straightjacket is required for the synaptic stabilization of cacophony, a voltage-gated calcium channel $\alpha_1$ subunit

Cindy V. Ly,1 Chi-Kuang Yao,2,3 Patrik Verstreken,2,3 Tomoko Ohyama,2 and Hugo J. Bellen1,2,3,4

1Department of Neuroscience, 2Department of Molecular and Human Genetics, 3Howard Hughes Medical Institute, and 4Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030

In a screen to identify genes involved in synaptic function, we isolated mutations in Drosophila melanogaster straightjacket ($stj$), an $\alpha_2\delta$ subunit of the voltage-gated calcium channel. $stj$ mutant photoreceptors develop normal synaptic connections but display reduced “on-off” transients in electroretinogram recordings, indicating a failure to evoke postsynaptic responses and, thus, a defect in neurotransmission. $stj$ is expressed in neurons but excluded from glia. Mutants exhibit endogenous seizure-like activity, indicating altered neuronal excitability. However, at the synaptic level, $stj$ larval neuromuscular junctions exhibit approximately fourfold reduction in synaptic release compared with controls stemming from a reduced release probability at these synapses. These defects likely stem from destabilization of Cacophony (Cac), the primary presynaptic $\alpha_1$ subunit in D. melanogaster. Interestingly, neuronal overexpression of cac partially rescues the viability and physiological defects in $stj$ mutants, indicating a role for the $\alpha_2\delta$ Ca$^{2+}$ channel subunit in mediating the proper localization of an $\alpha_1$ subunit at synapses.

Introduction

Neuronal voltage-gated calcium channels (VGCCs) mediate neuronal migration (Komuro and Rakic, 1998), neurite outgrowth (Rieckhof et al., 2003), synaptogenesis (Bahlis et al., 1998), neuronal excitability (Pietrobon, 2005), and neurotransmission (Smith and Augustine, 1988; Robitaille et al., 1990). VGCCs are comprised of a pore-forming $\alpha_1$ subunit associated with accessory subunits $\alpha_2$, $\delta$, and $\gamma$ (Takahashi et al., 1987; Tanabe et al., 1987). $\alpha_2\delta$ consists of two disulfide-linked subunits, $\alpha_2$ and $\delta$, derived from posttranslational cleavage of a single gene product (Ellis et al., 1988; De Jongh et al., 1990). Although $\delta$ is a minimal transmembrane domain that anchors the subunit to the plasma membrane, $\alpha_2$ is extracellular and heavily glycosylated, a modification important for regulating $\alpha_1$ activity (Jay et al., 1991; Gurnett et al., 1996; Sandoval et al., 2004).

Our understanding of how $\alpha_2\delta$ affects $\alpha_1$ pore subunits mostly derives from work in heterologous expression systems in which these subunits were coexpressed and biophysical parameters assessed by whole cell recording. Four $\alpha_2\delta$ homologues exist in vertebrates. Although several studies describe a role for $\alpha_2\delta_{1-3}$ in modulating the kinetics and voltage-dependence of channel gating (Singer et al., 1991; Felix et al., 1997; Klugbauer et al., 1999; Herlitze et al., 2003), others found no effect for $\alpha_2\delta$ in regulating these properties (Mikami et al., 1989; Gao et al., 2000). Research also suggests that $\alpha_2\delta_{1,2}$ increases Ca$^{2+}$ currents (Singer et al., 1991; Felix et al., 1997; Klugbauer et al., 1999; Gao et al., 2000; Canti et al., 2005), and $\alpha_2\delta$ overexpression in nonneuronal cells enriches N-, P/Q-, and L-type channels at the plasma membrane (Felix et al., 1997; Canti et al., 2005). However, no current enhancement is observed when $\alpha_2\delta_1$ is coexpressed with R-type channels (Qin et al., 1998). Though these studies highlight the potential effects of $\alpha_2\delta$ on VGCCs, in vivo studies based on loss-of-function data should reveal the contribution of $\alpha_2\delta$ to regulation of native channels.

Ducky mice that harbor mutations in $\alpha_2\delta_2$ have spike-wave seizures and are ataxic (Barclay et al., 2001; Brill et al., 2004; Donato et al., 2006). Also, dissociated ducky mutant Purkinje cells exhibit reduced Ca$^{2+}$ currents (Barclay et al., 2001; Donato et al., 2006). Notably, gabapentin, an antiepileptic drug also used to...
treat neuropathic pain, binds specifically to α₂δ (Gee et al., 1996), an interaction thought to reduce neurotransmission in these pathological conditions. Therefore, a better understanding of how α₂δ subunits affect neurotransmission may shed insight into the mode of action of gabapentinoid drugs as well as VGCC function.

