cause and effect, respectively. Increased synaptic excitation of motoneurons is indicative that the presynaptic interneurons that drive the motor output are more excitable, perhaps due to a similarly increased $I_{Nap}$ in these cells (although as these interneurons remain unidentified this is not yet testable). The voltage-gated Na$^+$ ($VgNa^+$) current in most neurons comprises both a rapidly inactivating transient $I_{Nat}$ and a smaller, but persistent, $I_{Nap}$ component (Catterall 2000). Whilst our functional understanding of the former is
comprehensive (underlying the depolarising phase of the action potential), the
function of the latter is less clear. $I_{\text{Nap}}$ is activated in the subthreshold voltage
range and is believed to contribute to plateau generation, pacemaker activity
and increased firing frequencies (Li and Bennett 2003; Li et al. 2004; Nikitin et
al. 2006; Tazerart et al. 2008).

Why then are $sda$ motoneurons, which have an increased $I_{\text{Nap}}$ not more
excitable than WT? Our previous work has identified homeostatic mechanisms
in *Drosophila* motoneurons which are capable of regulating intrinsic membrane
conductances to compensate for changing synaptic excitation (Baines et al.
2001). In particular, *Drosophila* motoneurons dynamically regulate expression of
$V_{\text{gNa}}^+$ channels, through control of translation of *paralytic* mRNA (that encodes
the sole $V_{\text{gNa}}^+$ channel in flies), in order to dampen or potentiate action
potential firing (Mee et al. 2004; Muraro et al. 2008). Based on these
observations, we would predict that exposure to heightened synaptic excitation
(observed in $sda$) would be compensated for by a reduction in ability to fire
action potentials in response to a fixed depolarizing input (Baines 2003; Baines
et al. 2001). Our observations of reduced action potential firing in these
motoneurons in response to injection of fixed depolarising current is, therefore,
validation of such homeostatic mechanisms. Unexpectedly, however, in $sda$ we
observed no change in $I_{\text{Nat}}$ and, indeed, an increased $I_{\text{Nap}}$. Regardless of these
uncertainties, analysis of endogenous synaptic drive in $sda$ aCC/RP2 clearly
show longer-lived plateau depolarizations that elicit increased action potential
firing. We conclude that the homeostatic mechanism which mediates the
apparent reduction in membrane excitability in $sda$ motoneurons, if present, is
not sufficient to fully compensate for the greatly increased synaptic drive that
these neurons are exposed to in this mutant.

This raises the interesting question of whether bs-behavior in sda is due to a
failure of homeostatic compensation. While this remains unclear, it seems likely
that such mechanisms, which act to fine-tune neuronal excitability, are
particularly important during embryonic development when neural networks first
form. Alterations in the balance of excitation and inhibition at these early stages
may initiate, through self-reinforcing cycles of abnormal activity, inappropriate
network activity that results in increased incidence of seizure-like activity
postembryonically (Blumenfeld et al. 2008). Our observations that the presence
of phenytoin during embryogenesis has either antiepileptic (sda) or proconvulsive
(WT) consequences provides support for such a hypothesis. It is possible that
reduction of \( I_{\text{Nap}} \) in backgrounds where it is elevated (i.e. sda) is sufficient to
reduce abnormal activity, whilst reduction below normal levels (ie. WT) might
conceivably evoke such an imbalance of activity. In the sda background, at
least, the mechanism of action of phenytoin is seemingly cell type-specific. The
aCC and RP2 motoneurons differ in a number of ways: target muscle, size of
synaptic terminal (1b/1s), resting potential, threshold and delay to 1st spike
(Schaefer et al.). Thus, it is perhaps not surprising that each neuron achieves a
reduction in \( I_{\text{Nap}} / I_{\text{Nat}} \) ratio through a different mechanism: a reduction of \( I_{\text{Nap}} \)
in aCC while an increase in \( I_{\text{Nat}} \) in RP2. It seems unlikely, however, that these
changes are brought about as a direct consequence of phenytoin either
blocking \( I_{\text{Nap}} \) or potentiating \( I_{\text{Nat}} \) because the animals from which we record
have developed in the complete absence of drug. More likely is that the
presence of phenytoin during neural development has contained the
hyperexcitability present in sda and this has allowed individual neuron types to
set an appropriate $I_{\text{Nap}}$ / $I_{\text{Nat}}$ ratio.

