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To cite this article: Derek Dean, Hannah Weinstein, Seema Amin, Breelyn Karno, Emma McAvoy, Ronald Hoy, Andrew Recknagel, Casey Jarvis & David Deitcher (2017): Extending julius seizure, a bang-sensitive gene, as a model for studying epileptogenesis: Cold shock, and a new insertional mutation, Fly, DOI: 10.1080/19336934.2017.1402993

To link to this article: https://doi.org/10.1080/19336934.2017.1402993

Accepted author version posted online: 10 Nov 2017.
Published online: 08 Dec 2017.
Extending *julius* seizure, a bang-sensitive gene, as a model for studying epileptogenesis: Cold shock, and a new insertional mutation

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\section*{ABSTRACT}

The bang-sensitive (BS) mutants of *Drosophila* are an important model for studying epilepsy. We recently identified a novel BS locus, *julius* seizure (*jus*), encoding a protein containing two transmembrane domains and an extracellular cysteine-rich loop. We also determined that *jus*\textsuperscript{iso7.8} is a previously identified BS mutation, is an allele of *jus* by recombination, deficiency mapping, complementation testing, and genetic rescue. RNAi knockdown revealed that *jus* expression is important in cholinergic neurons and that the critical stage of *jus* expression is the mid-pupa. Finally, we found that a functional, GFP-tagged genomic construct of *jus* is expressed mostly in axons of the neck connectives and of the thoracic abdominal ganglia. In this Extra View article, we show that a MiMiC GFP-tagged *jus* is localized to the same nervous system regions as the GFP-tagged genomic construct, but its expression is mostly confined to cell bodies and it causes bang-sensitivity. The MiMiC GFP-tag lies in the extracellular loop while the genomic construct is tagged at the C-terminus. This suggests that the alternate position of the GFP tag may disrupt Jus protein function by altering its subcellular localization and/or stability. We also show that a small subset of *jus*-expressing neurons are responsible for the BS phenotype. Finally, extending the utility of the BS seizure model, we show that *jus* mutants exhibit cold-sensitive paralysis and are partially sensitive to strobe-induced seizures.

\section*{Introduction}

Epilepsy is a neurological disorder characterized by recurrent seizures and affecting about 50 million people worldwide [1]. There are many classes of epilepsies and their etiology and phenotype manifestations vary widely. Seizures can be broadly classified as generalized or focal. Generalized seizures involve the entire brain while focal seizures originate from a focus and may remain more localized or may spread to include a larger brain region. Most epileptic seizures occur unpredictably and do not have specific triggers. However, some epilepsies are initiated by particular stimuli or activities such as reading, hot water, startle, or more commonly flashing lights [2]. Approximately 10\% of epileptics are sensitive to strobe lights as compared to less than 0.5\% of healthy individuals [3].

Human epilepsies that have known genetic causes most often affect voltage and ligand gated ion channel function (channelopathies), but others affect neurotransmitter release, synaptic transmission, lysosomal storage, mitochondrial function or neural migration [4]. Collectively, however, they represent a small fraction of the cases of epilepsy. Anti-epileptic drugs (AEDs) can control most epilepsies but, stubbornly, approximately 30\% of epileptics are not helped by any combination of AEDs even as the number of such drugs have increased [5]. Even when AEDs are deemed effective, they are designed to treat the symptoms of epilepsy, not its underlying causes, and they often have significant side effects [5]. AEDs show little utility in preventing epileptogenesis, i.e. the development of epilepsy. How a brain is altered to become seizure-prone during epileptogenesis remains largely mysterious.

The “bang-sensitive” (BS) class of behavioral mutants in *Drosophila* has led to the identification of
genes that cause seizures when triggered mechanically or electrically. The bang-sensitives most closely mimic generalized, tonic-clonic seizures of humans [6]. One class of BS mutants affect mitochondrial function, possibly through energy or metabolic perturbations. These include tko [7], kdn, and sesB [8]. Another class, affecting ion channels, includes the allele of the para gene, para\textsuperscript{bosl}. This mutant, affecting a voltage-gated sodium channel gene, results in a very strong BS phenotype that relates to human epilepsies with genetic causes [9]. More recently knock-ins of human sodium channel mutations have provided a model even more related to specific human epilepsies [10]. However, until very recently, there was no Drosophila developmental model of epileptogenesis.