In a screen for genes affecting synaptic function, we identified straightjacket (stj), which encodes a Drosophila melanogaster α₂δ similar to vertebrate α₂δs. stj mutants exhibit a severe reduction in Ca²⁺-dependent evoked neurotransmitter release that stems from a presynaptic role for stj based on in situ hybridization studies, enhancer trap expression, and analysis of spontaneous release at mutant synapses. Furthermore, we observe a reduction of the primary presynaptic D. melanogaster α₁ subunit, Cacophony (Cac), at mutant synapses, indicating that the synaptic defects result from a failure to properly localize synaptic Ca²⁺ channels.

Results

stj mutants display electroretinogram (ERG) defects

In a forward genetic screen designed to isolate genes involved in synaptic function, we isolated three alleles in one complementation group. Using the eyFLP system (Stowers and Schwarz, 1999; Newsome et al., 2000), we made flies homozygous for randomly induced ethyl methanesulfonate mutations in the visual system that were otherwise heterozygous in the body, thus circumventing the lethality associated with many mutations affecting synaptic transmission. Mutant flies were behaviorally screened for a response to light in a phototaxis assay (Benzer, 1967). Flies with reduced phototaxis responses were crossed, and progeny with homozygous mutant eyes were subjected to ERG recordings (Pak et al., 1969), extracellular field recordings of the photoreceptor (PR) response to light. Although all mutants initially failed to phototax, this defect failed to persist in later generations (unpublished data), as observed for other mutants isolated in similar screens (Verstreken et al., 2003, 2005). However, in response to a light stimulus, stj mutants exhibit a reduced depolarization in the ERG, which suggests a defect in phototransduction, as well as a lack of “on–off” transients (Fig. 1A, arrowheads), indicating a failure to induce a postsynaptic response (Pak et al., 1969). To determine whether the ERG defect stems from a pre- or postsynaptic requirement for the disrupted gene (the eyFLP system creates homozygous mutant PRs as well as mutant postsynaptic cells; Hiesinger et al., 2006),
we used the ey3.5FLP system, which only drives FLP recombinase in presynaptic PRs (Chotard et al., 2005; Mehta et al., 2005). As shown in Fig. 1, the “on” transients remain absent, suggesting that the affected gene is required presynthetically (Fig. 1 B). Because failure to evoke a postsynaptic response may derive from impaired synaptic development or synaptic function, we performed electron microscopy to examine the lamina where R1-R6 PRs synapse with the postsynaptic monopolar cells to form cartridges with stereotyped organization (Kirschfeld, 1967; Clandinin and Zipursky, 2000). Similar to controls (Fig. 1, C and F), stj1 (Fig. 1, D and G) and stj2 (Fig. 1, E and H) eyFLP mutant cartridges predominantly possess six PR terminals per cartridge (Kirschfeld, 1967; Clandinin and Zipursky, 2000). We observe no other obvious morphological defects at these synapses. These data suggest that mutants have defective postsynaptic responses because of aberrant synaptic communication, not aberrant neuronal development.

**stj encodes a D. melanogaster αδ subunit**

stj mutant third instar larvae are immobile with the exception of some head movements. Given the paralytic and ERG phenotypes, we sought to identify the gene affected in stj mutants. We used molecularly defined P elements (Zhai et al., 2003) to map stj to the 50C cytological interval of chromosome 2R. Deficiencies within the interval were then used to confirm and refine the mapping position. All three stj alleles failed to complement Df(2R)CX1 [49C1-4;50C23-D2] and Df(2R)Exel7128 [50C5;50C9], which spans a region <100 kbp (Fig. 2 A). Using P elements within the 50C5–50C9 interval, we performed fine mapping and found that a P element inserted in CG12295, P{KG06941} yielded zero recombinants out of 10,000 flies (Fig. 2 A), which suggests that CG12295 corresponds to stj. Sequencing of CG12295 revealed a point mutation in stj1, a Glu133Ala transition, altering a residue conserved in human and mouse homologues, and a short C-terminal transmembrane region (TM, line). Mutations associated with the two characterized stj alleles are indicated. (D) αδ phosphorylation fly αδs to nematode, mouse, and human homologues. (E) Lethal phase analysis of stj alleles. Extent of survival: L1, first instar; L2, second instar; P, pupae; UA, uncoordinated adult; −, not determined. Circles denote rescue to healthy adults by genomic construct. (F) Rescue of ERG defects by genomic stj construct. Arrowheads indicate the position of on–offs.
chemotaxis receptors, are thought to mediate binding to small molecules such as amino acids (Anantharaman and Aravind, 2000). BLAST searches revealed three putative homologues of αβδ in the *D. melanogaster* genome compared with four in mammalian species. CG12295 most closely resembles human αβδ3 (33% identical and 60% similar) and αβδ3 (31% identical and 59% similar; Figs. 2 D and S1). The VWA domain is particularly conserved, with 44% identity to αβδ3 and 48% identity to αβδ4. In addition, the Cache domain is 49–50% and 45% identical to vertebrate αβδ3 and αβδ4, respectively. αβδ3 and αβδ4 are less extensively characterized relative to other isoforms. Though no mutants currently exist for αβδ4, mutations in αβδ4 in mice and humans underlie PR dysfunction and progressive blindness (Wycisk et al., 2006a,b).