Perhaps the most striking observation that we report is that $I_{\text{Nap}}$ is increased in
$sda$ motoneurons, whilst $I_{\text{Nat}}$ remains unchanged. This is surprising because
both components represent different gating-modes of the same ion channel
(Mee et al. 2004; Vais et al. 2000). The *Drosophila* VgNa$^+$ channel, encoded by
just a single gene (*paralytic*) exhibits extensive alternative splicing (Lin et al.
2009; Loughney et al. 1989). We have shown recently that splicing of two
mutually exclusive membrane spanning exons located in domain III53-4 of the
channel protein (termed exons K and L) directly influence the magnitude of $I_{\text{Nap}}$
but not $I_{\text{Nat}}$ (Lin et al. 2009). Splice variants containing exon $k$ have a
significantly reduced persistent current compared to variants containing exon $L$.
Thus, we predict that a shift in splicing to favour inclusion of exon $L$ would result
in an increase in $I_{\text{Nap}}$ whilst $I_{\text{Nat}}$ remains unchanged. Verification of this
hypothesis will require isolation of splice variants from *sda* CNS.

In summary, human epilepsy is complex to both diagnose and treat because it
can arise from a multitude of causes, many of which remain unknown. This lack
of understanding may well underlie the fact that a significant number of patients
are refractory to drug intervention (Schmidt 2002). The use of mammalian
animal models for the development of novel antiepileptics, whilst appropriate for
modelling human epilepsy, has significant limitations. Chief amongst these are
cost and the extensive infrastructure required to screen large numbers. A
promising alternative is *Drosophila*. It has been estimated that the fly genome
contains ~75% of all human disease genes (Bier 2005), which include a number
of identified genes that when mutated lower seizure threshold (Song and Tanouye 2008). Additionally, *Drosophila* has long been used for high-throughput screens and a recent study has exploited this significant advantage to screen for novel antiepileptics (Stilwell et al. 2006). Our analysis of *sda* suggests that heightened seizures in this mutant are due, at least in part, to increased network activity and, in particular, an increased \( I_{\text{Nap}} \).

Acknowledgements

We are grateful to Prof. Mark Tanouye (Berkeley, CA) for support during the early stages of this work. We thank Dr Kevin O'Dell (Glasgow) for *sda* flies. We are also grateful to Waldemar Ockert for larval tracking data. Finally, we thank members of the Baines group and Louise Parker (Berkeley) for critical discussion and help in the writing of this paper. This work was funded by the BBSRC (UK).


Segal MM, and Douglas AF. Late sodium channel openings underlying epileptiform activity are preferentially diminished by the anticonvulsant phenytoin. *Journal of neurophysiology* 77: 3021-3034, 1997.


Figure 1. Both sda adult and larvae display a bs-phenotype.

A) Seizure-like activity can be induced in adult flies using mechanical shock. The Mean Recovery Time in sda is, however, significantly longer compared to wildtype (WT, 0.64 ± 0.3 vs. 40.5 ± 3.6 sec, n = 190 and 120). B) Electroshock of third instar larvae (50 V, 3 sec) is sufficient to elicit seizure-like episodes. The Mean Recovery Time is, again, significantly increased in sda (24.9 ± 3.4 vs. 164.9 ± 20 sec, n = 40).

Figure 2. sda motoneurons are exposed to increased synaptic excitation.

A, B) Typical examples of SRCs recorded in third instar (L3) aCC/RP2 (traces from RP2 in this instance) in wildtype (WT) and sda. Scale bars 200 pA / 2 sec. C) The mean amplitude of SRCs recorded is significantly greater in sda (55.6 ± 3.2 vs. 68.9 ± 5.4pA/pF). D) Duration of SRCs are also significantly increased in sda (1.79 ± 0.3 vs. 2.7 ± 0.2 sec). E) Frequency of SRCs observed are significantly reduced in sda (14.4 ± 2.1 vs. 6.0 ± 1.4 per min). For all means, n ≥ 8. F,G) Cumulative probability plots that better show the distribution of individual current amplitudes (F) and duration (G) for the averaged WT and sda data shown in C and D respectively.

Figure 3. Endogenous synaptic drive elicits greater excitation in sda.

A,B) Typical current clamp recordings, in which the membrane voltage is free to change, show sustained depolarisations that arise in aCC/RP2 due to presynaptic excitatory input (recordings shown from aCC in this instance). Notably these depolarisations are slightly larger and significantly longer in duration and fire considerably more action potentials (arrows) in sda.
motoneurons (B) compared to their WT counterparts (A). Scale bar = 10mV / 100 msec.

Figure 4. The persistent voltage-gated Na⁺ current (I_{Nap}) is elevated in sda motoneurons.

