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Mutations in a *Drosophila* $\alpha_2\delta$ Voltage-Gated Calcium Channel Subunit Reveal a Crucial Synaptic Function

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Voltage-dependent calcium channels regulate many aspects of neuronal biology, including synaptic transmission. In addition to their α_1 subunit, which encodes the essential voltage gate and selective pore, calcium channels also contain auxiliary $\alpha_2\delta$, β, and γ subunits. Despite progress in understanding the biophysical properties of calcium channels, the *in vivo* functions of these auxiliary subunits remain unclear. We have isolated mutations in the gene encoding an $\alpha_2\delta$ calcium channel subunit ($d\alpha_2\delta$-3) using a forward genetic screen in *Drosophila*. Null mutations in this gene are embryonic lethal and can be rescued by expression in the nervous system, demonstrating that the essential function of this subunit is neuronal. The photoreceptor phenotype of $d\alpha_2\delta$-3 mutants resembles that of the calcium channel α_1 mutant *cacophony* (*cac*), suggesting shared functions. We have examined in detail genotypes that survive to the third-instar stage. Electrophysiological recordings demonstrate that synaptic transmission is severely impaired in these mutants. Thus the $\alpha_2\delta$ calcium channel subunit is critical for calcium-dependent synaptic function. As such, this *Drosophila* isoform is the likely partner to the presynaptic calcium channel α_1 subunit encoded by the *cac* locus. Consistent with this hypothesis, cacGFP fluorescence at the neuromuscular junction is reduced in $d\alpha_2\delta$-3 mutants. This is the first characterization of an $\alpha_2\delta$-3 mutant in any organism and indicates a necessary role for $\alpha_2\delta$-3 in presynaptic vesicle release and calcium channel expression at active zones.

Key words: **calcium channel; synaptic transmission; bouton; active zone; neuromuscular junction; *Drosophila***

Introduction

Calcium channels have well established roles in synaptic transmission, cell excitability, intracellular signaling, and disease (Jen, 1999; Collin et al., 2005). Voltage-gated calcium channels have a unique responsibility for converting electrical changes across the plasma membrane into intracellular changes in calcium concentration. Molecularly, they contain a pore-forming α_1 subunit that confers many of the basic properties of the ion channel, including its voltage-sensitive gating, selectivity for calcium, and pharmacological properties (De Waard et al., 1996). However, calcium channels also contain $\alpha_2\delta$ and β subunits that can have a substantial influence on the properties of calcium channels when expressed in heterologous systems (Arikkath and Campbell, 2003). Both $\alpha_2\delta$ and β subunits can markedly increase surface expression of the channels (Gurnett et al., 1996; Wiser et al., 1996; Bichet et al., 2000; Felix, 2005) and can also influence the gating properties of the channel (Arikkath and Campbell, 2003; Bernstein and Jones, 2007). The β subunit is entirely intracellular and is the target for several pathways that modulate calcium channel function (Dolphin, 2003). The $\alpha_2\delta$ subunit, in contrast, lacks an intracellular domain. This subunit consists of two polypeptides that are transcribed as a single transcript and posttranslationally cleaved into the α_2 and δ chains, which remain linked by a disulfide bond (Klugbauer et al., 2003). The α_2 portion is entirely extracellular and heavily glycosylated, whereas the δ chain also includes a C-terminal transmembrane domain (Gurnett et al., 1996). In addition, there is a γ subunit whose role is controversial and that need not assemble with the calcium channel complex (Kang and Campbell, 2003). Although we have learned much about the biophysical properties of calcium channels, the roles of the auxiliary subunits in regulating calcium channels *in vivo* is less clear.

Some insights into the role of these accessory subunits *in vivo* come from a series of spontaneously occurring mutations in mice. These include mutations in a β subunit in *lethargic* (Burgess et al., 1997), an $\alpha_2\delta$ subunit in *ducky* (Barclay et al., 2001; Brodbeck et al., 2002) and in a spontaneous variant of C57BL/10 strain mice (Wycisk et al., 2006), as well as a γ subunit in *stargazer* (Letts et al., 1998; Held et al., 2002; Moss et al., 2002). Interestingly, each of these mutants displays ataxia and some form of epilepsy. Moreover, the $\alpha_2\delta$ calcium channel subunit has been shown to be a target of the anti-epileptic drug gabapentin (Stahl, 2004), although the role of this subunit in the disease remains unclear.

One complication in the genetic analysis of these accessory subunits has been the presence of multiple isoforms in the genome. With regard to $\alpha_2\delta$ subunits, the number of genes in an organism's genome has remained relatively constant: there are

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three $\alpha_2\delta$ isoforms in worms and flies and four isoforms in mammals; these have been classed as $\alpha_2\delta$-1,2,3, and 4 (Littleton and Ganetzky, 2000; Qin et al., 2002). In mammals, the $\alpha_2\delta$-1 subunit is expressed ubiquitously, whereas the $\alpha_2\delta$-2 subunit, the subunit mutated in *ducky*, is expressed in the brain, kidney, heart, and testes. The $\alpha_2\delta$-3 subunit is expressed only in brain (Gurnett et al., 1996; Marais et al., 2001). In *ducky* mice, loss of the $\alpha_2\delta$-2 subunit decreases the amplitude of calcium currents in Purkinje cells, in which it is highly expressed, but not in all neurons (Barclay et al., 2001; Brodbeck et al., 2002). Purkinje cells also have abnormal morphologies. At neuromuscular junctions, however, *ducky* mutations have little effect on transmitter release (Kaja et al., 2007). Loss of the $\alpha_2\delta$-4 subunit causes abnormalities in the outer plexiform layer of the retina (Wycisk et al., 2006). At present, it is uncertain which α_1 calcium channel assembles with which $\alpha_2\delta$ subunit *in vivo*. In heterologous systems, various combinations promote channel expression (Gurnett et al., 1996), but their associations may be less promiscuous *in vivo*. Thus, it has not been determined whether synaptic calcium channels also require an $\alpha_2\delta$ subunit and, if so, what significance that subunit would hold for the physiology of the synapse.

