Seizure suppression through manipulating splicing of a voltage-gated sodium channel

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Seizure can result from increased voltage-gated persistent sodium current expression. Although many clinically-approved antiepileptic drugs target voltage-gated persistent sodium current, none exclusively repress this current without also adversely affecting the transient voltage-gated sodium current. Achieving a more selective block has significant potential for the treatment of epilepsy. Recent studies show that voltage-gated persistent sodium current amplitude is regulated by alternative splicing offering the possibility of a novel route for seizure control. In this study we identify 291 splicing regulators that, on knockdown, alter splicing of the Drosophila voltage-gated sodium channel to favour inclusion of exon K, rather than the mutually exclusive exon L. This change is associated with both a significant reduction in voltage-gated persistent sodium current, without change to transient voltage-gated sodium current, and to rescue of seizure in this model insect. RNA interference mediated knock-down, in two different seizure mutants, shows that 95 of these regulators are sufficient to significantly reduce seizure duration. Moreover, most suppress seizure activity in both mutants, indicative that they are part of well conserved pathways and likely, therefore, to be optimal candidates to take forward to mammalian studies. We provide proof-of-principle for such studies by showing that inhibition of a selection of regulators, using small molecule inhibitors, is similarly effective to reduce seizure. Splicing of the Drosophila sodium channel shows many similarities to its mammalian counterparts, including altering the amplitude of voltage-gated persistent sodium current. Our study provides the impetus to investigate whether manipulation of splicing of mammalian voltage-gated sodium channels may be exploitable to provide effective seizure control.

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Abbreviations: AED = antiepileptic drug; CDK = cyclin-dependent kinase; DmNav = Drosophila melanogaster voltage-gated sodium channel; INaP = voltage-gated persistent sodium current; INaT = transient voltage-gated sodium current; JAK/STAT = Janus tyrosine kinase/signal transducer and activator of transcription; mTOR = mammalian target of rapamycin; NaP = voltage-gated sodium channel

Introduction

Although mutations in more than 60 genes have been linked to epilepsy (Noebels, 2003), a principle commonality underlying seizure generation is neuronal hyperexcitability. Multiple lines of evidence implicate an abnormal increase in neuronal voltage-gated persistent sodium current (INaP) directly contributes to hyperexcitability and, as such, this current component is an attractive target for antiepileptic drug (AED) design (Chen et al., 2001; Stafstrom, 2007). However, to date, no clinically approved AEDs are available to selectively target INaP without also impacting...
transient voltage-gated sodium current \( (I_{\text{NaT}}) \), which is critical for normal action potential firing.

It is well established that voltage-gated sodium \( (\text{Na}_v) \) channels rapidly inactivate after brief openings following depolarization of the neuronal membrane. What is less well understood is the mechanism through which inactivated channels briefly reopen to mediate \( I_{\text{NaP}} \) (Chen et al., 2001; Stafstrom, 2007). Regardless of these uncertainties, it is known that although \( I_{\text{NaP}} \) carries considerably less current than \( I_{\text{NaT}} \), its presence can have a profound influence on membrane excitability as it is able to keep a neuronal membrane depolarized for long periods of time (Yue et al., 2005). Indeed, the relative potency of clinically used AEDs such as phenytoin, valproate and lamotrigine almost certainly derive from their ability to potently reduce this conductance, in addition to inhibiting \( I_{\text{NaT}} \) (Chao and Alzheimer, 1995; Taverna et al., 1998; Spadoni et al., 2002).

