julius seizure, a *Drosophila* Mutant, Defines a Neuronal Population Underlying Epileptogenesis

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ABSTRACT Epilepsy is a neural disorder characterized by recurrent seizures. Bang-sensitive *Drosophila* represent an important model for studying epilepsy and neuronal excitability. Previous work identified the bang-sensitive gene *slamdance* (*sda*) as an allele of the *aminopeptidase N* gene. Here we show through extensive genetic analysis, including recombination frequency, deficiency mapping, transposon insertion complementation testing, RNA interference (RNAi), and genetic rescue that the gene responsible for the seizure sensitivity is *julius seizure* (*jus*), formerly *CG14509*, which encodes a novel transmembrane domain protein. We also describe more severe genetic alleles of *jus*. RNAi-mediated knockdown of *jus* revealed that it is required only in neurons and not glia, and that partial bang-sensitivity is caused by knockdown in GABAergic or cholinergic but not glutamatergic neurons. RNAi knockdown of *jus* at the early pupal stages leads to strong seizures in adult animals, implicating that stage as critical for epileptogenesis. A C-terminal-tagged version of Jus was generated from a fosmid genomic clone. This fosmid fusion rescued the bang-sensitive phenotype and was expressed in the optic lobes and the subesophageal and thoracic abdominal ganglia. The protein was primarily localized in axons, especially in the neck connectives, extending into the thoracic abdominal ganglion.

KEYWORDS bang-sensitive; GABAergic; cholinergic; epilepsy; *slamdance*

EPILEPSY is a debilitating neurological disorder characterized by unprovoked recurrent seizures and increased seizure susceptibility, affecting 50 million people worldwide (World Health Organization 2016). Despite its remarkable prevalence, there are few effective treatments for the disease. Current antiepileptic drugs (AEDs) act in relatively nonspecific ways, even though there are a myriad of different seizure types and etiologies. AEDs may provide initial relief, but many patients gradually develop resistance to the drugs and approximately one-third of patients are refractory to any combinations of AEDs. The inadequacy of current treatments for epilepsy reflects our lack of understanding of epileptogenesis, how a brain becomes seizure prone.

A variety of model organisms have been used to study the genetic causes of epilepsy, including zebrafish (Cunliffe 2016) and *Drosophila* (Song and Tanouye 2008). Behavioral screens

in *Drosophila* have identified mutants and the underlying genes that cause a “bang-sensitive” (BS) behavioral phenotype, that is, they paralyze and seize following mechanical or electrical stimulation (Pavlidis *et al.* 1994; Lee and Wu 2002; Fergestad *et al.* 2006; Parker *et al.* 2010). One such BS mutant, *slamdance*^{iso7.8} (*sda*^{iso7.8}), although otherwise behaviorally normal, exhibits neuronal hyperexcitability and paralysis for ~20 sec when subjected to mechanical shock (Zhang *et al.* 2002). Genetic analysis attributed the *sda* phenotype to a mutation in the *Drosophila* homolog of the human *aminopeptidase N* gene. Electrophysiological analysis has shown that the mutation causes seizure sensitivity by lowering the voltage threshold (Zhang *et al.* 2002). Voltage clamp studies on first and third instar larvae reveal that this may be caused by increased persistent sodium current in motoneurons, causing longer plateau depolarizations and a greater number of action potentials compared to wild-type flies (Marley and Baines 2011).

The *aminopeptidase N* gene of *Drosophila* is located on 3R at polytene bands 97D7–97D9 (Attrill *et al.* 2016). Although Zhang *et al.* (2002) performed a thorough genetic analysis of the mutant with existing x-ray-induced deficiencies, we observed a higher than expected recombination frequency

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(14.7%) between *sda*^{iso7.8} and the transposon insertion line *P[GawB]386Y*, which has an insertion site near the gene *amon* at polytene bands 97C4–97C5 and *sda*^{iso7.8} complemented molecularly defined deficiencies from the *aminopeptidase N* locus. This suggested that the *sda* locus had been mislocalized to *aminopeptidase N*. Here we demonstrate that the *sda*^{iso7.8} is an allele of an uncharacterized protein-coding gene *CG14509*, which we name *julius seizure* (*jus*), after the Roman emperor who reportedly suffered from epilepsy (Hughes 2004). *jus* is located at polytene bands 98F10–99A1, over 2.2 Mbp away from the *aminopeptidase N* gene. This claim is supported with deficiency mapping, transposon insertions, recombination frequencies, RNA interference (RNAi), and genetic rescue. A comparison of seizure recovery durations of different alleles and RNAi-mediated knockdowns indicate that the *sda*^{iso7.8} allele is likely a weak hypomorph of *jus*. RNAi-directed knockdown of *jus* revealed that it is required in neurons and not glia, and that both GABAergic and cholinergic neurons are important for the bang-sensitive phenotype. *Jus* expression is critical during the early pupal stage as RNAi-mediated knockdown at that time interval results in strongly bang-sensitive adults. A fosmid-based GFP fusion of *jus* shows that the protein is expressed in neurons of the optic lobe and strongly in selected neurons of the subesophageal (SOG) and thoracic abdominal ganglia (TAG).