In a forward genetic screen for mutations affecting synaptic transmission, we have isolated mutations in the *Drosophila* $\alpha_2\delta$-3 calcium channel subunit. This subunit ($d\alpha_2\delta$-3) is essential for viability in *Drosophila* and shares many of the phenotypes described in mutations of the α_1 calcium channel subunit, *cacophony*. We demonstrate a critical role for $d\alpha_2\delta$-3 in synaptic function in both photoreceptors and motorneurons.

Materials and Methods

Drosophila stocks and genetics. The *y*, *w*; *FRT42D*, *GMR-hid*, y^+, *cl 2R/CyO; EGUF/EGUF* stock used in the mutagenesis was previously described (Stowers and Schwarz, 1999). The *FRT42D* chromosome (Xu and Rubin, 1993), *elav-Gal4*, *mhc-Gal4*, *daughterless-Gal4*, and all other stocks were obtained from the Bloomington Stock Center (Bloomington, IN) unless otherwise noted.

EGUF-hid screen. Briefly, this screen engineered heterozygous flies whose eyes were homozygous for a mutagenized chromosome arm 2R (Stowers and Schwarz, 1999). We screened such flies for defects in photoreceptor synaptic transmission by behavioral and electrophysiological assays (phototaxis and electroretinogram, ERG). The results of this screen were previously described (Dickman et al., 2005). Eleven complementation groups were isolated with more than one allele that lacked light-evoked "on/off transients" in the ERG, a phenotype attributable to defects in transmission at the first synapse in the underlying lamina neuropil (Coombe, 1986). The photoreceptors themselves, however, could be activated by light, as indicated by the presence of a sustained component in the ERG.

Immunohistochemistry. Larvae were dissected in HL-3, fixed in 4% paraformaldehyde or Bouin's fixative in ice-cold buffer (Dickman et al., 2006) and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Larvae were imaged with a Zeiss (Oberkochen, Germany) LSM 510 laser scanning confocal microscope and 63× 1.4 NA objective using separate channels and processed using the LSM software or Adobe Photoshop. For bouton counting, larvae were grown on standard grape media at 25°C in uncrowded conditions (~40 larvae per plate) until the late third instar (determined by age, wandering behavior, and lack of food in the gut). Third-instar larvae were dissected in HL3 media, fixed for 30 min in 4% PFA, and stained. These animals were then viewed using a Nikon (Tokyo, Japan) E800 fluorescent microscope. All bouton counts were done blind to the genotype of the larva, and only neuromuscular junctions on muscle 6/7 of segment A2 were analyzed. Bouton number was normalized to muscle 6/7 surface area. Cy5-, Cy3-, or FITC-conjugated anti-HRP (1:100) was obtained from Jackson ImmunoResearch (West Grove, PA). Mouse anti-nc82 (1:100) was obtained from Drs. E. Buchner and A. Hofbauer, Germany (Wagh et al., 2006). FITC- or Cy3-conjugated secondary antibodies (1:200) were obtained from Jackson ImmunoResearch.

Genetic rescue. An expressed sequence tag construct (SD03196) containing the entire open reading frame of $d\alpha_2\delta$-*3* cDNA was obtained from Invitrogen (Carlsbad, CA). Two primers were generated against the $d\alpha_2\delta$-*3* cDNA: A *Bgl*II site was engineered into the forward primer and an HA tag in frame with the C-terminal end of $d\alpha_2\delta$-*3* and a *Kpn*I site were engineered into the reverse primer. The PCR product containing the tagged open reading frame was cloned into pUAST (Brand and Perrimon, 1993). Transformant flies were generated by standard methods and one insertion was mapped to the second chromosome. This was recombined with the $d\alpha_2\delta$-3^{DD106} and $d\alpha_2\delta$-3^{DD196} chromosomes. The following genotypes could survive to adulthood: [$d\alpha_2\delta$-3^{DD106}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD196}; *daGal4*], [$d\alpha_2\delta$-3^{DD106}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD196}; *elav-Gal4*], [$d\alpha_2\delta$-3^{DD106}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD196}; *daGal4/elavGal4*], [$d\alpha_2\delta$-3^{DD196}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD106}; *daGal4*], [$d\alpha_2\delta$-3^{DD196}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD106}; *elavGal4*], [$d\alpha_2\delta$-3^{DD196}, *pUAS-*$d\alpha_2\delta$-*3/*$d\alpha_2\delta$-3^{DD106}; *daGal4/elavGal4*].