A) Analysis of individual voltage-gated conductances in first instar (L1) sda aCC/RP2 are shown relative to wildtype (WT, which are normalised to 100%). Of the conductances isolated, both I_{Ca} and I_{Nap} are statistically different. Values shown are: I_{Kf} 92.0, I_{Ks} 104.1, I_{Ca} 65.4, I_{Nat} 105.8, I_{Nap} 159%. n ≥ 8. B-D)

Overlaid traces for I_{K}, I_{Ca} and I_{Na} from WT (black lines) and sda (grey lines), normalised for cell capacitance and evoked by a depolarising step from -90mV to +40mV (I_{K}); to +10mV (I_{Ca}) and to -20mV (I_{Na}). The I_{Na} traces have been scaled to have the same size I_{Nat}. Scale bars 20 pA/pF (B,C) and 10 pA/pF (D) / 10 msecs. E) Membrane excitability of third instar aCC/RP2, determined by injection of constant current (4pA steps, 0-48pA/500msec) shows a decrease in action potential firing in sda. Control is WT. All values shown are means (± SE) for n ≥ 8. Representative traces showing the firing of action potentials by successively greater depolarizing current injections (12, 20 and 24pA/500msec) in a WT aCC neuron are shown. Dotted lines above each trace denote 0mV (i.e. spikes do not overshoot), Scale bar 10 mV and 100msec. A-D show data from first instars and E shows action potential firing from third instar larvae.

Figure 5. Phenytoin both reduces I_{Nap} in motoneurons and rescues seizure.
A) Feeding third instar sda larvae with phenytoin (PHY 0.4mg/ml) does not affect the peak amplitude of the transient voltage-gated Na\(^+\) current (I\(_{\text{Nat}}\)). Values shown are: WT 34.0 ± 3.1; WT/PHY 28.5 ± 3.8; sda 31.4 ± 3.4; sda/PHY 30.5 ± 6.9; pA/pF. B) PHY significantly reduces the peak amplitude of the persistent Na\(^+\) current (I\(_{\text{Nap}}\)) in sda. Perplexingly, PHY ingestion significantly increases I\(_{\text{Nap}}\) in WT. Values shown are WT 7.5 ± 0.8; WT/PHY 12.2 ± 1.2; sda 14.2 ± 2; sda/PHY 6.8 ± 1.5 pA/pF. C) Shows I\(_{\text{Nap}}\) / I\(_{\text{Nat}}\) ratios derived from the individual data points used to derive means in A and B. PHY significantly reduces this ratio in sda, but increases it in WT. Values shown are: WT 23.8; WT/PHY 50.7; sda 45; sda/PHY 21%; n ≥ 7 for all means. D) Feeding of PHY (0.4mg/ml) is sufficient to ameliorate seizures induced in sda as evidenced by a significant reduction in Mean Recovery Time. By contrast, feeding of PHY to WT increases Mean Recovery Time (i.e. has a pro-convulsive effect). Values shown are: WT 44.5 ± 5.3; WT/PHY 64.3 ± 7.7; sda 161.5 ± 15.4, sda/PHY 58.3 ± 8.4 sec. n ≥ 20 for all means.

Figure 6. Phenytoin rescues SRC amplitude and duration in sda.

A), SRC amplitudes, corrected for capacitance, are shown for both wildtype (WT) and sda fed phenytoin (PHY 0.4mg/ml) which is sufficient to significantly reduce SRC amplitude in both genetic backgrounds. The largest reductions are seen in sda, where mean amplitude in the presence of the drug is less than in unfed WT. Values shown are: WT 55.6 ± 3.2; WT/PHY 41.2 ± 5.5; sda 68.9 ± 5.5; sda/PHY 38.4 ± 5.5 pA/pF. B) Feeding PHY to either WT or sda also significantly reduces SRC duration. Again the largest reduction being seen in sda (WT 1.79 ± 0.3; WT/PHY 1.1 ± 0.1; sda 2.7 ± 0.2; sda/PHY 0.94 ± 0.14 sec) C) PHY has no statistically significant effect on SRC frequency, (WT 14.4 ± 2.2;
WT/PHY 10.9 ± 2.3; sda 6.0 ± 1.4; sda/PHY 3.3 ± 1.06 per min). n ≥ 7 for all means.

Figure 7. Seizure severity correlates to the ratio of $I_{Nap} / I_{Nat}$.

A) Manipulation of the $I_{Nap} / I_{Nat}$ ratio, achieved by feeding rATX to wildtype (WT) third instars, shows a clear relationship between the magnitude of ratio and the Mean Recovery Time for seizure-like activity evoked by electroshock. All Mean Recovery Times shown (10mM to 100mM rATX) are significantly increased relative to WT (i.e. no rATX). Thus, we suggest that based on this data, an $I_{Nap} / I_{Nat}$ ratio of ~35% represents the minimum level above which we predict a bs-phenotype. N ≥ 20 and 6 (time and ratio, respectively).