As homozygotes, the three *stj* alleles failed to survive beyond the early larval stages. However, when placed over *Df(2R)Exel7128* or in trans to one another, these larvae arrest as pupae and some emerge as uncoordinated adults, suggesting that these alleles may contain extraneous second site mutations that contribute to the homozygous lethality (Fig. 2 E). Of note, when over deficiency, both *stj*1 and *stj*2 alleles have similar lethal phases compared with the truncation mutant *stj*3, indicating that both may constitute null or severe hypomorphic alleles.

To ascertain that the defects stem from loss of αβδ, we introduced a 28.6-kbp genomic transgene in P[acman] (Venken et al., 2006) and neurally expressed a full-length UAS-FLAG-*stj*-HA cDNA transgene in the mutants. The genomic construct rescued *stj*/*Df* and transheterozygote mutant combinations to adulthood (Fig. 2 E, circles). Note that some *stj*2/*Df* and *stj*2/*Df* animals elose occasionally as adults but are severely uncoordinated (unpublished data) and unable to fly, whereas rescued adults walk and fly normally (unpublished data). In addition, the genomic transgene restored the physiological defects observed by ERG. Similar to *eyFLP* mutants (Fig. 1 A), *stj*/*Df* and *stj*2/*Df* adult escapers also had reduced depolarization and loss of on-off transients. However, these ERG anomalies were corrected in the rescued adults (Fig. 2 F). The genomic *stj* transgene also restored on-off transients in *eyFLP* *stj*1 and *eyFLP* *stj*2 mutants (not depicted).

Furthermore, when we used *C155-GAL4* to drive expression of UAS-FLAG-*stj*-HA panneuronally in *stj*2/*Df* and *stj*2/*Df* mutants, we also recovered viable adults. Thus, *stj* is required in the nervous system. Together, these findings show that *stj* is a crucial neuronal gene necessary for proper synaptic communication.

### *stj* is expressed in neurons

To determine where *STJ* mRNA is expressed, we performed in situ hybridization on whole mount embryos. As shown in Fig. 3 A, the *STJ* message is expressed in the embryonic nervous system starting at stages 11 and 12 and is highly enriched in the brain and ventral nerve cord (VNC) in late stage embryos. A sense probe fails to label the embryonic brain (Fig. 3 B), indicating that the signal is specific to *STJ*. This is consistent with data showing that *STJ* mRNA is abundant in the brain and thoraco-abdominal ganglion of adult flies but is not detected in other adult tissues (www.FlyAtlas.org; Chintapalli et al., 2007). Thus, the *STJ* message is highly expressed in the *D. melanogaster* nervous system.

Given the paralysis in late larval stages, we turned to the third instar nervous system. We attempted to generate antibodies but were unsuccessful. However, we obtained an enhancer trap line (*NP1574*) that contains a GAL4 driver (Kyoto Institute of Technology Drosophila Genetic Resource Center; Hayashi et al., 2002) within the 5′ untranslated region of *stj* (Fig. 2 B). Hence, in combination with a UAS reporter, GAL4 expression of this enhancer trap may reveal the expression pattern of *stj*.