Materials and Methods

Fly stocks

Fly stocks were maintained on a glucose, yeast, and cornmeal agar media at 22–25° in plastic vials unless otherwise noted. The *slamdance*^{iso7.8} mutant was a gift from M. A. Tanouye. Fly stocks were obtained from either the Bloomington Stock Center at Indiana University or from the Vienna *Drosophila* RNAi Center (VDRC). When applicable, *w*¹¹¹⁸ (BL #5905) was used as a control.

Rescue constructs

To assemble the rescue construct, a *PvuI*–*AgeI* fragment containing the *jus* open reading frame (restriction enzymes from New England Biolabs, Ipswich, MA) was purified from the GH11945 cDNA clone (obtained from the *Drosophila* Genomics Resource Center (DGRC), GenBank accession no. AY128418) ligated with T4 DNA ligase (New England Biolabs) into *PacI*–*AgeI* digested pUAS-c5-attB (Daniels *et al.* 2014) and sent to Bestgene (Chino Hills, CA) for transformation at the 68A4 docking site on chromosome 3. The rescue line will be referred to as UAS-*jus*. The UAS-*jus* transgene was recombined with *sda*^{iso7.8} for rescue assays. *w*; UAS-*jus*, *sda*^{iso7.8} flies were crossed to *P{GawB}elav^{C155}*; *sda*^{iso7.8} or *w*; *P{y[+t7.7] w[+mC]=GMR55G02-GAL4}attP2*, *sda*^{iso7.8} and vortex tested as described below. For fosmid transgenic generation, clone CBGtg9060B0572D was obtained from Source BioScience (Nottingham, UK) containing the genomic locus of *jus* and tagged at the C terminus with TY1, GFP, V5,

BLRP, and 3×Flag (Sarov *et al.* 2016). The fosmid was sent to Genetivision (Houston, TX) for injection and transformation at the VK1(2R)59D3 docking site. Individual lines were screened for V5 and Flag-tagged immunoreactivity. One line was selected and it will be referred to as *Fos{jus-TGVBF}*. For genomic rescue experiments, *w*; *Fos{jus-TGVBF}*/CyO; *PBac[WH]CG14509^{f04904}/+* animals were crossed to *sda*^{iso7.8} animals. *w*+ F₁ animals were selected so that all animals tested were *PBac[WH]CG14509^{f04904}/sda*^{iso7.8} and CyO+ animals (no fosmid) were compared to CyO– animals (fosmid containing) in vortex testing (see below).

Vortex testing

Adult flies, at least 3 days posteclosion, were anesthetized with carbon dioxide and placed in vials containing food and 10 flies each. Twenty-four hours after CO₂ exposure, the flies were tested for bang sensitivity by vortexing the vial for 10 sec using the Vortex Genie 2 (VWR Scientific, Radnor, PA) at maximum speed. The number of flies that seized in response to the stimulus was recorded. Recovery times were determined by recording the interval elapsed for 50% of the flies to right themselves following the cessation of vortexing. For *jus* alleles, RNAi with different GAL4s, and rescue experiments with complementary DNA (cDNA) and genomic constructs, at least 20 flies of each genotype were tested, but in most cases, 60–80 flies were tested. For the RNAi developmental time course with GAL80^{ts}, 50% recovery time was determined by observing when animals awakened from seizures and attempted to stand. At least 30 flies were tested for each time point. For the fosmid rescue experiments, at least 60 flies were tested for each genotype.

Complementation testing

sda^{iso7.8} was mapped by crossing a series of overlapping molecularly defined deficiencies to *sda*^{iso7.8} homozygotes and vortex testing the *sda*^{iso7.8}/Df progeny for complementation. The deficiencies extended from the gene *rough* to the gene *claret* (~3,000,000 bp) on chromosome 3R. Crosses that yielded nearly 100% bang-sensitive progeny were considered to have failed to complement *sda*^{iso7.8}. Candidate genes were selected from the overlapping regions of the noncomplementing deficiencies. Fly stocks with transposable element insertion sites within the candidate genes were also crossed to *sda*^{iso7.8} and vortex tested for complementation. Transposon insertion lines that failed to complement *sda*^{iso7.8} were considered to have insertion sites within the same gene as the *sda*^{iso7.8} allele.