B) Representative voltage clamp recordings of $I_{Na}$ at varying concentrations of rATX. To better show the selective increase in $I_{Nap}$, all recordings have been scaled to have the same size $I_{Nat}$.

Figure 8. Maternal feeding of phenytoin reduces the ratio of $I_{Nap} / I_{Nat}$ in F1 progeny.

A) Feeding gravid sda females phenytoin (0.4mg/ml) reduces the $I_{Nap} / I_{Nat}$ ratio recorded in aCC/RP2 in their third instar progeny. From the point of egg-laying to recording, the progeny were not exposed to exogenous phenytoin. The plots show the distribution of ratios determined for WT (WT), sda and sda born from phenytoin-treated mothers (sda-treated). Vertical lines show the population mean (21, 45 and 29%, n = 8, 7 and 15, respectively). sda-treated is significantly different from sda (P ≤ 0.01), but not from WT (P > 0.05). B) shows the magnitude of both $I_{Nap}$ and $I_{Nat}$ recorded in the 15 cells from sda-treated mothers shown in (A). Magnitudes are compared to sda L3 born from untreated
mothers (values for both currents set to 100%). In 7/15 cells (data group 1) $I_{\text{Nap}}$ is reduced relative to $I_{\text{Nat}}$. In 7/15 cells (data group 2), $I_{\text{Nat}}$ is increased relative to $I_{\text{Nap}}$. Open circles represent recordings from aCC whilst closed circles denote RP2.

Supplementary video 1: Wildtype third instar larval response to electroshock: video shows a crawling third instar WT larvae receiving a 15V, 3s electrical shock to the cuticle via tungsten wires. Shock is administered between 6-9s and full recovery is considered to have occurred once free crawling resumes at 42 seconds. Timings are taken from the start of the video.

Supplementary video 2: sda third instar larval response to electroshock: video of crawling third instar sda larvae receiving a 15V, 3S electrical shock to the cuticle via tungsten wires. Shock administered between 20-23s and full recovery is considered to have occurred once crawling, with a full body peristaltic wave, resumes at 158s (2min 38s). Note prolonged lack of co-ordinated body movement post electroshock. Timings are taken from the start of the video.
Figure 1

A) Adult

B) Larvae

Mean Recovery Time (s)

WT  sda

**
Figure 2

A. WT: L3

B. sda: L3

C

D

E

F

G

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Figure 2
Marley and Baines
Figure 3

A. WT

B. sda
Figure 4

A sda (1st instar)

% of Control

IKf IKs ICa INat INap

B (1st instar)

IKf IKs

WT sda

C (1st instar)

INa INat INap

WT sda

D (1st instar)

ICa

E (3rd instar)

Action potentials

current injected (pA)

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Figure 4
A \( I_{\text{Nat}} \)

B \( I_{\text{Nap}} \)

C \( I_{\text{Nap}} / I_{\text{Nat}} \) ratio

D Mean Recovery Time (s)
Figure 6

A

Amplitude (pA/pF)

WT WT PHY sda sda PHY

B

Duration (s)

WT WT PHY sda sda PHY

C

Frequency (per min)

WT WT PHY sda sda PHY

Marley and Baines
Table 1: Maternal drug feeding suppresses seizure in sda.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MRT (sec)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>31.2 ± 3.8$^a$</td>
<td>40</td>
</tr>
<tr>
<td>WT + DMSO</td>
<td>86.5 ± 3.8$^a$</td>
<td>15</td>
</tr>
<tr>
<td>WT + PHY</td>
<td>187 ± 26.6$^a$</td>
<td>20</td>
</tr>
<tr>
<td>sda</td>
<td>203.5 ± 18$^b$</td>
<td>40</td>
</tr>
<tr>
<td>sda + DMSO</td>
<td>267.3 ± 41.4$^{b,c}$</td>
<td>15</td>
</tr>
<tr>
<td>sda + PHY</td>
<td>86.5 ± 12.0$^c$</td>
<td>20</td>
</tr>
</tbody>
</table>

Feeding phenytoin (PHY, 0.4mg / ml) to gravid females results in progeny that do not display increased seizures. Progeny were electroshocked at third instar and were never exposed to phenytoin, other than what might have been transferred to them from the adult fly. The number of flies tested for each genotype is stated (n). Wildtype (WT) and sda fed vehicle only (DMSO), exhibit increased Mean Recovery Time (MRT) indicative that DMSO has a proconvulsive effect. WT fed PHY show a greatly increased MRT indicative that this compound is proconvulsive when fed to normal non-bs animals (see Results text for more details). Letters show pair-wise significances, $P \leq 0.01^{a,c}$; $P \leq 0.05^b$. 