Our recent work identified \textit{sda}\textsuperscript{iso7.8} (now \textit{jus}\textsuperscript{ada iso7.8}) as an allele of \textit{julius seizure}, the previously uncharacterized CG14509 gene. \textit{jus} is expressed in a limited number of neurons during development, selective RNAi knockdown of \textit{jus} results in BS adults, and a developmentally defined stage is important for the severity of the \textit{jus} BS phenotype [12]. Thus, \textit{julius seizure} has many of the features with which to study the process of epileptogenesis.

In this Extra View article we show that an in frame, GFP-tag insertion in the extracellular domain of Jus results in a BS phenotype and a mislocalization of the protein from the axons to the neuronal cell bodies. The small intersection of \textit{jus} expression and that of a GAL4 line capable of genetic rescue identifies a small population of neurons that regulate overall excitability of the CNS during development. Furthermore, we have extended the BS model by demonstrating that cold shock can trigger paralysis in \textit{jus} mutants. We also demonstrate that strobe lighting can cause paralysis in \textit{jus} mutants, but provide the caveat that this phenotype is highly variable and requires multiple generations of selection to increase penetrance.

**Results and discussion**

In order to visualize expression of \textit{jus}, the MiMIC transposon between coding exons 2 and 3 was converted to a GFP-containing artificial exon using RCME [13]. The resulting protein has an in-frame GFP tag inserted in the extracellular loop of the protein (Fig. 1A). Hereon we refer to this allele as \textit{jus}\textsuperscript{GFSF}. Staining for GFP of \textit{jus}\textsuperscript{GFSF} adult brains revealed expression in the optic lobes, limited expression in the central brain surrounding the antennal lobes, and strong expression in select neurons of the 1\textsuperscript{st} and 2\textsuperscript{nd} segments of the thoracic abdominal ganglion (Fig. 1B). This Jus-GFP tagged protein was mostly localized to cell bodies, not axons. Vortex testing of homozygous \textit{jus}\textsuperscript{GFSF} adults revealed that this allele is 100% bang-sensitive (N = 40), indicating that the insertion of the GFP containing tag interrupts an important protein domain in Jus. The bang-sensitivity

**Figure 1.** MiMIC conversion to GFP trap and expression in the adult brain. A."GFP" indicates site in the Jus protein where the GFP, Flag, StrepII, and FlAsH tags are inserted. Transmembrane domains and intracellular domains are omitted for clarity and cysteine residues are highlighted in grey. Visualization of amino acid sequence was performed by TOPO2 ([http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py](http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py)) B. Confocal images of \textit{jus}\textsuperscript{GFSF} adult brain stained with synaptic marker anti-brp (magenta) or anti-GFP (green). GFP expression is evident in the eye, optic lobes, near the antennal lobes, and strongly in segments of the thoracic abdominal ganglia. Scale bar is 124 \( \mu \text{m} \).
caused by $jus^{GFSTF}$ and the differing subcellular localization of the MiMiC tag from the C-terminally tagged genomic fosmid construct previously reported [12] suggests that mislocalization may be partially responsible for the mutant phenotype caused by the MiMiC insertion. This mislocalization may stem from the failure of $Jus^{GFSTF}$ to bind to axonal-targeting proteins such as X11L [14]. Alternatively, insertion of GFP in the extracellular domain may reduce the stability of Jus such that it degrades before it is transported to the axons. Future experiments will test these hypotheses.

While the MiMiC tagged protein likely does not represent the endogenous localization of Jus, its tendency to localize to cell bodies can be used more easily in colocalization studies than one that localizes to axons. We previously showed that a GAL4 line driven by genomic DNA from the $jus$ locus ($w; P[y^{+t7.7}]w^{+[mC]} = GMR55G02-GAL4)attP2$) was able to rescue the bang-sensitive phenotype of $jus^{eda iso7.8}$ [12]. To explore the neuronal overlap between the GAL4 expression and $jus^{GFSTF}$, we generated a line with a nuclear localized UAS-nls-mCherry reporter and $jus^{GFSTF}$ and crossed it to the GAL4 above. Interestingly, there was limited overlap between the GFP expression and nls-mCherry (Fig. 2). Upon closer examination, a small number of neurons in the antennal lobe (AL, Fig. 2A), subesophageal ganglion (SEG, Fig. 2A), and in the first two segments of the thoracic abdominal ganglion (Fig. 2B) show GFP cell body staining and nuclear mCherry expression. Overall, a rather limited number of neurons appear to be capable of rescuing the bang-sensitive phenotype.