Recombination experiments

sda^{iso7.8} virgin females were crossed to males from the transposon insertion line *w**; *P[GawB]386Y*. *sda*^{iso7.8}/*w**; *P[GawB]386Y* virgin females were then crossed back to *sda*^{iso7.8} males. The recombinant progeny with the genotype *w**; *P[GawB]386Y*, *sda*^{iso7.8}/*sda*^{iso7.8} could be identified as red eyed and bang sensitive. The number of recombinants were counted and multiplied by 2 to account for the other undetectable recombinant genotype + *sda*^{iso7.8}/+ +. The total number of recombinants was divided by the total number of

Table 1 Recombination frequency between *sda* and genetic markers

Genotype	Polytene locus	Number tested	% Recombination
<i>w[*]</i> ; <i>P{GawB}386Y</i>	97C5	599	14.7
<i>w[*]</i> ; <i>P{EPgy2}CG11873^{EY00432}</i>	98F6	52	3.8
<i>yw</i> ; <i>ry⁵⁰⁶</i> <i>P{SUPorP}CG11897^{KG04612}</i>	98F13	140	0
<i>w¹¹¹⁸</i> ; <i>PBac{RB}CG11898^{e03595}</i>	98F13	160	1.3
<i>Dr</i>	99B3	545	1.4

progeny, and then multiplied by 100 to give the total percentage of recombination. The same procedure was repeated to calculate the percentage of recombination between *sda^{iso7.8}* and the transposon insertion line *P{y[+mDint2] w[BR.E.BR]=SUPor-P}CG11897^{KG04612}*, *w^{*}*; *P{EPgy2}CG11873^{EY00432}*, *w¹¹¹⁸*; *PBac{RB}CG11898^{e03595}*, and the dominant marker *Dr*.

RNAi

Virgin females from the RNAi lines *P{GD352}v4434* (VDRC), *P{GD352}v4435* (VDRC), and *P{y[+t7.7] v[+t1.8]=TRiP.JF03192}attP2* RNAi line was crossed to

JF03192}attP2 (BL#28764) were crossed to males from the GAL4 driver line *P{GawB}elav^{C155}* (BL#458). Bang-sensitive RNAi/GAL4 heterozygotes indicated successful RNAi knockdown. Since the *P{GawB}elav^{C155}* GAL4 driver is x-linked, only female progeny from the cross were RNAi/GAL4 heterozygotes. The male progeny, which contained only the RNAi gene or *P{GawB}elav^{C155}*, were vortex tested as a control. In most of the subsequent crosses, the *P{y[+t7.7] v[+t1.8]=TRiP.JF03192}attP2* RNAi line was crossed to *Chat-GAL4* (BL#6798), *repo-GAL4* (BL#7415), *VGAT-GAL4*

Figure 1 Deficiency mapping of *sda^{iso7.8}* by bang sensitivity. (A) Chromosome 3R from 25.6 to 29.8 M and the deficiencies (*Dfs*) from 96E to 99B (modified from FlyBase), which were crossed to *sda^{iso7.8}*. Numbers below deficiencies in red are those crossed to *sda^{iso7.8}* in B. (B) Percentage of bang-sensitive flies of *sda^{iso7.8}*/*Df*. Failure to complement is observed for *Dfs* 9–11. * indicates deficiencies that failed to complement *sda^{iso7.8}* and ** indicates a deficiency that complements *sda^{iso7.8}* yet removes the former location of the *sda* locus. (C) The *Julius seizure* (*jus*) locus with indicated transposons and deficiencies is shown. *PBac{WH}CG14509^{f04904}* failed to complement *sda^{iso7.8}*.