Understanding the molecular machinery that regulates \( I_{\text{NaP}} \) is poor, which is partly because of the relative complexity generated by the presence of multiple sodium channel genes (SCN1A–SCN11A) in the mammalian genome, all of which show differing levels of this current (Goldin, 2001; Lin and Baines, 2015). Several lines of evidence suggest that \( I_{\text{NaP}} \) amplitude can be regulated by mRNA alternative splicing. For example, splicing at exon 5 in human SCN1A is mutually exclusive with the choice of either exons 5A or 5N (for adult and neonatal). Heterologous expression of human SCN1A-5N in HEK293T cells, produces channels that exhibit more rapid inactivation and reduced \( I_{\text{NaP}} \) compared to SCN1A-5A (Fletcher et al., 2011). Alternative splicing in this region (exon 5 or 6) is also observed in SCN2A, SCN3A, SCN8A and SCN9A in both humans and mice (Sarao et al., 1991; Yarowsky et al., 1991; Gustafson et al., 1993; Kasai et al., 2001; Raymond et al., 2004). Intriguingly, the observed increased inclusion of exon 6N in both Scn2a and Scn3a following electrical or kainite-induced seizure in adult rat hippocampus implies a correlation between splicing and seizure generation (Gastaldi et al., 1997; Aronica et al., 2001). A somewhat clearer picture of how splicing affects \( I_{\text{NaP}} \) has emerged from studies in Drosophila melanogaster (Lin et al., 2009, 2012). In contrast to mammals, insects contain only one \( \text{Na}_v \) channel homologue, encoded by paralytic (\( Dm\text{Na}_v \), currently known as \( \text{para} \) ) (Feng et al., 1995). Splicing at exon 25 in \( Dm\text{Na}_v \) mirrors that observed at exon 5 in SCN1A: one of a pair of mutually-exclusive exons (termed K and L in the fly) encodes region S3–4, which contributes to the voltage sensor. Channels containing exon L exhibit significantly larger \( I_{\text{NaP}} \) compared to those containing exon K, with no change in \( I_{\text{NaT}} \) (Lin et al., 2009). Increased inclusion of exon L, along with an enlarged \( I_{\text{NaP}} \) in motoneurons, is characteristic of bang-sensitive mutants (e.g. \( \text{sda} \) and \( \text{eas} \)) that exhibit lower seizure threshold and increased seizure duration in response to electric shock (Lin et al., 2012). Splicing of exon 25 is, moreover, activity-dependent with activity increasing inclusion of exon L, which in turn increases action potential firing leading to a reinforcing positive feedback. Manipulating splicing to increase exon K expression uncouples this feedback cycle, reduces \( I_{\text{NaP}} \) and rescues seizure-like behaviour in these same seizure mutants (Lin et al., 2012).

Splicing at exon 25 is modified by pasilla, a K homology (KH) domain-containing RNA binding protein (Park et al., 2004; Lin et al., 2012). Knockdown of pasilla expression increases inclusion of exon K, decreases \( I_{\text{NaP}} \) and, importantly, provides effective rescue of seizure (Lin et al., 2012). Thus, understanding the regulatory mechanisms that orchestrate splicing in \( \text{Na}_v \) transcripts may be exploitable for the design of AEDs that have high specificity for targeting \( I_{\text{NaP}} \). The mammalian homologues of pasilla, NOVA1 and NOVA2, also regulate SCN alternative splicing (Ule et al., 2003, 2006). Like pasilla, NOVA recognizes YCAY motifs located in introns (which flank both exon 5/6 in mammalian SCNs and exon 25 in \( Dm\text{Na}_v \)). Moreover, a number of observations link NOVA function with epilepsy. Mesial temporal lobe epilepsy has been associated with an upregulation of NOVA2 and SCN1A-5N transcript abundance (Heinzen et al., 2007). Perturbation of NOVA steady-state levels in \( \text{Nova}^{2+/-} \) heterozygous mice gives rise to cortical hyperexcitability and to spontaneous generalized seizure discharge (Eom et al., 2013). NOVA localization shifts from primarily nuclear to cytoplasmic within hours after pilocarpine-induced seizure (Eom et al., 2013). These, and additional, observations highlight an important and perhaps exploitable relationship between SCN mRNA splicing, NOVA and epilepsy. The conservation of function between pasilla and NOVA offers the opportunity to use the tractability of Drosophila to rapidly identify underlying signalling pathways.

In this study, we generated luciferase-based mini-genes to report splicing at exon 25 in \( Dm\text{Na}_v \). Expression in S2R+ cells and exposure to a Drosophila double-stranded RNA library identified 291 genes that, on knockdown, increased inclusion of exon K (sufficient to reduce \( I_{\text{NaP}} \)). Expression of RNA interference (RNAi) \( \text{in vivo} \) shows that knockdown of 95 of these genes provides significant behavioural rescue of induced-seizure in two bang-sensitive mutants. We further show that small molecule inhibitors of the protein products of some of the targeted genes are effective anticonvulsants.