Electrophysiology. 10–20 larvae were raised at 25°C, separated from heterozygous siblings as second-instar larvae and grown on agar apple plates with yeast coating (Loewen et al., 2001). Third-instar larvae were dissected in saline then bathed in modified HL-3 saline with the following ionic concentrations (in mM): NaCl 70, KCl 5, $MgCl_2$ 10, $NaHCO_3$ 10, sucrose 115, Trehalose 5, HEPES 5, pH7.2, and $CaCl_2$ as indicated. Current clamp recordings were performed on muscle 6 or 7 in abdominal segments A2 or A3 with 10–20 MΩ electrodes. The nerve was cut and suction electrodes (fire polished to a diameter of ~5 μm and filled with bath solution) were used for stimulation. Only muscles with resting potentials more hyperpolarized than −60 mV were considered for analysis. Data were collected using a Digidata and Axopatch 200B amplifier. pClamp 9 (Molecular Devices, Union City, CA), Excel (Microsoft, Seattle, WA), and miniAnalysis (Synaptosoft, Decatur, GA) were used for data analysis. Quantal content was determined by dividing the amplitude of the evoked junctional potentials (EJP) by the average mini amplitude (before stimulation) and correcting for nonlinear summation (Martin, 1955) in the case of high calcium conditions.

cacGFP quantification. Neuronal expression of cacGFP (Kawasaki et al., 2004) was obtained using the following stocks: (1) UAScacGFP/elavGal4, 2) $d\alpha_2\delta$-3^{k10814}/$d\alpha_2\delta$-3^{DD106}; UAScacGFP/elavGal4, and (3) $d\alpha_2\delta$-3^{DD196}*/Df(2R)7128;* UAScacGFP/elavGal4. Larvae were dissected, fixed in 4% paraformaldehyde and mounted as described above. Wild-type (wt) and $d\alpha_2\delta$-*3* mutant larval synapses were immunostained with the neuronal membrane marker HRP and boutons were selected for imaging and analysis based on morphology and HRP staining alone. Larvae were imaged with a Zeiss LSM 510 laser-scanning confocal microscope and 63× 1.4 NA objective. A single optical slice was taken, with the focal plane chosen on the basis of anti-HRP staining of the neuronal boutons. Wild-type and mutant images were taken with the same settings within one experiment (although conditions and settings changed between experiments, and therefore results between experiments cannot be compared). Images were analyzed using MetaMorph Imaging software (Molecular Devices). Images were first filtered using a low-pass 10 × 10 pixel filter to get rid of noise. Local average background intensity measurements were taken, and subtracted from the image, after which the image was thresholded at 2× average background intensity. Puncta were defined as contiguous regions within boutons that were above threshold, and the average and integrated intensity of each punctum was measured.

Results

Isolation and identification of mutants in the $d\alpha_2\delta$-*3* calcium channel subunit

In the course of a forward genetic screen of chromosome 2R for mutants in synaptic transmission (Dickman et al., 2005), we isolated a complementation group displaying severe defects in the electroretinogram (ERG) response to light (Fig. 1*A*) when photoreceptors were made homozygous for the mutation (Stowers and Schwarz, 1999). In particular, the presence of sustained components but lack of "on/off transients" in ERG responses sug-