Table 2 Complementation testing of *sda*^{iso7.8} with transposons from 98F12 to 99A1

Gene disrupted	Genotype tested	% Bang-sensitive progeny
CG14516	<i>P[EPg]CG14516^{HP31652}/sda</i>	0
Atg14	<i>P[EPgy2]Atg14^{EY14568}/sda</i>	0
elF2D	<i>PBac[WH]elF2D^{f04128}/sda</i>	0
CG11897	<i>P[SUPor-P]CG11897^{ICG04612}/sda</i>	0
CG11880	<i>P[EPgy2]CG11880^{EY00989}/sda</i>	0
CG14509	<i>PBac[WH]CG14509^{f04904}/sda</i>	100
CG11898	<i>PBac[RB]CG11898^{e03595}/sda</i>	0
alpha-Man-1b	<i>PBac[WH]α-Man-1b⁰⁷²²¹/sda</i>	0
yem	<i>P[epGY2]yem^{EY23024}/sda</i>	0
CG14509	<i>Mi[MIC]CG14509^{MI11213}/sda</i>	7.5
CG14509	<i>Mi[ET1]CG14509^{MB12140}/sda</i>	0

(BL#58409), and OK371-GAL4 (BL#26160). For developmental studies, an *Act5C-GAL4* (BL#3954) construct was recombined with the *P{y[+t7.7] v[+t1.8]=TRiP.JF03192}attP2* RNAi line. The resulting line was crossed to *w[*]*; *P{w[+mC]=tubP-GAL80^{ts}; TM2/TM6B, Tb* (BL#7108) and the resulting progeny were incubated at either 22° or 29° at different developmental stages. *P{w[+mC]=tubP-GAL80^{ts}+/+; Act5C-GAL4, P{y[+t7.7] v[+t1.8]=TRiP.JF03192}attP2/TM2* or *TM6B, Tb, Hu* animals were selected and then vortex tested as described above. More than 30 animals were tested for each time point.

Electrophysiological methods

Physiological recordings were performed on the giant fiber (GF) pathway essentially as described (Howlett and Tanouye 2009). Flies were immobilized in dental wax and a small hole was made through the cuticle of the thorax with a sharpened tungsten needle to reveal the dorsal longitudinal flight muscle (DLM). Two uninsulated sharpened tungsten electrodes were inserted through the head cuticle into the brain and another into the abdomen as a ground and fourth sharpened 2.5 M Ω insulated recording electrode (MicroProbes, Gaithersburg, MD) was inserted through the opening of the thorax into the flight muscle. Stimuli were generated by a Grass S88 stimulator and recordings were picked up by an A-M Systems AC 1800 amplifier (Sequim, WA) at a gain of 100 times and filter settings of between 10 Hz and 5 kHz. Signals were digitized by a Digidata 1322a (Molecular Devices, Sunnyvale, CA) and recorded with pCLAMP8 software (Molecular Devices). GF threshold and high-frequency stimulation protocols were as described (Howlett and Tanouye 2009). Seizure intervals were measured from the last spike of the initial seizure to the first spike of the recovery seizure.

Antibody staining

Fos{jus-TGVBF} flies were briefly anesthetized in CO₂, then dipped in ethanol and dissected in Ca²⁺-free HL3 saline, fixed, and stained as described (Lawton *et al.* 2014). After staining, brains were postfixed for 15 min, rinsed, then mounted in Vectashield (Vector Labs, Burlingame, CA). Mouse anti-V5 was used at 1:250 and secondary donkey anti-mouse Alexa-488 (Invitrogen, Waltham, MA) was used

at 1:1000. Brains were imaged on an inverted Zeiss LSM 880 confocal microscope with C-Achroplan 32 $\times$ /0.85 Corr M27 water lens. Images are maximal projections of confocal stacks as processed by Zen (Peabody, MA) or Imaris software v8.2.0 (Bitplane, Concord, MA).

Western blot

Ten heads from *Fos{jus-TGVBF}* were ground up in loading buffer, heated to 95° for 3 min, loaded onto a 10% polyacrylamide gel, electrophoresed, and blotted onto Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA). Blots were blocked in 5% nonfat dry milk PBS with 0.05% Tween 20 (PBST), probed with anti-Flag M2 (Sigma-Aldrich, St. Louis, MO) at 1:1000 dilution in PBST with 1% BSA, washed and probed with donkey anti-mouse HRP (Jackson Immuno-research, West Grove, PA) at 1:5000 dilution, washed in PBST, and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Blots were exposed to BioMAX Light Film (Sigma-Aldrich) for 15 sec.

Statistics

One-way ANOVA with Tukey's multiple comparison's test or the Student's *t*-test for pairwise comparisons were used as appropriate. Significance testing and graphing was performed with GraphPad Prism 8 software (La Jolla, CA).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains are available upon request.