![Figure 2](image-url)

**Figure 2.** Overlap of GMR55G02-GAL4>nls-mCherry expression with $jus^{GFSTF}$. A. Overlap of mCherry expression (magenta) and $jus^{GFSTF}$ (green) in the brain is highlighted by arrows. Most of the overlapping neurons are in the brain surrounding the antennal lobes (AL) and in the subesophageal ganglion (SEG). B. Overlap of mCherry expression and $jus^{GFSTF}$ in the thoracic abdominal ganglion (T). Arrows highlight overlap in neurons in the first (T1) and second (T2) segments of the TAG. Scale bars indicate 50 μm.
In a deliberate effort to expand the available sensory modalities that can precipitate a seizure of bang-sensitive mutants, we first investigated if a train of brief light flashes from a strobe light could provoke seizures in *jus* 

\[ \text{sda iso7.8} \] mutants. Initial efforts resulted in a small percentage of flies paralyzing. But upon selecting for strobe sensitive flies for several generations, a more sensitized stock was generated. This sensitized stock was used in the following study. 250 *sensitized stock* was generated. This sensitized stock was subjected to 10s of strobe resulting in precipitating 194 flies to seizure (78%). The flies collapsed after 1 to 25 s and shook violently for 10–20 s before becoming still for another 20–30 s, righting themselves, and resumed walking after about a minute. The flash rate of the strobe light required to precipitate seizure varied from 10–50 Hz. This is a significant finding because unlike the mechanically-jarring vortex stimulation, flashing light from a strobe is relatively non-intrusive. More importantly, strobe induced seizure aligns fly seizure with human epilepsy, where strobe-induced seizures are common in Dravet syndrome, Jeavons syndrome, and juvenile myoclonic epilepsy [2]. Strobe-sensitive BS mutant flies are not restricted to only *jus* 

\[ \text{sda iso7.8} \] flies. We have found strobe sensitivity in other BS mutant lines of *Drosophila* including bang-senseless [1] (para 

\[ \text{bss} \]) (Hoy & Tanouye, in preparation). However, we suggest several caveats when conducting strobe experiments: (1) The strobe-sensitive phenotype appears to be highly sensitive to genetic background; for example, our *y*w' 

\[ \text{fl} \] 

\[ \text{jus} \] 

\[ \text{sda iso7.8} \] strain exhibited paralysis in only 9.1% of cases after strobe treatment (N = 187). (2) Carbon dioxide anesthetization makes *jus* mutants refractory to paralysis by strobe lighting for far longer than to paralysis by vortexing or cold shock. We recommend allowing flies to recover from carbon dioxide overnight before assaying with strobe. (3) Finally, we have observed that strobe sensitivity can be enriched by selection for the phenotype. However, the selection process requires several generations.

Previous studies showed that extreme temperatures can also affect bang-sensitive mutants [15,16]. Heating flies mutant for *bss*, *eas*, or *jus* 

\[ \text{sda iso7.8} \] reversibly protected against bang-sensitivity. Also, in the absence of mechanical shock, cold shock can induce seizures in *bss* and *eas* mutants and heat shock can induce seizures in *bas* and *tko* mutants. We had observed that exposure of *jus* 

\[ \text{sda iso7.8} \] mutants to refrigerated diet induced seizures (unpublished observations).

Therefore, we sought to characterize the cold-sensitivity of *jus* mutants in more detail. We exposed vials of wild type controls and various *jus* mutants to 14, 10, and 2°C ice baths for 1 minute, then monitored their recovery from paralysis in a second water bath that had been set at room temperature (Fig. 3). *jus* mutants were paralyzed at higher temperatures than controls were (Fig. 3A,B). At 2°C, a temperature that paralyzed wild type flies as well (Fig. 3C), *jus* mutants took longer to recover from paralysis than controls (Fig. 3F).

To independently confirm that the cold-sensitivity of *jus* mutants was due to loss of *jus* function, as well as to identify the neuronal subpopulations responsible for the cold-sensitive phenotype, RNAi of *jus* was induced by a variety of neuronal GAL4 drivers (Fig. 4). RNAi-*jus* expression with *clav*, VGA*-, VGlut*, and ChAT-GAL4 drivers all provided significant cold-sensitivity relative to flies carrying only the GAL4 or RNAi construct. Of the neurotransmitter-specific GAL4s, ChAT-GAL4 was the most effective RNAi-*jus* driver for causing cold-sensitive paralysis, just as it was for inducing bang-sensitivity [12]. In the previous study we had not observed bang-sensitivity when the RNAi-*jus* contract was expressed with a glutamatergic driver (V-glut-GAL4), but did more recently when we raised flies at 29°C to increase GAL4 function (data not shown). Therefore, *jus* appears to affect cold-sensitive paralysis and bang-sensitive paralysis in overlapping subpopulations of neurons.