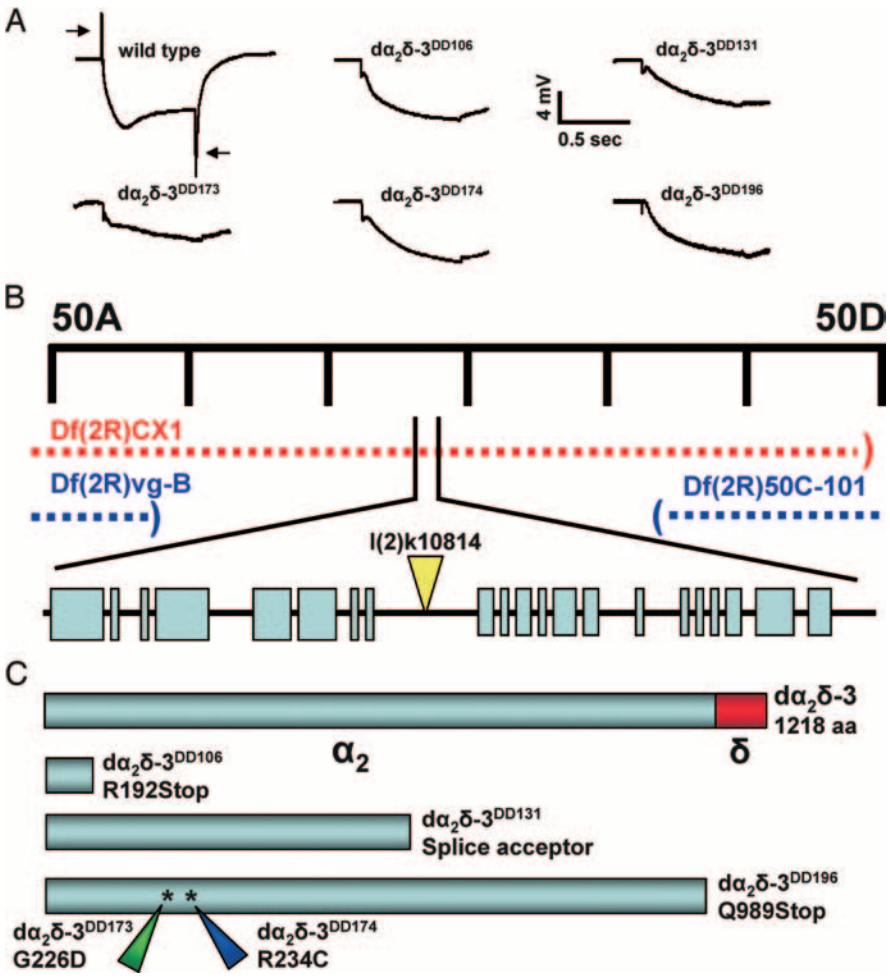

Figure 1. Identification of mutants in the $d\alpha_2\delta$-*3* calcium channel subunit. ***A***, Representative ERG recordings of recombinant photoreceptors in control (*y, w; FRT42/FRT42, GMR-hid, cl; EGUF/+*) and five $d\alpha_2\delta$-*3* alleles (*y, w; FRT42,* $d\alpha_2\delta$-*3/FRT42, GMR-hid, cl; EGUF/+*). *EGUF* is a chromosome carrying *ey-GAL4* and *UAS-FLP* transgenes. The $d\alpha_2\delta$-*3* mutant eyes lack "on/off transients". ***B***, Mapping of $d\alpha_2\delta$-*3* mutants. A transposon insertion line (*k10814*) and a deficiency chromosome *Df(2R)CX1* failed to complement the lethality of all five $d\alpha_2\delta$-*3* alleles, but those alleles were viable in combination with the deficiencies *vg-B* and *50C-101*. ***C***, Molecular analysis of $d\alpha_2\delta$-*3* mutations. $d\alpha_2\delta$-*3* open reading frames were sequenced in the mutants, and molecular lesions in all five were identified as shown.

gested an inability to properly activate second order neurons (Coombe, 1986). 5 lines were independently isolated that failed to complement each other for lethality. The ERGs of this complementation group differed from other synaptic mutants isolated in this and other screens (Stowers and Schwarz, 1999; Dickman et al., 2005) in that the wave form of the sustained components was abnormally slow in its onset and recovery (Fig. 1). A similar phenotype appears in mutants in the α_1 calcium channel subunit *cacophony* (Smith et al., 1998b).

This complementation group was mapped to the 49C-50D chromosomal region on the basis of the failure of chromosomal deficiency *CX1* to complement the 5 alleles (Fig. 1*B*). A transposon insertion line in the region, *k10814*, also failed to complement these mutants for lethality. Inverse PCR and sequencing revealed the transposon to be inserted within an intron of an uncharacterized gene (*CG12295*) with homology to the $d\alpha_2\delta$-*3* subunit. The predicted transcription unit covers >14 kB of genomic DNA and 21 exons with a protein product of 1218 amino acids. The *Drosophila* genome contains three genes predicted to encode $d\alpha_2\delta$ subunits and these are homologous to three of the isoforms described in mammals (Littleton and Ganetzky, 2000). The gene containing the insert in *k10814* corresponds to the $d\alpha_2\delta$-*3* isoform and predicts a protein with 31% overall sequence identity with rat $\alpha_2\delta$-3. Expressed sequence tags from the *Drosophila* Genome Project show that two different transcripts arise from the locus, only differing in their 5′ untranslated regions. The open reading frame was sequenced in each mutant, revealing a molecular change in each of the alleles (Fig. 1*C*). The $d\alpha_2\delta$-3^{DD106} allele encodes an early stop codon at the 192nd amino acid and is thus a likely null mutation. The *DD196* allele encodes a late stop codon, creating a nonsense mutation at amino acid 989. Both *DD173* and *DD174* alleles are missense mutations in the α_2 domain, and *DD131* likely creates a splicing error midway through the open reading frame. Despite the variety of molecular lesions in the $d\alpha_2\delta$-*3* gene, all 5 of the $d\alpha_2\delta$-*3* mutants (*DD106*, *DD131*, *DD173*, *DD174*, and *DD196*) die as well formed, late-stage embryos when homozygous or when in combination with each other or either of two different deficiencies, *Df(2)CX1* or *Df(2)Ex7128*. However, the *k10814* insertion, when homozygous or in combination with either deficiency, survives to third-instar stages in standard conditions or to adulthood when raised apart from heterozygous siblings (Loewen et al., 2001); these adults are ataxic, unable to fly, and die a few days after eclosion. This allele is therefore likely to be a hypomorphic mutation, perhaps resulting in a reduction in wild-type levels of the protein.

To confirm that lethality was the specific result of a loss of $d\alpha_2\delta$-*3* function and to determine in what tissues $d\alpha_2\delta$-*3* function is required, we generated a rescue construct with a $d\alpha_2\delta$-*3* cDNA under the control of UAS elements (Brand and Perrimon, 1993). Adult viability could be restored with ubiquitous expression of $d\alpha_2\delta$-*3* using *daughterless*-Gal4 or with neuronal expression using *elav*-Gal4, but not with muscle expression using *MHC*-Gal4 (data not shown). Thus the only essential function of $d\alpha_2\delta$-*3* is in the nervous system.