Results

Complementation testing and recombination mapping

In our initial experiments, three lines of evidence suggested that the previous mapping of *sda*^{iso7.8} to 97D had been inaccurate. First, initial recombination of *sda*^{iso7.8}, with a predicted nearby *w+* marked *P*-element at polytene band 97C4-5 revealed nearly a 15% recombination rate, suggesting that the mutation is distant from the reported 97D7-9 location. Second, recombination with *w+* *P*-element and the dominant

Figure 2 Recovery times of *jus* alleles. The 50% recovery times of allelic combinations of *jus* are shown. The original *sda*^{iso7.8} is a fairly weak hypomorph, while *PBac[WH]CG14509^{f04904}* is a stronger hypomorph but not a null as revealed by the longer recovery time of *PBac[WH]CG14509^{f04904}/Df*. The strongest allelic combination of *PBac[WH]CG14509^{f04904}/Df(3R)BSC501* has a >10-fold recovery time as compared to that of *sda*^{iso7.8}. Significant differences were observed between all allelic combinations (indicated with **** $p < 0.0001$) except for *sda*^{iso7.8} vs. *sda*^{iso7.8}/*PBac[WH]CG14509^{f04904}* and *sda*^{iso7.8}/*Df(3R)501* vs. *sda*^{iso7.8}/*Df(3R)874*. Error bars are $\pm$ SEM.

marker *Dr* indicated the *sda* locus was very near 99F12–99A (Table 1). Third, the large, molecularly defined deficiency *Df(3R)ED6255* spanning the reported *sda* locus aminopeptidase *N* complemented the *sda*^{iso7.8} allele (Figure 1, A and B).

To remap the *sda* locus, a series of molecularly defined deficiencies spanning from polytene bands 96E to 99B were crossed to *sda*^{iso7.8} (Figure 1A). Figure 1B shows the percentage of bang-sensitive flies for each *sda*^{iso7.8}/*Df* combination. The deficiencies *Df(3R)ED6310*, *Df(3R)BSC874*, and *Df(3R)BSC501* all failed to complement *sda*^{iso7.8} and had an overlapping region between 3R: 29,138,895 and 3R: 29,191,671. Candidate genes with high levels of expression in the nervous system were chosen from this region for further investigation.

We then crossed *sda*^{iso7.8} to flies containing transposon insertions within the candidate genes. The transposon/*sda*^{iso7.8} transheterozygotes were vortex tested for behavioral complementation. Table 2 shows the percentage of bang-sensitive progeny for each genotype. The only stock that failed to complement *sda*^{iso7.8}, *PBac[WH]CG14509^{f04904}*, contained an insertion site in an intron near the 5' end of *CG14509*. Two other fly stocks, *Mi[MIC]CG14509^{MI1213}* and *Mi[ET1]CG14509^{MB12140}*, each with different transposon insertion sites within *CG14509*, largely complemented *sda*^{iso7.8} with

Figure 3 Bang sensitivity of different RNAi lines in combination with different GAL4s. (A) Percentage of flies that are bang sensitive due to RNAi knockdown. *OK371* is expressed in glutamatergic neurons, *Chat* in cholinergic, *vGAT* in GABAergic, and *elav* panneurally. *Repo* is expressed only in glia and *Act5C* is expressed strongly and ubiquitously. RNAi refers to Bloomington stock, *P[y+7.7] v[+t1.8]=TRIP.JF03192*attP2, and RNAi lines 4434 and 4435 refer to VDRC stocks, *P[GD352]v4434* and *P[GD352]v4435*, respectively. (B) The 50% recovery time of flies that seize in A. Significant differences are indicated with * $P = 0.0112$, ** $P = 0.0019$, and **** $P < 0.0001$. Error bars are $\pm$ SEM.

7.5 and 0% bang sensitivity, respectively, suggesting that they do not significantly disrupt the expression of *CG14509* (Table 2). Hemizygous combinations of *sda*^{iso7.8} with *Dfs* resulted in significantly longer recovery times than *sda*^{iso7.8} homozygotes (Figure 2). These data strongly point to *CG14509* as the gene responsible for the bang sensitivity. The coding sequence of *CG14509*, which we now refer to as *Jus*, consists of a protein of ~50 kDa with two predicted transmembrane