### Materials and methods

#### Mini-gene construction

Genomic DNA was extracted in 50μl extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl and 200 μg/ml proteinase K) and incubated at 37°C for 30 min. \( Dm\text{Na}_v \) genomic DNA, spanning exon 24 to exon 26, was amplified by PCR (Phusion® High-Fidelity DNA Polymerase, New England Biolabs) that consisted of the following in a total volume of 50μl: 20 pmol primers, dNTPs at 0.2 mM each, and 1×...
Phusion HF buffer with 1.5 mM Mg$^{2+}$. Forward primer (5'-gactgctgtgaccATGGCATAGAAGTCTAGTCGGCGC-3'), located at exon 24, introduced a KpnI site and a translational initiation codon. Reverse primer (5'-gttggcgcgcgcgcgcgctgATC TAAATATTTTCCAGCAAAAAGCTG-3'), located at exon 26, introduced an XbaI and NotI sites. Cycling conditions were: initial denaturation at 98°C for 5 min; 35 cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 4 min; a final extension step at 72°C for 10 min. The PCR product was digested with KpnI and NotI and ligated into pBluescript® II KS vector (Stratagene Inc). A Luciferase reporter gene, renilla or firefly, was inserted in-frame to the 3' end of exon 26. Both renilla and firefly genes were PCR amplified and XbaI and NotI sites introduced at the 5' and 3' ends, respectively. The primer pairs (5' to 3') are: renilla, gcggccgcTTATTGTTCATTTTTGAG TATCCAGAA and gttggcgcgcgcgcgcgctgTTATGGTTCATTTTTGAG AACTCGCTC; firefly, gcggccgcTTATTGTTCATTTTTGAG TATCCAGAA and gttggcgcgcgcgcgcgctgTTATGGTTCATTTTTGAG AACTCGCTC. To report K exon expression, (K-renilla mini-gene) a termination codon was inserted in exon L by site-directed mutagenesis. In the same way, a termination codon was introduced at the 5' end of exon 24, introduced a KpnI site and a translational initiation codon. The 5'-ATG 0-3-384 well plates) for 48 h and followed by co-transfection (Effectene®, QIAGEN) of K-renilla and L-firefly mini-genes (10 ng each) for a further 48 h. The transfection procedure is as described in the manufacturer’s instructions (QIAGEN). S2R+ cells were lysed with 0.35% Triton™ X-100 in BL buffer (50 mM HEPES, 0.5 mM EDTA, 0.36 mM phenylacetic acid and 0.07 mM oxalic acid) and coelenterazine-h (3 μM, Promega) added to measure K-renilla luciferase activity. Renilla-luciferase activity declined completely after 10 min and β-Luciferin (0.46 mM, Molecular Probes) was then added to measure L-firefly luciferase activity. A Varioskan™ flash plate reader (Thermo Scientific) was used to measure luminescence.

**Determination of exon inclusion**

The determination of ratio of exon K to exon L inclusion in DmNa, from whole CNS is described in Lin et al. (2012).

**Quantitative PCR**

Quantitative PCR was performed using SYBR Green I real-time PCR method (Roche, LightCycler® 480 SYBR® Green I Master). The Ct values, as defined by the default setting, were measured using a LightCycler® 480 II real-time PCR (Roche) using a thermal profile of 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 10 s at 60°C, and 10 s at 72°C. Single-product amplification was confirmed by post-reaction dissociation analysis. PCR primers were designed with the aid of LightCycler® Probe Design Software 2.0 (v1.0) (Roche). Primer sequences (5' to 3') are listed in Supplementary Table 1. Relative gene expression was calculated using the 2-ΔΔCt, where ΔCt was determined by subtracting the average Rp49 Ct value from that for each gene.

**Fly stocks**

Flies were maintained on standard cornmeal medium at 25°C. Bas$^1$ and bss$^1$ were gifts from Dr Kevin O’Dell (University of Glasgow). Wild-type was Canton-S. The UAS-RNAi pasilla (stock no. 33.426) was obtained from Bloomington and all other UAS-RNAi lines (Supplementary Table 2) were obtained from the Vienna Drosophila Resource Centre. Bas$^1$, Gal4$^{Cba}$ and bss$^1$, Gal4$^{Cba}$ were derived by crossing Bas$^1$ (bang sensitive) or bss$^1$ (bang sensless) with Cha$^{B19}$-Gal4 (gift from Dr Paul Salvaterra, City of Hope, USA).

**Behavioural screening on bang sensitive mutants**

Twenty virgin females of bas$^1$, Gal4$^{Cba}$ were crossed with five UAS-RNAi males. Because bas$^1$ is on the X chromosome and heterozygous bas$^1$/+ females show significantly reduced mean recovery time (28.3 ± 4.3 s), we used bas$^1$/Y hemizygous males (232.7 ± 26.2 s) for the behavioural screening. Flies (2–3 days old) were tested at least 1 day after collection to ensure total recovery from CO₂-anaesthesia. Flies were transferred to an empty vial and left to recover for 30 min before mechanical shock by vortexing the vial at maximum speed for 10 s. Mean recovery time was calculated from the average time taken for all 10 flies to recover from paralysis to standing. At least three replicates were performed for each RNAi line. Values were compared to control flies (bas$^1$/Y; Gal4$^{Cba}$/+) by ANOVA with Tukey’s post-test. Results were deemed significant at either *P < 0.05 or **P < 0.01. In the same way, we cross virgin females of bss$^1$, Gal4$^{Cba}$ with UAS-RNAi males and the F1 male flies (bas$^1$/Y; Gal4$^{Cba}$/UAS-RNAi) were tested.
Acute exposure of chemical inhibitors