Synaptic overgrowth in $d\alpha_2\delta$-*3* mutants

The similarity of the ERG responses to those of *cacophony*, an α_1 subunit that is needed for triggering calcium-dependent neurotransmitter release at the neuromuscular junction (Kawasaki et al., 2000), raised the possibility that $d\alpha_2\delta$-*3* also functioned at the neuromuscular junction. We therefore examined the phenotype of $d\alpha_2\delta$-*3* at the well studied neuromuscular junctions on muscles 6 and 7 of third-instar larvae. *cac* hypomorphs that can survive to this stage have been reported to have decreases in synaptic strength at this synapse, and fewer synaptic boutons (Rieckhof et al., 2003). Because $d\alpha_2\delta$-3^{k10814} mutants can also survive to third-instar stages, we examined their phenotype at this synapse.

We found a 15% increase in bouton number in $d\alpha_2\delta$-$3^{k10814/DD106}$ mutants compared with controls (199 ± 6. com-

pared with 175 ± 6 boutons per junction; mean ± SEM, $p < 0.05$, $n = 25$ and 22) (Fig. 2*A*,*B*). In addition, *dα$_2$δ-3* mutants exhibited a 23% decrease in muscle size compared with controls ($74 \times 10^3 \pm 1 \times 10^3$ compared with $96 \times 10^3 \pm 3 \times 10^3$ μm^2; $p < 0.001$) (Fig. 2*C*). In wild-type animals, muscle size and bouton number increase together during development, probably so that an increase in synaptic strength can match the decrease in input resistance of the muscle fiber as it grows (Lnenicka and Keshishian, 2000). Normalizing bouton number to muscle size may therefore be appropriate as a means to offset changes in boutons secondary to changes in muscle size. In the present study, this normalization yielded a 50% increase in bouton number per muscle area in *dα$_2$δ-3* mutants compared with controls ($2.7 \times 10^{-3} \pm 0.1 \times 10^{-3}$ compared with $1.8 \times 10^{-3} \pm 0.1 \times 10^{-3}$ boutons per μm^2; $p < 0.001$) (Fig. 2*B*). In contrast, *cac^{NT27}* mutants (Rieckhof et al., 2003) did not exhibit an increase in bouton number with or without normalizing to muscle size (data not shown). To control for the genetic background of the P-element containing chromosome, we recorded in heterozygous conditions (*y, w/+; dα$_2$δ-3^{k10814}/FRT42D*) and found no difference from *y, w/+; Canton-S/FRT42D*. We attempted to rescue the increase in bouton number by neuronally expressing dα$_2$δ-3 or cacGFP in *dα$_2$δ-3$^{k10814/DD106}$* mutants, however neither manipulation rescued the bouton number phenotype, although expression of dα$_2$δ-3 in the nervous system did significantly improve muscle size.