In agreement with our in situ hybridization data, the GAL4 driver is expressed in the brain and VNC in adults (Fig. 3 C) and larvae (Fig. 3, D–I′′), but is not present during early embryogenesis (not depicted). Therefore, we refer to *NP1574-GAL4* as *stj-GAL4*. Given the visual processing defects observed in *stj* *eyFLP* mutants (Fig. 1 A), we assessed *stj-GAL4*-driven GFP expression in the visual system. Cytoplasmic GFP is evident in PR axons and terminals and in the optic lobes of the adult brain, which is consistent with a role for *stj* in visual processing (Fig. 3 C). In third instar larvae, we detected *stj-GAL4*-driven GFP signal predominantly in a subset of cells in the VNC (Fig. 3 D) and salivary glands (not depicted). Here, GFP-positive cells are coalesced with the panneuronal marker Elav (Fig. 3 E; O’Neill et al., 1994) but not Repo (Fig. 3 F; Muhligh-Veresen et al., 2005), a glial marker. However, *stj-GAL4* is expressed only in a subset of neurons within the VNC, some of which colocalize with Even-skipped (Fig. 3 G; Patel et al., 1994), a motor neuron marker. These motor neurons send axonal projections that form synapses outlined by the pre- and postsynaptic marker Dlg (Parnas et al., 2001) on body wall muscles 6/7 (Fig. 3, H and H′). Also, some GFP-positive neurons coincide with cell bodies labeled with anti-GABA (Fig. 3, I–I′′). GABA is synthesized by glutamate decarboxylase (GAD) found exclusively in inhibitory neurons, and, notably, GABA and GAD are present in cell bodies of the *D. melanogaster* nervous system (Buchner et al., 1988). Thus, *stj* is also present in a subpopulation of inhibitory interneurons. Therefore, *stj* is expressed in a discrete subset of neurons in the third instar larva, including motor neurons and inhibitory interneurons.

To examine the subcellular localization of Stj, we drove UAS-FLAG-*stj*-HA panneuronally using *C155-GAL4* in *stj*/*Df* mutants and labeled using antibodies to Syb (Fig. 3 J′), a synaptic marker, and HA (Fig. 3 J) to detect Stj. Unlike Cac, a presynaptic VGCC subunit that localizes to puncta corresponding to active zones (Kawasaki et al., 2004), Stj shows extensive colocalization with Syb and is distributed throughout the synapse (Fig. 3 J′′).

### *stj* mutants are hyperexcitable

Disruption of vertebrate and invertebrate VGCCs have been shown to predispose organisms to epileptic events. Mouse mutants that affect various VGCC subunits, including α2A (*tottering*), β3 (*lethargic*), and αδ3 (*ducky*), exhibit epileptic phenotypes (Burgess and Noebels, 1999). Furthermore, hypomorphic mutants in *cac* display seizure-like activity at elevated temperatures (Rieckhof et al., 2003), and *stj* (CG12295) expression is dynamically regulated in *D. melanogaster* seizure mutants (Guan et al., 2005). We therefore explored whether mutations in *stj* might also affect neuronal excitability by recording the endogenous
pressed in motor neurons. Intriguingly, the loss of stj in a discrete subset of neurons, particularly GABAergic neurons, may contribute to neuronal hyperexcitability in these mutants by altering the balance of excitation and inhibition in the neuronal circuit subserving locomotion. Of note, GABA blockade has been shown to lead to seizure-like activity in flies (Stilwell et al., 2006). Together, this suggests that stj mutants show defects at both the network and synaptic levels.

**stj mutants have defects in evoked neurotransmission**

To assess neurotransmitter release at stj synapses, we performed additional electrophysiological recordings at the larval neuromuscular junction (NMJ). We recorded from muscles 6/7 of dissected third instar larvae with intact VNCs at elevated temperature, a common paradigm for assessing seizure-like activity in *D. melanogaster* (Budnik et al., 1990; Rieckhof et al., 2003). Controls often exhibit rhythmic activity (Fig. 4, A and E). However, though burst events are relatively rare in stj/Df and stj2/Df mutants, activity trains often last 30 s or longer (Fig. 4, B, C, and E). In addition, a genomic αδ transgene restores rhythmic CPG activity in stj/Df and stj2/Df mutants, indicating that these defects are specific to loss of stj (Fig. 4, D–E; and not depicted). Notably, mutant bursts are also lower in amplitude compared with the control (Fig. 4, B and C). The reduced amplitude of events is consistent with stj being expressed in motor neurons. Intriguingly, the loss of stj in a discrete subset of neurons, particularly GABAergic neurons, may contribute to neuronal hyperexcitability in these mutants by altering the balance of excitation and inhibition in the neuronal circuit subserving locomotion. Of note, GABA blockade has been shown to lead to seizure-like activity in flies (Stilwell et al., 2006). Together, this suggests that stj mutants show defects at both the network and synaptic levels.
mutations over deficiency and in trans show a marked reduction with 1.5 mM Ca\(^{2+}\) for control (A; of endogenous CPG activity performed at elevated temperature (36 °C) GMR-lacZ; FRT42D iso stj 1 /Df(2R)Exel7128 lacZ; FRT42D iso stj 2 /Df(2R)Exel7128 (TTX) to suppress evoked release. The mean mEJP amplitudes with respect to synaptic function (Fig. 5 B). Furthermore, when we introduce a genomic rescue construct in mutants over deficiency and in trans to one another, the reduced EJP amplitudes are restored (Fig. 5 B), which indicates that the loss of stj is solely responsible for the exocytic defect.