Groups of 10 young adult male flies (bas^1/Y) within 8 h of eclosion were placed in an empty vial containing filter paper soaked with sucrose (5%) and drug. Flies were kept in the vial for 24 h at which point the filter paper was removed. Flies were left to recover for 30 min before being vortexed. Mean recovery times and statistical significance were determined as described above. The chemical inhibitors and the solvent used were: phenytoin (D4505, Sigma) dissolved in H2O/0.1 N NaOH solution (3:1); dipyriramole (D9766, Sigma) and rapamycin (10798668, Fisher Scientific) dissolved in ethanol; etoposide (E1383, Sigma) dissolved in ethanol/DMSO solution (5:1); isothionate (PZ0199, Sigma) and antipain dihydrochloride (A6191, Sigma) dissolved in H2O. These solvents were also fed to the respective control (bas^1/Y) flies and did not show significant effect to mean recovery time.

Electrophysiology

Methods used to identify anterior corner cell motorneurons and isolate and record sodium currents are described in Marley and Baines (2011).

Results

Mini-gene reporters for splicing of exons K and L

To identify regulators of splicing at exon 25 (i.e. exons K or L) in DmNav, we constructed two mini-gene reporters (Fig. 1A). Each reporter, driven by an actin promoter, contains DmNav genomic DNA spanning exon 24 to exon 26 connected in-frame to a luciferase reporter gene (renilla or firefly) and a translational initiation codon artificially introduced in exon 24. In K-renilla, a termination codon was introduced in exon L, such that inclusion of exon K leads to expression of a mRNA encoding a renilla-fusion protein, while inclusion of exon L results in a truncated, and non-functional, transcript. In the same way, a termination codon was introduced in exon K in L-firefly, such that inclusion of exon L expresses a mRNA encoding a firefly-fusion protein, while inclusion of exon K results in a truncated protein. The ratio between renilla:firefly luciferase activities effectively reports the K:L ratio.

To determine functionality of the mini-gene cassettes, we transfected them into S2R+ cells and confirmed both renilla and firefly luciferase activity (Fig. 1B and C). Knockdown of pasilla predictably altered the K:L ratio to favour increased inclusion of K (K:L 1.9 ± 0.2) compared to untreated (which was set at 1) or control double-stranded RNA treated cells (0.8 ± 0.1) (Fig. 1B-D). RNAl-mediated knockdown of pasilla also results in reduced expression of both renilla and firefly luciferase reporters to 46% and 25% (n = 5, P < 0.01), respectively, compared with untreated cells (Fig. 1B and C). Indeed, this was a common effect noted with many of the double-stranded RNAs that we tested (the reduction is quantified in Supplementary Table 2). Regardless of effect to expression level, our results confirm that S2R+ cells have the required machinery to splice exons K and L in DmNav, and that the mini-genes effectively report this splicing event.

A genome-wide RNAi screen to identify regulators of splicing

Using a Drosophila double-stranded RNA genome-wide library (Heidelberg 2, BKN) (Horn et al., 2010), we treated S2R+ cells with ~21 000 double-stranded RNAs (~98.8% coverage, covering ~14 000 protein encoding genes and ~1000 non-coding genes) for 48 h, followed by co-transfection of K-renilla and L-firefly mini-genes for a further 48 h. The ratio of K-renilla:L-firefly was then determined. We performed two replicates of screening and used criteria (K:L ratio ≥ 1.9 and Z-score > 1.5) to identify double-stranded RNAs that exhibited a similar or greater effect than double-stranded RNA pasilla. We identified 299 double-stranded RNAs (291 genes, ~1.4% of the genome) which satisfied these criteria (Supplementary Table 2). Gene Ontology Annotation (Boyle et al., 2004; Camon et al., 2004) classifies these into 11 categories, including transcription/translation, post-transcriptional/post-translational modification, cell signalling, cell cycle, metabolism, oogenesis, cellular scaffolding and ion transport (Fig. 2). Twenty-one per cent of the target gene products (i.e. proteins) are involved in post-transcriptional modification, including mRNA alternative splicing, polyadenylation and mRNA localization. This represents an enrichment compared to the genome, which contains 2.8% of genes involved in RNA processing (Fig. 2) (Boyle et al., 2004). Notably, we identified pasilla validating our screen methodology. Furthermore, some transcripts, for example Not1 (CG1884) and crowded by cid (CG5970), were hit twice by double-stranded RNAs (BKN20186 and BKN25930, BKN27434 and BKN46065, respectively) targeted to different regions.