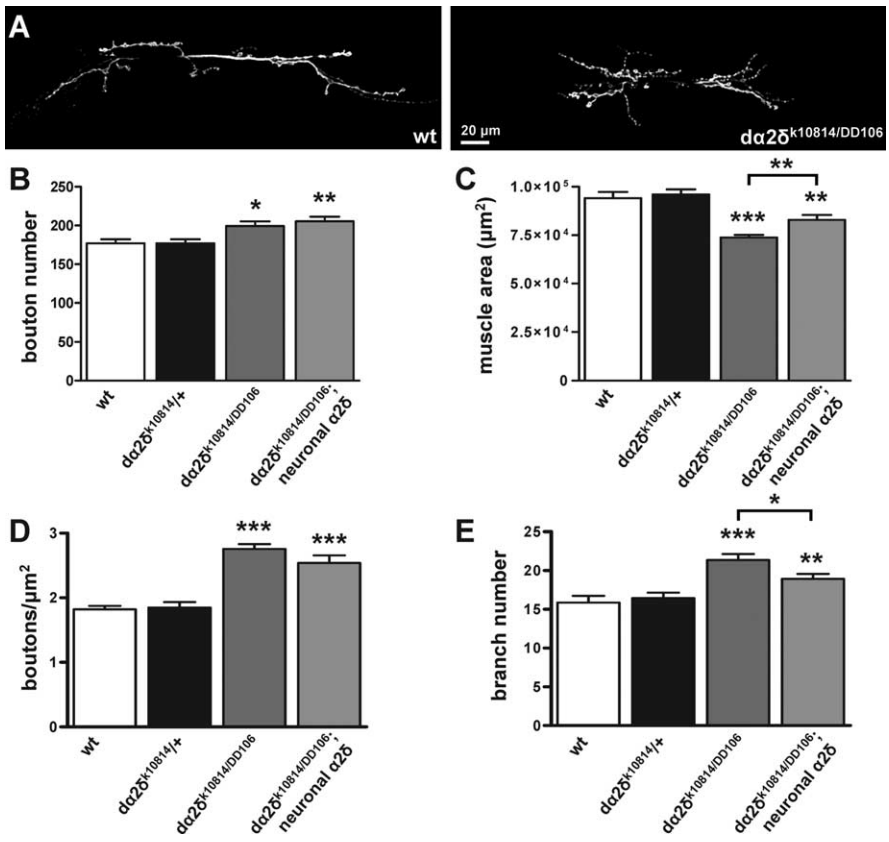

Figure 2. Synaptic overgrowth in *dα$_2$δ-3* neuromuscular junctions. ***A***, Representative larval neuromuscular junctions at muscles 6 and 7 immunostained with the pan-neuronal marker anti-HRP in wild-type (*y, w/+; +/FRT42D*) and *dα$_2$δ-3* mutants (*y, w/+; dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}*) showing additional boutons and more branching in *dα$_2$δ-3* mutant synapses. Both panels are at the same magnification. ***B–E***, Quantification of bouton number (***B***), muscle size (***C***), bouton number normalized to muscle surface area (***D***), and branch number (***E***) in those genotypes along with the following additional genotypes: *y, w/+; dα$_2$δ-3^{k10814}/FRT42D* and *y, w/+; dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}, UASdα$_2$δ-3-HA; elavGal4/+* (neuronal *dα$_2$δ*). For wild type, 20 neuromuscular junctions were analyzed from 10 larvae; in heterozygotes, 22 junctions from 11 larvae; in mutants, 25 junctions from 15 larvae; in neuronal *dα$_2$δ*, 24 junctions from 12 larvae. *p* values are relative to wild type, unless otherwise indicated, and are unadjusted for multiple comparisons; however, significance was confirmed using one-way ANOVA and a Student's–Newman–Keuls *post hoc* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

In addition to the increase in bouton number, we found a 30% increase in branch number in *dα$_2$δ-3$^{k10814/DD106}$* mutants (21 ± 0.8. compared with 16 ± 0.7 branches per junction; mean ± SEM, $p < 0.001$) (Fig. 2*A*,*E*). Neuronal expression of dα$_2$δ-3 in *dα$_2$δ-3$^{k10814/DD106}$* mutants significantly rescued this phenotype. Thus dα$_2$δ-3 may play a modest role in determining synaptic bouton number and the extent of terminal branching.

Reduced transmitter release in *dα$_2$δ-3* synapses

The anatomical alterations at *dα$_2$δ-3^{k10814}* third-instar neuromuscular junctions were accompanied by substantial electrophysiological changes. Initial recordings were made in HL-3 saline with 0.3 mM Ca^{2+} and 10 mM Mg^{2+}, conditions in which calcium influx and release are far from saturation and changes in synaptic strength are therefore readily apparent. Wild-type larvae (w^{1118}) were compared with heterozygous mutants (*y, w; dα$_2$δ-3^{k10814}/FRT42D*) to control for changes in the genetic background and to two mutant allelic combinations (*dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}* and *dα$_2$δ-3^{k10814}/Df(2R)CX1*). Miniature EJP (mEJP) amplitudes in the mutant genotypes (1.13 ± 0.04 mV, mean ± SEM, $n = 47$, in *dα$_2$δ-3^{k10814}/Df(2R)CX1* and 1.13 ± 0.14 mV, $n = 11$, in *dα$_2$δ-3^{k10814}/dα2δ-3^{DD106}*) were comparable with those in wild type (1.12 ± 0.43 mV. $n = 35$) or the heterozygotes (1.06 ± 0.19 mV, $n = 7$), but mEJP frequencies were reduced in *dα$_2$δ-3* mutants (1.67 ± 0.10 Hz in *dα$_2$δ-3^{k10814}/Df(2R)CX1* and 1.71 ± 0.18 Hz in *dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}*, compared with 2.67 ± 0.23 Hz in w^{1118} or 2.76 ± 0.48 Hz in heterozygotes) (Fig. 3*A*,*B*). The amplitudes of EJPs in *dα$_2$δ-3* mutants were reduced by 85% (2.83 ± 0.74 mV, $n = 22$, in *dα$_2$δ-3^{k10814}/Df(2R)CX1* and 5.18 ± 1.23 mV, $n = 11$, in *dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}* compared with 18.61 ± 2.35 mV, $n = 11$, in w^{1118} or 16.43 ± 2.02 mV, $n = 7$, in heterozygous controls) (Fig. 3*C*,*D*). Given that quantal size, as determined from the amplitude of spontaneous minis, was unchanged, the quantal content of evoked responses must have been similarly reduced in *dα$_2$δ-3* mutant synapses (Fig. 3*D*). Consistent with a reduction in quantal content, nerve stimulation frequently failed to elicit an evoked response in *dα$_2$δ-3^{k10814}/Df(2R)CX1* mutants: the synapses had a 48.2 ± 1.43% failure rate compared with 2.86 ± 0.61% in control larvae. We also found the reduction in quantal content to be apparent over a range of calcium concentrations (Fig. 3*E*).

To test whether the reduction in transmission was attributable to alterations in *dα$_2$δ-3* expression, we drove *dα$_2$δ-3* specifically in the nervous system in a mutant background (*dα$_2$δ-3^{k10814}/dα$_2$δ-3^{DD106}, UAS-dα$_2$δ-3; elavGAL4/+*). This restored robust