In addition to exocytic defects, a reduction in EJP amplitudes similar to controls, indicating that there are no deleterious effects on exocytosis. These results suggest that stj loss of function has predominant deleterious effects on exocytosis.

At synapses, Ca\(^{2+}\) is a key regulator of vesicle fusion and the amount of neurotransmitter released (Katz and Miledi, 1969). To explore the relationship between Ca\(^{2+}\) entry and evoked release at stj synapses, we measured EJPs, counting failures, at different extracellular Ca\(^{2+}\) concentrations from 0.1 to 1 mM Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{ext}). At every [Ca\(^{2+}\)]\text{ext} studied, EJP amplitudes in the mutant were reduced compared with controls (Fig. 5 I). To examine [Ca\(^{2+}\)] sensitivity, we corrected for nonlinear summation of EJPs (Martin, 1955), determined quantal content, and generated a logarithmic plot of quantal content versus low [Ca\(^{2+}\)]\text{ext} (Fig. 5 J).

We also examined control and stj mutant synapses for paired pulse facilitation, an enhancement of neurotransmitter release caused by elevation of residual Ca\(^{2+}\) in the nerve terminal (Zucker and Regehr, 2002). We applied two stimuli spaced 20, 50, and 100 ms apart and recorded EJPs in 1 mM Ca\(^{2+}\). The extent of facilitation was expressed as the paired pulse ratio (PPR), EJP\(2/EJP1\). When the pulse interval is 100 ms, there is no significant difference between the PPR in control and mutant animals. However, at pulse intervals of 50 and 20 ms (Fig. 5 K), stj /Df (Fig. 5 J) and stj /Df (not depicted), indicating a reduction in synaptic Ca\(^{2+}\) sensitivity.

junction (NMJ). Motor neurons were severed to prevent endogenous stimulation. We then stimulated control and mutant motor axons at 1 Hz in 1 mM Ca\(^{2+}\) and measured excitatory junctional potentials (EJPs) from the muscle. Compared with controls, all stj mutations over deficiency and in trans show a marked reduction in EJP amplitude (Fig. 5, A and B), revealing a severe defect in Ca\(^{2+}\)-regulated exocytosis. Notably, stj heterozygotes (stj /+) have EJP amplitudes similar to controls, indicating that there are no prominent dominant-negative effects associated with these mutations with respect to synaptic function (Fig. 5 B). Furthermore, when we introduce a genomic rescue construct in mutants over deficiency and in trans to one another, the reduced EJP amplitudes are restored (Fig. 5 B), which indicates that the loss of stj is solely responsible for the exocytic defect.

In addition to exocytic defects, a reduction in EJP amplitude may also reflect impairments in vesicular neurotransmitter loading or postsynaptic receptor function. To examine these possibilities, we measured spontaneous miniature EJPs (mEJPs) in stj mutants in 0.5 mM Ca\(^{2+}\) and 10 mM tetrodotoxin (TTX) to suppress evoked release. The mean mEJP amplitudes between control and mutants are not different (Fig. 5, C and D), nor are the distributions of mEJP event amplitudes (Fig. 5, F–H), which suggests that vesicle loading and postsynaptic receptor function are intact. In addition, we did not observe a difference in mEJP frequency between controls and stj mutants (Fig. 5 E).

stj mutants exhibit a mild NMJ overgrowth but normal synaptic bouton ultrastructure