Behavioural screen to verify RNA interference targets influence seizure

The unidentified bas^1 Drosophila mutation exhibits seizure-like behaviour when adult flies are exposed to strong sensory stimuli (e.g. vortexing) (Grigliatti et al., 1973; Parker et al., 2011). As previously stated, manipulations that increase inclusion of exon K rescue seizure-like behaviour in bang-sensitive mutants (Lin et al., 2012). To test whether knockdown of the 291 genes, identified in our double-stranded RNA screen, similarly rescue seizure in a bang-sensitive mutant, we performed a behavioural screen by expressing UAS-RNAi constructs in cholinergic neurons.
We individually determined the mean recovery time of 265 RNAi candidates, which are currently available from the Vienna Drosophila Resource Centre. As expected, knockdown of pasilla significantly rescues seizure duration (133.3 ± 91 versus 1415 ± 244 versus 614 ± 39 s, untreated versus control double-stranded RNA versus pasilla double-stranded RNA, respectively) and L-firefly (18 ± 0.1 ± 858 versus 23 ± 0.1 ± 1274 versus 4552 ± 548 units, untreated versus control double-stranded RNA versus pasilla double-stranded RNA, respectively) in S2R + cells. pasilla double-stranded RNA altered the K:L ratio to favour increased inclusion of K (K-renilla:L-firefly ratio 1.9 ± 0.2) compared to untreated (which was set at 1) or control double-stranded RNA treated cells (0.8 ± 0.1). Control double-stranded RNA used is BKN21565 (CG11360), known to regulate splicing of DmNav exons 11 and 12 but not exon 25 (Park et al., 2004). Values (n = 3, mean ± SEM) were compared by a Student’s t-test and results were deemed significant at **P ≤ 0.01. dsRNA = double-stranded RNA.

The principle excitatory neurotransmitter of the insect CNS in \textit{bas} is L-glutamate. We individually determined the mean recovery time of 265 RNAi candidates, which are currently available from the Vienna Drosophila Resource Centre. As expected, knockdown of pasilla significantly rescues seizure duration (133.3 ± 91 versus 1415 ± 244 versus 614 ± 39 s, untreated versus control double-stranded RNA versus pasilla double-stranded RNA, respectively) and L-firefly (18 ± 0.1 ± 858 versus 23 ± 0.1 ± 1274 versus 4552 ± 548 units, untreated versus control double-stranded RNA versus pasilla double-stranded RNA, respectively) in S2R + cells. pasilla double-stranded RNA altered the K:L ratio to favour increased inclusion of K (K-renilla:L-firefly ratio 1.9 ± 0.2) compared to untreated (which was set at 1) or control double-stranded RNA treated cells (0.8 ± 0.1). Control double-stranded RNA used is BKN21565 (CG11360), known to regulate splicing of DmNav exons 11 and 12 but not exon 25 (Park et al., 2004). Values (n = 3, mean ± SEM) were compared by a Student’s t-test and results were deemed significant at **P ≤ 0.01. dsRNA = double-stranded RNA.
mutation. This mutant carries a missense (hypomorphic) mutation of DmNav, and exhibits the most severe seizure-like phenotype of any bang-sensitive Drosophila mutant (Parker et al., 2011b). Ninety-five RNAi lines, including RNAi pasilla, rescue seizure behaviour in bss\(^{1}\) (Fig. 5). In general, RNAi lines that effectively rescued mean recovery time in bas\(^{1}\) are similarly effective in bss\(^{1}\). The degree of seizure rescue observed in both genetic mutants (i.e. line of best fit) shows a relationship that is significantly different to zero at \(P \leq 0.01\) (zero representing a horizontal, no correlation, line) (Fig. 5). Of the 97 UAS-RNAis we tested, 95 lines (98%) significantly rescued mean recovery time (\(P \leq 0.05\)) in both bas\(^{1}\) and bss\(^{1}\) mutants. According to Gene Ontology annotation (Boyle et al., 2004; Camon et al., 2004), 20% of these 95 RNAis are classified into post-transcriptional modification category (Fig. 2). Twelve UAS-RNAi lines produced particularly strong rescue in both bang-sensitive mutants (>60% rescue, identified as solid circles in Fig. 5): the most effective amongst these were Cell division cycle 5 ortholog (CG6905), Syncrip (CG17838), CG5418 and eIF4AIII (CG7483) (Supplementary Table 3). Genes that, when knocked down, potently rescue seizure duration in both mutants are likely to work through well-conserved pathways and may, therefore, be optimal candidates to take forward to mammalian seizure studies.