The bang-sensitive paralysis of *jus* mutants is nearly fully penetrant [11], but the same phenotypes induced by strobe or cold can be incompletely penetrant and sensitive to experimental parameters. This could provide opportunities to better understand *jus* function and to develop *jus* as a model for studying epileptogenesis. For example, moderate cold shock induces paralysis at intermediate frequencies for some genotypes (Fig. 3B). This could provide an efficient, sensitized tool for genetic screens and enable us to better understand the mechanism behind each element of the seizure phenotype.

**Experimental procedures**

**Fly Stocks**

Fly stocks were maintained on yeast and glucose media at 22–25°C in plastic vials. The *slam-dance* 

\[ \text{sda iso7.8} \] mutant strain was a gift from Mark Tanouye. All other fly stocks were obtained from the

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\[ \text{sda iso7.8} \] mutant strain was a gift from Mark Tanouye. All other fly stocks were obtained from the
Bloomington Stock Center. When applicable, the w^{118} (BL#5905) and y^{1}w^{1} (BL#1495) strains were used as controls. GAL4 drivers were: elav-GAL4 (a pan-neural driver, BL#458), VGAT-GAL4 (a GABAergic driver, BL#26160), VGlut-GAL4 (a glutamatergic driver, BL#26160), and ChAT-GAL4 (a cholinergic driver, BL#6798), For RNAi of jus we used the P{TRiP.JF03192}attP2 line (here referred to as RNAi-jus; BL#28764). Other strains used in this study are listed below.

Figure 3. Mutations in jus exacerbate cold-sensitive paralysis. 1–3 day old adult flies were subjected to cold shocks at 14°C (A, D), 10°C (B, E) or 2°C (C, F) as described in the Experimental procedures, and percent of total exhibiting paralysis (A–C) and mean recovery time from paralysis (D–F) were scored. y^{1}w^{1} and w^{118} were used as wild type controls because y^{1}w is the genetic background for our jus^{sda iso7.8} strain (abbreviated here as 'sda iso7.8') and w^{118} is the genetic background for the jus^{GFSTF}, jus^{f04904} and Df(3R) BSC501 ('DF') strains. (A) 14°C cold shocks did not cause paralysis of any controls, but caused high rates of paralysis in flies with two mutant copies of jus. jus^{sda iso7.8} homozygotes were an exception to this trend, showing no paralysis at this temperature. However, they exhibited significant paralysis in the hemizygous condition (sda iso7.8/Df). (B) As at the higher temperature, 10°C cold shocks caused high rates of paralysis in flies with two mutant copies of jus, and at this temperature there were also moderate levels of paralysis in flies heterozygous for all mutant alleles except for jus^{GFSTF} (compare GFSTF/+ to the other heterozygotes). Interestingly, the y^{1}w^{1} control strain also exhibited moderate rates of paralysis at this temperature while w^{118} flies were not paralyzed. (C) 2°C cold shock caused paralysis of all flies that were tested. In (A–C), * indicates p < 10^{-5}, Fisher’s exact test. Heterozygotes were tested relative to their wild type background strain. Flies with two mutant copies of jus were tested relative to their background wild type strain as well as relative to each of their alleles in the heterozygous condition. (D, E) Recovery time from paralysis after cold shock at 14°C and 10°C, respectively. Flies homozygous or hemizygous for the jus^{GFSTF} and jus^{f04904} alleles exhibited the longest recovery times. (F) Although cold shocks at 2°C paralyzed all genotypes that we tested, flies with two mutant copies of jus took a significantly longer time to recover than all of their associated controls (again jus^{sda iso7.8} homozygotes were an exception). In (D–F), error bars indicate SEM, and + indicates p < 0.05 for a Student’s t-test—experimental groups were compared to controls as described above for the Fisher’s tests. For all of these cold shock experiments, N = from 7 up to 57 flies for each genotype-temperature combination, an exception being N = 5 for jus^{GFSTF} homzygotes at 2°C (‘f04904/Df’) due to difficulties with obtaining large numbers of flies with this genotype.
MiMIC RCME

For GFP-trap generation, the MiMIC insertion line (BL#55592) was crossed to phiC31 and the resulting embryos were injected with plasmid pBS-KS-attB1-2-PT-SA-SD-1-EGFP-FlAsH-StrepII-TEV-3xFlag [13] and yw embryos were selected and screened by PCR for the correct orientation of plasmid by Bestgene (Chino Hills, CA). Individual lines were screened for GFP expression and Flag-tag immunoreactivity. This line will be referred to as jusGFSTF.