Synapse growth is regulated both by synaptic Ca\(^{2+}\) entry and neuronal hyperexcitability, we assessed whether NMJ morphology might be altered by labeling control and mutant larvae with the pre- and postsynaptic membrane marker Hrp (Parnas et al., 2001) and the presynaptic membrane marker Dlg (O’Neill et al., 1994). Relative to controls (Fig. 6, A and C), stj mutants (Fig. 6, B and C) exhibit a significant but mild synaptic overgrowth of the NMJ on muscles 6/7 in proportion to muscle size, reflected by a proportional increase in bouton number per muscle area (Fig. 6 D). This may be caused by the effect of hyperexcitability on synapse growth or a compensatory response to reduced synaptic transmission.
To more closely examine single boutons, ultrastructural analyses were performed on control (Fig. 6, E and G) and mutant (Fig. 6, F and H) boutons. However, these studies revealed no differences in vesicle density (Fig. 6 I), synaptic length (Fig. 6 J), active zone density (Fig. 6 K), number of active zone–associated vesicles (Fig. 6, L), or vesicle size (Fig. 6, M and N). Notably, the subsynaptic reticulum (SSR) surrounding the boutons appears more disordered in mutant boutons. However, even when SSR is almost entirely absent, as in dpix mutants (Parnas et al., 2001), synaptic function is only mildly affected. Thus, our SSR defect is not likely to contribute to the impaired synaptic release in stj mutants. These data indicate that though NMJ synapses are slightly overgrown in stj mutants, most aspects of bouton architecture are intact.

Cac is reduced at stj synapses

Given the similarity of stj and cac phenotypes (Smith et al., 1998; Rieckhoff et al., 2003), we determined whether Cac is properly localized in stj mutants. When expressed solely in neurons, cacEGFP (Kawasaki et al., 2004) rescues the embryonic lethality as well as synaptic function of cac null mutants and localizes to synaptic active zones. Therefore, we expressed cacEGFP in control and mutant neurons using C155-GAL4. We visualized C155-GAL4–driven expression of CacEGFP (green) at third instar NMJs (Fig. 7, A and D) and costained with Bruchpilot (Brp; Fig. 7, A’ and D’, magenta), an active zone marker (Wucherpfennig et al., 2003), and Dlg to outline synapses (not depicted). Because native CacEGFP fluorescence is weak, we amplified the signal using tyramide enhancement.
this suggests that CacEGFP can properly traffic to active zones independently of stj but requires stj to ensure appropriate levels of Cac at the synapse. To determine whether the reduction in synaptic CacEGFP might stem from global mistrafficking or reduced stability of Cac in stj mutants, we also looked at the VNC (Fig. 7, B and E) and axonal projections (Fig. 7, C and F) of control and mutant larvae overexpressing cacEGFP. We found that CacEGFP was distributed similarly within cell bodies throughout the VNC in control (Fig. 7 B) and mutant (Fig. 7 E) animals. Furthermore, the signal intensities of CacEGFP within the VNC of control and mutant animals are not different (Fig. 7 J). In addition, levels of CacEGFP are similar within axonal projections (Fig. 7 K). Hence, the data indicate that stj is not crucial for the global stabilization or axonal transport of Cac but rather plays a more discrete role in stabilizing Cac locally at synapses. Neuronal overexpression of cac partially rescues stj phenotypes

Interestingly, when we expressed cacEGFP panneuronally in stj/Df and stj/Df mutant backgrounds, we observed a partial rescue
stj mutant larvae, which barely move (unpublished data). To determine whether neuronal expression of cacEGFP affects the function of stj mutant NMJs, we recorded EJPs in 1 mM Ca\(^{2+}\) at 1 Hz from C155-GAL4; UAS-cacEGFP/+ and C155-GAL4; stj\(^{1}/Df\) larvae (Fig. 8, C and D). Neuronal expression of cacEGFP alone did not alter the EJP amplitude compared with controls (Fig. 8 D). However, although C155-GAL4; stj\(^{1}/Df\) and C155-GAL4; stj\(^{2}/Df\) larvae have reduced EJP amplitudes compared with controls, the response is increased relative to stj\(^{1}/Df\) and stj\(^{2}/Df\) (Fig. 8, C and D). In addition, we...
investigated the effect of cacEGFP overexpression on the hyper-excitability observed in stj mutants by recording endogenous CPG activity from C155-GAL4; stj1/Df, UAScacEGFP. Unlike stj1/Df, mutants overexpressing cacEGFP rarely exhibit activity bursts lasting 30 s or more (Fig. 8, E and F). Thus, panneuronal expression of cacEGFP can partially rescue the viability and functional defects associated with stj loss of function. Together, this further indicates that stj genetically interacts with cac.