### Rescue of seizure by small molecule inhibitors

The ability of known AEDs to suppress seizure in Drosophila provides further validation that this insect model is appropriate to identify and evaluate new anticonvulsant compounds (Reynolds et al., 2004; Marley and Baines, 2011). Our double-stranded RNA screen has identified a number of genes, the protein products of which are already the subject of study for novel AED design. These include PDE11, RAPTOR (TOR-signalling), TOPO II, cyclin-dependent kinase 4 (CDK4) and CG11110. All significantly reduced bas\(^{1}\) seizure duration to 133.3 ± 4.4, 66.9 ± 11.2, 105.1 ± 43.2, 158.6 ± 39.6, 89.7 ± 29.3 and 39.2 ± 21.6 s, respectively. Values (mean ± SD for \(n = 5\)) were compared by ANOVA with Tukey’s post-test and results were deemed significant at *\(P \leq 0.05\) or **\(P \leq 0.01\).

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**Figure 2** DmNav splicing regulators. Two hundred and ninety-nine double-stranded RNAs (targeting 291 genes, ~1.4% of the genome) increased inclusion of exon K in DmNav, (black bar). Ninety-five of these corresponding UAS-RNAi lines rescue seizure duration in both seizure mutants, bang sensitive\(^{1}\) and bang senseless\(^{1}\) (white bar). These genes can be classified into 11 categories according to Gene Ontology annotation (Boyle et al., 2004; Camon et al., 2004). The percentages with categories are indicated. See Supplementary Table 2 for details of these genes. dsRNA = double-stranded RNA.

**Figure 3** UAS-RNAi lines rescue induced-seizure duration of bas\(^{1}\) mutant flies. Flies were subjected to a mechanical shock (10 s vortex) and the mean recovery time (MRT) was measured. Bas\(^{1}\)/Y and bas\(^{1}\)/Y;Gal4\(^{Chw}\)/+ (denoted bas/Gal4) male flies show similar mean recovery times (232.7 ± 26.2 and 238.8 ± 31.5 s, respectively). UAS-RNAi lines shown knockdown gene expression of pasilla, phosphodiesterase 11 (PDE11), raptor, topoisomerase II (topo II), cyclin-dependent kinase 4 (CDK4) and CG11110. All significantly reduced bas\(^{1}\) seizure duration to 133.3 ± 4.4, 66.9 ± 11.2, 105.1 ± 43.2, 158.6 ± 39.6, 89.7 ± 29.3 and 39.2 ± 21.6 s, respectively. Values (mean ± SD for \(n = 5\)) were compared by ANOVA with Tukey’s post-test and results were deemed significant at *\(P \leq 0.05\) or **\(P \leq 0.01\).
anticonvulsive in Drosophila. To do so, we identified known chemical inhibitors and fed these to bas\(^1\) mutant flies. Drugs used were dipyridamole (phosphodiesterase inhibitor), rapamycin (inhibit TOR-signalling), etoposide (Topo II inhibitor), isethionate (CDK4 inhibitor), and antipain (serine-type peptidase inhibitor). Exposure of adult bas\(^1\) flies to these drugs, 24 h before testing, show that each is sufficient to produce a dose-dependent and significant reduction in seizure duration comparable to phenytoin, a potent anticonvulsant in both flies and mammals (Fig. 6). The amount of drug that each fly ingested was not measured and is, therefore, unknown. That these drugs, which target the protein products of the genes identified in our screen, are effective anticonvulsants not only validates our screen, but provides significant confidence that we have identified many additional, but as yet uncharacterized proteins that may prove to be exploitable for novel AED design.