Figure 4 Differences in electrically evoked seizures of *jus* alleles. Recordings of flight muscles following high-frequency stimulation of the brain result in a primary seizure immediately following the stimulus, followed by a recovery seizure tens of seconds later. (A, top) The response of *sda^{iso7.8}* to stimulus. The interval between primary seizure and recovery seizure is ~30 sec. (bottom) The response of *PBac[WH]CG14509^{f04904}* to stimulus. The interval between primary seizure and recovery seizure is ~70 sec. (B) Average seizure threshold between *sda^{iso7.8}* and *PBac[WH]CG14509^{f04904}* was not found to be significantly different (unpaired, two-tailed *t*-test, $P = 0.0877$). (C) Quantification of interval between primary and recovery seizure is displayed. *PBac[WH]CG14509^{f04904}* has a significantly longer interval between seizures as compared to *sda^{iso7.8}* (unpaired, two-tailed Student's *t*-test, **** $P < 0.0001$). $n = 9$ for *PBac[WH]CG14509^{f04904}* and $n = 16$ for *sda^{iso7.8}*. Error bars are $\pm$ SEM.

domains and an extracellular loop with multiple cysteine residues, presumably forming disulfide bonds. The extracellular loop is the only region expected to possess any significant organized secondary structure (Kelley *et al.* 2015).

Genetic rescue

To further confirm that the defect in the *sda^{iso7.8}* allele is due to a mutation in *jus*, genetic rescue was performed. Using a UAS-*jus* rescue construct and the panneuronal driver, *elav-gal4*, *sda^{iso7.8}* homozygotes were rescued 100% in a vortex test assay ($n = 20$). Similar results were obtained in rescuing *sda^{iso7.8}/Df* mutants (97% rescue, $n = 36$). The GAL4 line, *w; P{y[+t7.7]w[+mC]}=GMR55G02-GAL4}attP2*, from the genomic locus of *jus* as the driver, was able to rescue 85% of the flies from bang sensitivity ($n = 61$). Since UAS-*jus* rescued the *sda^{iso7.8}* allele, we attempted to identify the mutation responsible in *sda^{iso7.8}* for the bang-sensitive phenotype. However, we failed to detect any mutation affecting the coding sequence by exon sequencing of *jus*.

RNAi

If the bang-sensitive phenotype results from a loss of *jus* function, it is predicted that knocking down *jus* expression would lead to the seizure phenotype. We tested three different RNAi lines: *P{GD352}v4434*, *P{GD352}v4435*, and *P{y[+t7.7]v[+t1.8]}=TRiP.JF03192}attP2*. Crossing each RNAi line to *P{GawB}elav^{C155}* resulted in 100% bang sensitivity when both transgenes were present, as compared to 0% in the control without the GAL4 driver. Expression of RNAi with a glial driver did not cause bang sensitivity. Expression in subsets of neurons using either GABAergic or cholinergic drivers resulted in varying percentages of bang-sensitive adults, but expression using a glutamatergic-specific GAL4, *OK371*, had

no effect (Figure 3A). Similar results were obtained with another independent glutamatergic driver (data not shown). Using the strong *Act5C-GAL4* in combination with *P{y[+t7.7]v[+t1.8]}=TRiP.JF03192}attP2* resulted in bang-sensitive flies with extremely long recovery times (Figure 3B), ~10 times greater than the *sda^{iso7.8}* allele.

Seizure phenotype

One of the hallmarks of bang-sensitive mutants is the induction of the phenotype with electrical stimulation (Song and Tanouye 2008). High-frequency stimulation of the *sda^{iso7.8}* resulted in an initial seizure followed by a recovery seizure, as has been previously reported (Zhang *et al.* 2002). Similar seizures were observed in the transposon *PBac[WH]CG14509^{f04904}* homozygous line (Figure 4A). The seizure thresholds of each genotype did not differ significantly ($7.75 \text{ V} \pm 0.3227$ for *sda^{iso7.8}* and $8.67 \text{ V} \pm 0.3727$ $P = 0.0786$, two-tailed Student's *t*-test). One striking difference in the *PBac[WH]CG14509^{f04904}* line was the interval between the initial and the secondary seizure. *sda^{iso7.8}* homozygotes had a mean interval of 29 ± 4.3 sec, while *PBac[WH]CG14509^{f04904}* had a significantly longer mean interval of 56 ± 14.0 sec ($P = 0.0002$, *t*-test, one tail, unpaired), consistent with its longer recovery time following vortexing.