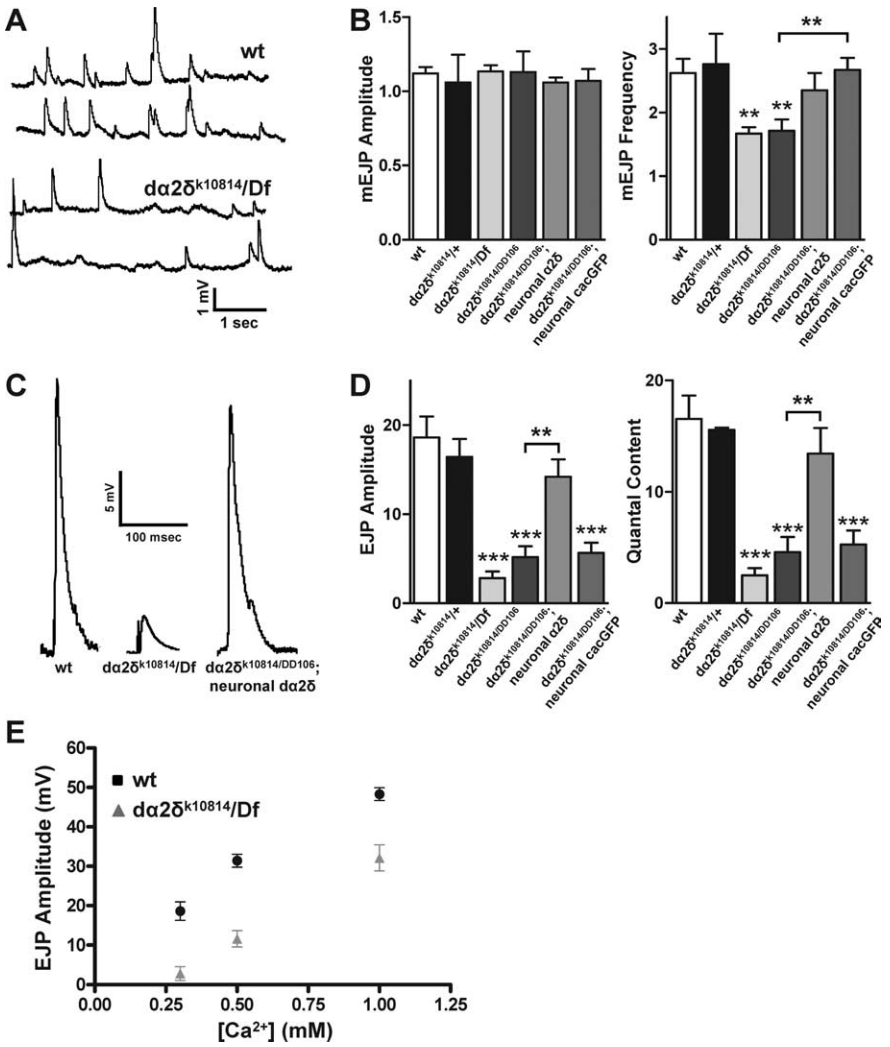

Figure 3. Reduced synaptic efficacy at $d\alpha_2\delta$-*3* neuromuscular junctions. Recordings were made from third-instar neuromuscular junctions from wild-type larvae (w^{1118}), heterozygous and homozygous mutant larvae of the indicated genotypes, and homozygous mutant larvae with restored neuronal expression of $d\alpha_2\delta$-*3* ($d\alpha_2\delta$-$3^{k10814}/d\alpha_2\delta$-$3^{DD106}$, UAS$d\alpha_2\delta$-*3*-HA; *elav-Gal4/+*) or *cac* ($d\alpha_2\delta$-$3^{k10814}/d\alpha_2\delta$-$3^{DD106}$, UAS*cac-GFP*; *elavGal4/+*). ***A***, Representative mEJP events recorded from neuromuscular junctions in wild-type and mutant ($d\alpha_2\delta$-$3^{k10814}/Df(2R)CX1$) larval synapses. ***B***, Quantification of mEJP amplitude and frequency. mEJP frequencies are slightly reduced at $d\alpha_2\delta$-*3* synapses, but quantal size is unchanged. ***C***, Representative EJPs. ***D***, Quantification of EJP amplitudes. Amplitudes are reduced by 85% in $d\alpha_2\delta$-*3* mutants but restored by neuronal expression of the UAS$d\alpha_2\delta$-*3*-HA transgene. ***E***, EJP amplitude is reduced across several calcium concentrations in $d\alpha_2\delta$-*3* mutants. *p* values are relative to wild type unless otherwise indicated and were obtained using one-way ANOVA and a Tukey's *post hoc* test. $^{**}p < 0.01$; $^{***}p < 0.001$.

synaptic function to these synapses so that mEJP frequency, EJP amplitude, and quantal content were no longer significantly different from controls (Fig. 3) (mEJP amp = 1.06 ± 0.03 mV; mEJP freq = 2.35 ± 0.27 Hz; EJP amp = 14.21 ± 1.94 mV; $n = 8$).

Together, these experiments demonstrate a critical requirement for the d$\alpha_2\delta$-3 subunit in evoked synaptic release at the neuromuscular junction. The extent to which this allele alters the efficacy of release at a given active zone could be different from the change in quantal content if the number of active zones is altered in the mutant. As described above, the neuromuscular junctions of $d\alpha_2\delta$-*3* larvae have more boutons than control larvae. To determine whether this increase in bouton number represented a parallel increase in the number of active zones, neuromuscular synapses were immunolabeled with the monoclonal antibody nc82, a well characterized marker of active zones in *Drosophila* (Wagh et al., 2006). Despite the increase in bouton number, $d\alpha_2\delta$-*3* mutants exhibited a 20% decrease in nc82 puncta per neuromuscular junction compared with controls (932 ± 33 compared with 1165 ± 41; $p < 0.001$, $n = 14$ and 16) (Fig. 4). When normalized to the number of boutons per synapse, this translated to a 40% decrease in nc82 puncta per bouton in the mutants (3.76 ± 0.09 compared with 5.99 ± 0.22; $p < 0.001$) (Fig. 4). Counts are from 14 NMJs from 7 control larvae and 16 NMJs from 8 mutant larvae ($p < 0.001$). Assuming that this represents a true decrease in active zone number rather than just a mislocalization of the nc82 epitope, the 20% decrease in the total number of active zones is still insufficient to explain the 85% decrease in quanta released per stimulus. Thus the mutation of $d\alpha_2\delta$-*3* has caused not only a net decrease in transmitter release, but also a decrease in release per active zone.