Dipyridamole decreases INaP and exon L inclusion

The seizure phenotype characteristic of bang-sensitive mutants (i.e. sda and eas) is associated with increased inclusion of exon L in DmNav and increased INaP in central motor neurons (Marley and Baines, 2011; Lin et al., 2012; Lin and Baines, 2015). Similarly, bas\(^1\) exhibits an increased INaP compared to wild-type. A persistent to transient current (P:T) ratio was measured by whole-cell voltage-clamp from the anterior corner cell (aCC) motorneuron (comparing INaT produced at 0 mV to INaP at \(+30\) mV) in bas\(^1\) and determined to be 53.1 ± 2.4% compared to 39.4 ± 3.4% in wild-type (\(P \leq 0.01\)). Feeding dipyridamole (0.4 mg/ml) to bas\(^1\) larvae significantly reduced the P:T ratio (30.9 ± 9.2%, \(P \leq 0.01\)), through a specific reduction of INaP (Fig. 7A and B). Increased INaP expression correlates with increased exon L inclusion in bas\(^1\) neurons (98.9 ± 1.0% versus 87.8 ± 3.6%, bas\(^1\) versus wild-type, \(P \leq 0.01\)). Exposure of bas\(^1\) larvae to dipyridamole also rescued exon L inclusion to wild-type levels (88.1 ± 1.4%, \(P \leq 0.01\), Fig. 7C). Thus, the anticonvulsive properties of dipyridamole are likely mediated through its ability to alter...
splicing of \textit{DmNav} to favour the K-exon variant that is associated with a smaller $I_{Nap}$. We have yet to determine if the other small molecule inhibitors described above act in a similar manner but, based on the action of dipyridamole, there is every reason to predict that they will.

**Discussion**

Despite an availability of numerous clinically-approved AEDs, 20–30% of epilepsy patients fail to respond to drug treatment (Sillanpaa and Schmidt, 2006; Loscher and Schmidt, 2011; Brodie et al., 2012). Even for those patients that respond, debilitating side-effects can, and often do, arise. A common and effective target of many AEDs is the Nav channel, but the inability of existing drugs to discriminate between reducing $I_{Nap}$ without also affecting $I_{Nat}$ limits their effectiveness. To date, no clinically-approved AED shows specificity for just $I_{Nap}$. A recent study fully illustrates the efficacy of seizure rescue achievable by selective block of $I_{Nap}$ (Anderson et al., 2014) indicative that this target is likely to produce better, and perhaps more tolerable, AEDs. Taking advantage of our previous demonstration that splicing selectively regulates $I_{Nap}$ in \textit{DmNav} (Lin et al., 2009), we now identify 95 genes that, on knockdown, result in significant rescue of seizure duration presumably through potent reduction of $I_{Nap}$. The protein products of these genes represent a valuable resource for the potential design of novel AEDs.

Of the 291 genes we identified, 13 belong to the Cyclin/ Cdk family. Moreover, seven of the corresponding RNAlines, \textit{Cdk1} (CG3563), \textit{Cdk2} (CG10498), CDC45L (CG3658), Cdc5 (CG6905), Cyclin B (CG3510), Cyclin D (CG9096) and \textit{Cdk4} (CG5072) significantly rescue seizure duration in both \textit{bas} and \textit{bas} mutants (Supplementary Table 3) indicative of common and exploitable mechanisms. We also show that acute feeding of isethionate, a CDK4 inhibitor, to \textit{bas} adult flies, rescues seizure duration. This over-representation implicates that cyclin/CDK function may be a tractable target for AED design. It is no surprise, therefore, that cyclin/CDKs have been implicated in epileptogenesis. For example, cyclin B1 upregulation is observed in the hippocampus of pentylenetetrazole (PTZ)-
kindled rats (Pavlova et al., 2006) and patients with temporal lobe epilepsy (Nagy and Esiri, 1998). Similarly, administration of kainite (KA) upregulates cyclin D1 expression in wild-type mice and loss of one copy of cyclin D1 (cyclin D1−/− heterozygous mice) prevents kainite-induced seizure (Liu et al., 1996; Timsit et al., 1999; Koeller et al., 2008). We also identified an unknown gene (CG31694), which regulates the JAK/STAT (Janus tyrosine kinase/signal transducer and activator of transcription) pathway (Muller et al., 2005). The JAK/STAT pathway is upregulated in pilocarpine- or kainite-induced status epilepticus, which results in temporal lobe epilepsy in rodents (Choi et al., 2003; Xu et al., 2011). Administration of the JAK/STAT inhibitor, WP1066, reduces the severity of pilocarpine-induced seizure and downregulates downstream target transcripts of JAK/STAT, including cyclin D1 (Grabenstatter et al., 2014). Our findings raise the possibility that seizure induction results in activation of JAK/STAT signalling, through regulation of cyclin/CDK expression.