Expression in the CNS

To identify the expression pattern of *jus*, a fosmid clone (Sarov *et al.* 2016) containing the full genomic region of *jus* with in-frame tags of GFP, 3 $\times$ Flag, and V5 at its C terminus (Figure 5A) was inserted at a docking site at the distal end of chromosome 2R. To assess the expression of this transgenic *Fos{jus-TGVBF}*, a Western blot from head extracts was

Figure 5 Expression and genetic rescue with C-terminally-tagged *jus* genomic construct. (A) Transgenic containing the genomic fosmid construct of *jus* inserted on 2R, *Fos{jus-TGVBF}*, is predicted to produce a full-length Jus tagged at the C terminus with 2XTY1, GFP, V5, BLRP, and Flag. The expected fusion is shown schematically. (B) Western blot of head extract of *Fos{jus-TGVBF}* probed with anti-Flag antibody detecting the tagged Jus protein. (C) Seizure recovery time of *sda^{iso7.8}*/PBac[WH]CG14509 animals with or without the presence of a single copy of *Fos{jus-TGVBF}*. Highly significant differences were observed in recovery time in animals with the *Fos{jus-TGVBF}* construct (**** $P < 0.0001$). Error bars are $\pm$ SEM. (D) Confocal images of *Fos{jus-TGVBF}* adult brain stained with anti-V5 antibody (green). Expression is evident in the neuropil of the lamina and medulla of the optic lobes (highlighted with arrowheads), neck connectives, and in the axons of the TAG. Bar, 100 μ m. (E) Higher magnification of the optic lobe shows expression in dendritic processes that extend across the medulla and in a cluster of cell bodies. Bar, 20 μ m. (F) Higher magnification of the neck connectives shows strong expression in the subesophageal ganglion (SOG) and in the axons connecting the brain and TAG. Bar, 20 μ m.

performed. Using an anti-Flag antibody, we detected the predicted size for the tagged Jus protein of ~ 90 kDa (Figure 5B). No Flag immunoreactive band was detected in *w¹¹¹⁸* head extract controls (data not shown). To test the functionality of Jus-TGVBF, we crossed the PBac transposon allele of *jus* to *sda^{iso7.8}* with or without the *Fos{jus-TGVBF}* rescue construct. While both sets of flies seized, the PBac[WH]CG14509⁰⁴⁹⁰⁴/*sda^{iso7.8}* animals containing a single copy of the *Fos{jus-TGVBF}* rescue construct recovered significantly faster than controls ($P < 0.0001$), indicating the C-terminally tagged Jus is functional (Figure 5C). To localize the tagged protein, an anti-V5 antibody was used to stain *Fos{jus-TGVBF}* adult brains. The brain showed expression in the optic lobes, SOG, and TAG (Figure 5, D–F). On the subcellular level, Jus-TGVBF is found abundantly in neuronal processes, especially axons. In the optic lobes, localization of Jus-TGVBF is found in synaptic layers

and in dendritic processes (Figure 5E), suggesting that Jus+ neurons are involved in visual processing. Similarly, strong Jus-TGVBF localization is observed in the neck connectives, which are composed of descending axons (Figure 5, D and F). In the SOG, strong protein localization is observed in selected cell bodies as well as in axons projecting to the TAG. In the TAG, most of the expression is found in axons that traverse T1, T2, T3, and the abdominal segment.

Critical period

To assess whether *jus* expression is required during development for the bang-sensitive phenotype, the *Act5c > RNAi* line was crossed to a *w*; *P{w[+mC]}=tubP-GAL80^{ts}* and incubated at 22°, then shifted to 29° at selected developmental stages. At continuous 29° incubation, GAL80^{ts} is inactivated and the RNAi is transcribed, resulting in 100% of the progeny being bang

Figure 6 Developmental RNAi effects on seizure severity. *Act5C > RNAi-jus + Tub-GAL80^{TS}* embryos starting at the GAL80^{TS} permissive temperature (22°) and switching to 29° to allow RNAi expression at indicated developmental times. (A) The percentage of bang-sensitive adults following the temperature treatments. (B) Recovery times are not statistically different for animals shifted to 29° at the second instar, three instar, or pupal stages, P1–P3. Pupal stages P4–P6 have a significant decrease in their recovery time as compared to all the earlier time points (**** $P < 0.0001$, ** $P = 0.0062$). Error bars are $\pm$ SEM.

sensitive with long severe seizures and extended time for full recovery. Continuous incubation at 22° resulted in the majority of the flies with bang sensitivity but with much more rapid recovery, likely owing to a combination of the strength of the *Act5C-GAL4* driver and some leakiness of GAL80^{TS} at 22° (Figure 6A). The recovery time of these animals was in a narrow time window (~20 sec), with animals fully recovering mobility upon awakening from seizures (Figure 6B). To define the critical time window

for *jus* expression, crosses were started at 22°, then progeny were shifted at the second instar, third instar, early pupal, or midpupal stages to 29°. All animals (100%) shifted to 29° at the second instar, third instar and the early pupal stage were bang sensitive as adults. Those shifted at the midpupal (P4–P6) and those raised completely at 22° show less than complete bang sensitivity (Figure 6A,B). Interestingly, animals that shifted to 29° as late as the early pupal stage had seizures that were statistically no different from those of second or third instar but differed significantly from animals shifted at the midpupal stage.