Discussion

Neuronal communication between synapses involves regulated exocytosis of neurotransmitter at presynaptic sites, a process triggered by regulated influx of Ca2+ through VGCCs (Smith and Augustine, 1988; Catterall, 1998; Robitaille et al., 1990). VGCCs are thought to be comprised of a protein complex consisting of a pore-forming α1 subunit along with several accessory subunits including β, γ, and α2δ (Takahashi et al., 1987; Tanabe et al., 1987). Here, we describe the isolation and characterization of D. melanogaster α2δ mutants named stj. stj mutants display seizure-like activity and a severe reduction in synaptic release. Our findings indicate that these defects stem from a failure to properly stabilize Cac, a presynaptic α1 pore subunit, at synapses.

Although four α2δ homologues exist in vertebrates, only three α2δ subunits are encoded in flies. The three D. melanogaster α2δ homologues are most similar to mammalian α2δ1 and α2δ2, which are the least characterized in vertebrates. Though mutants of vertebrate α2δ1 and α2δ2 have not yet been described, mutations in α2δ1 impair retinal function, resulting in a slowly progressing cone dystrophy in patients that leads to blindness (Wycisk et al., 2006a, b). Notably, PR function is disrupted in cac mutants (Smith et al., 1998), and we also find that stj mutants have abnormal ERGs, suggesting a conserved role for VGCCs in retinal signaling. Consistent with this finding, another study on the D. melanogaster α2δ subunit (i.e., stj and dαδ) published while this manuscript was in submission also reported aberrant PR signaling (Dickman et al., 2008). Notably, we find that PR morphology in the visual system is intact, suggesting that the defect is functional, not developmental, in nature.
Further insight into the in vivo role of α₂δ has been garnered through work on a spontaneous mouse mutant of α₂δ known as ducky. The three alleles of ducky, ducky (Barclay et al., 2001), ducky+/− (Donato et al., 2006), and Cacna2d2/+/− (Brill et al., 2004), exhibit spike-wave seizures reminiscent of absence epilepsy and are ataxic. Similarly, we also find that stj mutants have altered neuronal excitability and surmise that this may be caused by a selective loss of stj in a subset of neurons, possibly inhibitory interneurons. Consistent with this possibility, blockade of GABA receptors in D. melanogaster larvae has been shown to predispose to neuronal hyperactivity (Stilwell et al., 2006).

Five functionally distinct VGCC α2 subtypes encoded by 10 different genes are expressed in mammalian excitable tissues, L-, N-, P/Q-, R-, and T-types (Catterall, 2000). However, within the D. melanogaster genome, there are only four genes that encode α₂ subunits representing homologues of vertebrate N-, P/Q-, and R-type (cac, also known as dmcα1A; Smith et al., 1996), L-type (dmcα1D; Zheng et al., 1995), and T-type (dmcα1T) channels (CG15899; http://flybase.bio.indiana.edu/) and an invertebrate-specific α₂ subunit (dmα1αU, also known as narrow abdomen and halothane resistance; Nash et al., 2002). However, we focused on cac for the following reasons. First, mutations in dmα1αU are viable and predominantly affect diurnal locomotor activity patterns but exhibit no other obvious neurological defects (Nash et al., 2002). Second, dmcα1T has not been characterized in the fly and no phenotypic analysis is available. Third, dmcα1D is thought to primarily underlie muscle Ca²⁺ currents (Zheng et al., 1995; Ren et al., 1998). Although dmcα1D transcripts are expressed in the brain, the function of dmcα1D in the nervous system has not been established (Zheng et al., 1995). However, cac mutants display a marked reduction in synaptic release, similar to what is observed when stj is lost, and is likely the primary α₂ subunit involved in neurotransmission in flies (Smith et al., 1996; Kawasaki et al., 2000). In addition, cac mutants have ERG (Smith et al., 1998) and seizure (Rieckhof et al., 2003) phenotypes similar to stj mutants. The synaptic defects in cac mutants can be corrected by neuronal overexpression of cac cDNA, which suggests a requirement for this gene in the nervous system (Kawasaki et al., 2004). Consistent with this data, stj is predominantly expressed and required in the nervous system, as demonstrated by our ability to rescue the mutants with a neuronally driven stj transgene and in situ hybridization studies. Together, the neuronal localization and phenotypic similarities suggest that cac is a likely target for stj function.