Our screen identifies many additional genes that may prove exploitable for novel AED development. Notable amongst these are Pde11 (CG15159) and raptor (CG4320). Aberrant cAMP/cGMP levels are reported in human epilepsy and animal seizure models. For example, elevated cGMP and cAMP has been reported in the cerebral cortex, cerebellum and hippocampus following chemical-induced seizure (Ferrandelli et al., 1980; Kohno et al., 1997). Repeated injections of cAMP analogues into rat amygdala produced progressively more severe seizure behaviours similar to that induced by electrical kindling (Yokoyama et al., 1989). The role of phosphodiesterase inhibitors for the treatment of seizure is more controversial. For example, sildenafil, a phosphodiesterase-5 inhibitor, shows anti-convulsant action in the mouse 6-Hz psychomotor seizure model (Nieczym et al., 2013) but exhibits pro-convulsant activity in PTZ-induced mouse clonic seizure model (Montaser-Kounhsari et al., 2011). Inconsistency may derive from the expression of multiple phosphodiesterases in different brain regions (Domek-Lopacinska and Strosznajder, 2005), the ability of inhibitors to cross the blood–brain barrier (Liebenberg et al., 2005, 2012) and/or the dose of proconvulsants used for seizure induction (Bankstahl et al., 2012). In our screen, knock down of Pde11 increases DmNa+, exon K inclusion and UAS-RNAi-Pde11 expression rescues both bas1 and bss1 seizure duration. We also found that the phosphodiesterase inhibitor, dipyridamole, significantly reduced seizure duration. Dipyridamole produces a marked increase in the threshold for the onset of tonic extension in the PTZ-induced rodent seizure model (Akula et al., 2008).

The direct interaction of raptor and mTOR is required for mTOR signalling (Hara et al., 2002; Kim et al., 2002). mTOR is a serine/threonine kinase involved in the highly conserved PI3K-Akt signalling pathway. It has recently been reported that hyperactivation of mTOR signalling is followed by seizure induction in rat and mouse models (Waltereit et al., 2006; Grabenstatter et al., 2014). Administration of mTOR inhibitors, i.e. rapamycin, prevents the development of absence seizure in WAG/Rij rats (Russo et al., 2013), kindling seizure in Tsc1GRAP2KO mice (Zeng et al., 2008) and kainite-induced status epilepticus in rats (Macias et al., 2013). As such, the mTOR pathway has been identified as a ‘druggable’ target for the prevention of epileptogenesis (Lasarge and Danzer, 2014). In our screen, downregulation of raptor expression increased inclusion of DmNa+, exon K and reduced seizure duration of both bas1 and bss1. Furthermore, ingestion of rapamycin also effectively ameliorated bas1 seizure duration.

Identifying seizure suppressor genes in Drosophila has proven effective for identifying mechanisms underlying seizure and identifying novel targets for AED design (Kuebler et al., 2001; Hekmat-Scafe et al., 2005; Parker et al., 2011a). For example, topoisomerase 1 (top1) and gilgamesh mutant flies, as well as the topoisonerase 1 inhibitor, camptothecin, reduce the severity of bss1 seizure behaviour (Song et al., 2007; Howlett et al., 2013). In this study, the candidates of our screen are seizure suppressor genes which regulate a common downstream gene transcript, DmNa+. Knockdown of these genes is sufficient to rescue seizure behaviour of bang-sensitive mutants. However, the potential of the genes we identify here to become the basis for the design of novel AEDs goes beyond this study. The final choice will be dependent on many factors. These include how gene manipulation affects transcription/translation rates, in addition to splicing. Indeed, we see clear evidence for effects to transcription/translation of our mini-gene constructs but, importantly, identify many effective gene knockdowns that lack such an effect and only influence the splicing ratio to favour inclusion of exon K (Supplementary Table 2). We must also test for additional effects of gene knockdown in vivo including, but not limited to, effect to INaT and INaP. Na+ transcripts are heavily spliced and effects to other alternate exons and channel kinetics must be determined. Knockdown of pasilla affects splicing at DmNa+ exons 12, 22, 23 in addition to 25 (Lin et al., 2012). The change at exon 25 leads to increased inclusion of exon K which, in turn, reduces the amplitude of INaT without influence to INaT (Lin et al., 2009, 2012). Finally, understanding which of the genes we identify show increased transcription following treatments to induce seizure, or in bang-sensitive mutant backgrounds, should also be informative. The expectation is that these genes are upregulated during/after seizure. Indeed, Ebf1 (CG4954), shn (CG7734) and Relish (CG11992), which we identify in our screen, are all upregulated in fly seizure mutants (Guan et al., 2005). These follow-on studies, essential to narrow down our choice of genes to explore in detail, are readily achievable using Drosophila.

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**Supplementary material**

Supplementary material is available at Brain online.

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