Discussion

This study demonstrates that the *sda*^{iso7.8} phenotype is caused by a mutation in the protein coding gene, *CG14509*, which we designate *julius seizure* (*jus*). The only deficiencies that failed to complement *sda*^{iso7.8} during genetic mapping were *Df(3R)ED6310*, *Df(3R)BSC874*, and *Df(3R)BSC501*, which had an overlapping region between 3R: 29,138,895 and 3R: 29,191,671 (Figure 1). Of the candidate genes chosen from this region, only *CG14509* exhibited bang sensitivity when disrupted with transposon insertion *PBac[WH]CG14509^{f04904}* in combination with *sda*^{iso7.8}. Low recombination rates between *w+* marked transposons and the dominant marker *Dr* in the *jus* region are also consistent with *CG14509* being the *sda* locus. RNAi knockdown of *CG14509*, with three different RNAi lines: *P{GD352}v4434*, *P{GD352}v4435*, and *P{y[+t7.7] v[+t1.8]} = TRiP.JF03192* attP2 using a panneuronal GAL4 driver, provided confirming evidence. Genetic rescue of *sda*^{iso7.8} with a *jus* cDNA and genomic fosmid construct strongly supports the notion that *sda*^{iso7.8} is an allele of *jus*. Earlier mapping efforts of *sda* may have been misled by the use of x-ray-induced deficiencies that may have chromosomal aberrations outside of the 97D region.

The *Jus* protein is predicted to have intracellular N and C termini, two transmembrane domains and an extracellular loop containing 12 cysteines that likely form disulfide bonds. A proteomic study of *Drosophila* membrane proteins identified *CG14509* as a membrane protein (Khanna *et al.* 2010), consistent with its two predicted transmembrane domains. Though the protein bears no significant homology to vertebrate proteins and the only recognizable domain, the EB domain (Marchler-Bauer *et al.* 2015), has no described function, *jus* clearly has importance in regulating neuronal excitability during *Drosophila* development. It is possible that another protein, without detectable sequence homology, plays a similar functional role in vertebrates. Further studies will be directed at understanding which proteins and pathways are affected by *jus*.

Like other bang-sensitive mutants, seizures may be triggered by direct high-frequency stimulation of the brains of *jus* mutants. Here we show seizures in *sda*^{iso7.8} like those previously reported (Zhang *et al.* 2002) and the seizure phenotype in the transposon allele, *PBac[WH]CG14509^{f04904}*. Notably, the interval between the initial seizure and recovery seizure is significantly longer with the transposon allele. This difference is in agreement with the much longer recovery times following mechanical stimulation.

The expression of Jus-TCVBF observed in the optic lobes and in many descending axons in the neck is suggestive of a role in transmitting sensory information to the thoracic abdominal ganglion. Additional roles of Jus in sensory processing come from an earlier study in which a transposon allele in *CG14509* was found to have defective olfactory responses (Sambandan *et al.* 2006). The overall pattern of Jus neuronal expression is suggestive of a network of interconnected neurons that may function to stabilize excitability, analogous to the network of Fru+ neurons involved in all aspects of courtship (reviewed in Yamamoto 2007).

RNAi-mediated knockdown of *jus* in various neuronal populations was an effective approach to identify the neurons that regulate bang sensitivity. Panneural GAL4-driven RNAi expression results in strongly bang-sensitive flies, while glial or glutamatergic drivers have no effect. Both GABAergic and cholinergic drivers generate bang-sensitive adults, indicating that these neuronal types are important for the phenotype. For each of the neurotransmitter-specific GAL4 drivers tested, other independent GAL4 lines, under control of genomic DNA for vesicular transporters for GABA and acetylcholine, were also used to drive RNAi expression. Similar seizure susceptibility results were obtained (data not shown). Developmentally controlled expression of RNAi using GAL4/GAL80^{ts} indicated that preadult expression of *jus* is important for regulating neuronal excitability. This is in agreement with previous work that indicated that increasing neural activity at either the embryonic or pupal stages can result in a hyperexcitable phenotype (Giachello and Baines 2015). Interestingly, we identified the early pupal stage as important for determining the seizure severity. Further studies can define the neuronal processes impacted by a lack of *jus* during pupal development.

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