Work in heterologous expression systems has demonstrated that α₂δ subunits can increase Ca²⁺ current amplitudes approximately threefold by increasing the expression of α₁ on the membrane (Singer et al., 1991; Felix et al., 1997; Klugbauer et al., 1999; Gao et al., 2000; Canti et al., 2005). In addition, ducky mutant Purkinje cells exhibit a ∼35% reduction in P-type Ca²⁺ currents despite no changes in unitary Ca²⁺ currents (Barclay et al., 2001). However, evidence of channel mislocalization in ducky mice has not been demonstrated and the mechanism by which α₂δ loss leads to the defects in ducky mice remains unclear. Similar to Dickman et al. (2008), we observe a severe reduction in EJJP amplitude at stj mutant NMJ synapses. We find that this impairment is likely caused by a reduction in the highly Ca²⁺-dependent release probability because we observed increased facilitation at stj synapses and a rightward shift in the Ca²⁺ dependence of neurotransmitter release. The alternate study partially attributes the reduction in synaptic release to a reduced number of active zones, suggested by a decreased mEJP frequency and a reduction in Brp labeling (Dickman et al., 2008). However, we do not observe a loss of active zones in stj mutants. Notably, they compared their mutants to Canton-S to assess Brp labeling, whereas a different control (w¹¹¹⁸) was used for electrophysiological analyses. Because it was not reported whether these defects could be corrected by α₂δ transgene expression, we cannot exclude that some of the defects are due to genetic background.

However, we do find that CacEGFP is dramatically reduced at mutant synapses when expressed panneuronally, demonstrating a direct role for an α₂δ subunit in regulating the synaptic levels of an α₁ subunit in vivo, a finding also corroborated by Dickman et al. (2008). Interestingly, although CacEGFP is reduced at stj mutant synapses, it properly localizes to active zones, suggesting that the synaptic targeting of Cac does not depend solely on stj and requires other factors. In addition, we do not observe differences in CacEGFP distribution or signal in the VNC or axonal projections, indicating that the global stability and axonal transport of Cac are not affected by stj loss of function. Hence, stj most likely plays a discrete role in the synaptic stabilization of Cac.

Interestingly, we also find that neuronal overexpression of cac can partially rescue the viability and electrophysiological phenotypes observed in stj mutants, providing further evidence that they interact. In contrast, although Dickman et al. (2008) found that cacEGFP overexpression improves the survival of their mutants, they did not observe an improvement in EJP amplitude when cacEGFP is overexpressed in the mutants. To measure cacEGFP rescue activity at the NMJ, Dickman et al. (2008) performed electrophysiological recordings in 0.3 mM Ca²⁺, whereas our studies were done in 1 mM Ca²⁺. It is possible that at 0.3 mM Ca²⁺, the mutants, which display a right-shift in Ca²⁺ sensitivity, are operating in a subcooperative regime where the ameliorative effects of cacEGFP overexpression are masked. Notably, the ERG, hyperexcitability, and NMJ synaptic release phenotypes are improved in our mutants with neuronal cac overexpression. Hence, stj is required for the proper function and localization of Cac but this defect can be partially overcome with cac overexpression.

In summary, we have isolated novel mutations in stj, a neuronal D. melanogaster α₂δ subunit. Our studies define a predominant role for a D. melanogaster α₂δ in regulating neuronal excitability and neurotransmitter release by specifically stabilizing cac, a presynaptic VGCC α₂ subunit, at synapses. This work should facilitate further studies to illuminate the role of α₂δ as a therapeutic target and modulator of VGCC function.

**Materials and methods**

*D. melanogaster* strains and genetics

The isolation of 118 mutants on chromosome 2R, including *stj¹*, *stj²*, and *stj³*, has been described previously (Verstreken et al., 2002). The control genotype was *yw; P[ fly* + FRT]42D<sup>neoFRT</sup>* (FRT42D<sup>neoFRT</sup>). Flies with visual

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systems homzygous for stj were w y eyFP GMR-lacZ; FRT42D+stj/FRT42D+P[w+ w] y/47FAI [2J/c-R1]1. Mapping of stj is described in Fig. 2 A. P element stocks and Df(2R)CX1 (indicated in Fig. 2 A) were obtained from the Bloomington Drosophila Stock Center (Brizuela et al., 1994; Bellen et al., 1994; Parks et al., 2004). Df(2R)Exel7128 was obtained from the Exelixis stock collection (Thibault et al., 2004; stj, stj, and Df(2R)Exel7128 were maintained over CyO; K-GAL4 UAS-GFP transgenic animals were cultured on grape juice plates with yeast paste. NP1574-GAL4 was obtained from the Kyoto Institute of Technology Drosophila Genetic Resource Center (Hayashi et al., 2002) and was crossed to UAS-GFP, UAS-GFP,...
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