Seizure triggering

For strobe stimulation, we subjected vials of 10-20 jusda iso7.8 flies that were 4–10 days post-eclosion to 10 s of strobe, with a flash rate of the strobe light that was varied from 10–50 Hz. The effective stimulus was quickly determined by varying the strobe frequency over this range. The most effective strobe rate was about 15–30 Hz.

For mechanical testing, adult flies, 1–3 days post-eclosion, were anesthetized with carbon dioxide and placed in vials containing food and 10 flies each. 24 hours after CO₂ exposure, the flies were tested for bang sensitivity by vortexing the vial for 10 seconds using the Vortex Genie 2 (VWR Scientific) 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 [11].

For cold testing, adult flies, 1–3 days post-eclosion, were anesthetized with carbon dioxide and distributed into standard plastic fly vials with plastic wrap stretched over the top opening to form a seal. Flies were allowed to recover for at least 30 minutes before cold shock assays, and from here on, vials were handled one at a time and gently so as not to inadvertently induce seizures by mechanical stimulation. For cold shock, vials were submerged for one minute in an ice water bath that had been chilled to 2, 10, or 14°C, with just the plastic wrap and the very top of the vial above water. Vials were then warmed in a 21–23°C (room temperature) water bath until all the flies had righted themselves. Data were collected by positionining a smart phone camera immediately above the plastic wrap and recording videos of flies as they experienced cold shock and recovery.

Antibody staining

jusGFSTF flies were briefly anesthetized in CO₂, then dipped in ethanol and dissected in Ca²⁺ free- HL3 saline and fixed, stained, mounted as described [17]. Rabbit anti-GFP (Invitrogen) was used at 1:1000, anti-brp NC82 (Developmental Hybridoma Bank) at 1:50, and secondary Donkey anti-Rabbit Alexa488 (Invitrogen) and Donkey anti-mouse Cy3 (Jackson Immunoresearch) were used at 1:1000. Brains were imaged on an Inverted Zeiss LSM 880 confocal microscope with a C-Achroplan 32x/0.85 water immersion objective. For colocalization experiments, w; UAS-nls-mCherry/+; P[y+lht.7] w++; [+mC] = GMR55G02-GAL4attyP2, jusGFSTF/+ flies were generated to determine the degree of overlap of P[y+lht.7] w++; [+mC] = GMR55G02-GAL4attyP2 (BL#46070) and, jusGFSTF.

Figure 4. RNAi of jus in neurons exacerbates cold-sensitive paralysis. (A) Expression of RNAi-jus in post-mitotic neurons (with an elav-GAL4 driver), or in GABAergic, glutamatergic, and cholinergic subpopulations (VGAT, VGlut, and Chat respectively), caused cold-sensitive paralysis at 10°C, a temperature that almost never caused cold-sensitive paralysis in flies carrying only a GAL4 or RNAi construct. * p<10⁻², Fisher’s exact test of flies carrying both the RNAi-jus construct and a GAL4 construct vs. RNAi-jus-only and vs. GAL4-only controls. (B) Recovery time from paralysis after cold shock at 10°C. In (B), error bars indicate SEM, and ** indicates p<10⁻⁵ for a Student’s t-test comparing the same groups that were compared with the Fisher’s test in (A). N = 14 or more flies for all genotypes in these RNAi experiments.
Images are maximum intensity projections of confocal z-stacks by Zen or Imaris (Bitplane) software.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Mark Tanouye and the Bloomington *Drosophila* Stock Center for flies used in this study (NIH P40OD018537). The monoclonal antibody NC82 developed by E. Buchner was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Confocal microscopy was performed at the Cornell University Biotechnology Resource Center supported by NYSTEM CO29155 and NIH S10OD018516.

**Funding**

This work was supported by the Triad Foundation.

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