Overexpression of cacGFP rescues embryonic lethality in $d\alpha_2\delta$ mutants

The larval NMJ has been shown to use the α_1 subunit encoded by *cac* for triggering synaptic transmission (Smith et al., 1998a; Dellinger et al., 2000; Kawasaki et al., 2000, 2002; Peng and Wu, 2007). If d$\alpha_2\delta$-3 is necessary for α_1 expression and/or stability, overexpression of this α_1 subunit might compensate for loss of d$\alpha_2\delta$-3. We tested this possibility by expressing a GFP-tagged cac transgene (Kawasaki et al., 2004). Neuronal overexpression of cacGFP in $d\alpha_2\delta$-$3^{DD196}/Df(2)7128$ mutants rescued the embryonic lethality of this genotype, resulting in progeny that survived to early pupal stages (data not shown). However, larvae and pupae were considerably smaller than wild type and extremely sluggish.

We similarly tested whether overexpression of the α_1 calcium channel subunit cac in a mutant background could restore proper synaptic function to $d\alpha_2\delta$-$3^{k10814}/d\alpha_2\delta$-$3^{DD106}$ mutant synapses (Fig. 3). Although this rescued the change in mEJP frequency, it failed to increase EJP amplitude (mEJP amp = 1.07 ± 0.08 mV; mEJP freq = 2.67 ± 0.19 Hz; EJP amp = 5.65 ± 1.16 mV; $n = 13$). This finding may reflect a ceiling for α_1 channel function at the synapse in the absence of d$\alpha_2\delta$-3.

Synaptic cacGFP is reduced in $d\alpha_2\delta$-*3* mutants

To examine the hypothesis that the d$\alpha_2\delta$-3 subunit plays a role in ensuring the proper localization or expression level of the cac α_1 subunit, we compared the fluorescent intensity of the GFP-tagged *cac* transgene at the third-instar larvae of wild-type and $d\alpha_2\delta$-$3^{k10814}/d\alpha_2\delta$-$3^{DD106}$ mutant neuromuscular junctions. In wild-type third-instar larvae, cacGFP has been shown to mark active zones with fluorescent puncta that represent small clusters of this channel subunit (Kawasaki et al., 2004) (Fig. 5). We analyzed two parameters of this fluorescence: their average fluores-

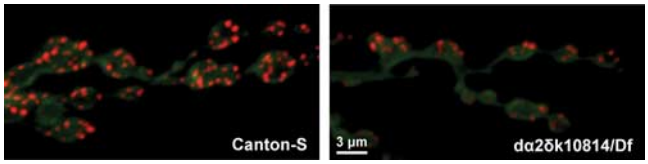

Figure 4. Active zones appear to be decreased in $d\alpha_2\delta$-*3* synaptic terminals. Wild-type and $d\alpha_2\delta$-*3* mutant ($d\alpha_2\delta$-3^{k10814}/*Df(2R)CX1*) larval synapses immunostained with the active zone marker nc82 (red) and the neuronal membrane marker anti-HRP (green). nc82 puncta per neuromuscular junction were reduced by 20% in the mutant (932 ± 33 compared with 1165 ± 41; $p < 0.001$; $n = 14$ and 16), and nc82 puncta per bouton were reduced by 40% (3.76 ± 0.09 compared with 5.99 ± 0.22; $p < 0.001$). Counts are from 14 NMJs from seven control larvae and 16 NMJs from eight mutant larvae. $p < 0.001$.

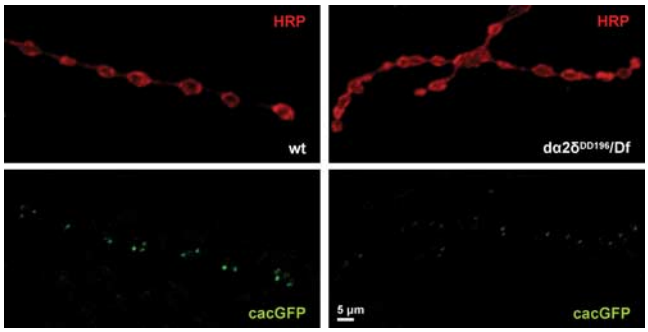

Figure 5. cacGFP intensity is reduced in $d\alpha_2\delta$-*3* synaptic terminals. Neuronal expression of cacGFP was achieved at wild-type (*UAScacGFP/elavGal4*) and $d\alpha_2\delta$-*3* mutant larval synapses. cacGFP puncta (green) in wild-type and mutant synapses immunostained with the neuronal membrane marker anti-HRP (red) and imaged at the same gain settings. cacGFP fluorescence was determined as either the average intensity of pixels within puncta or the integrated intensity of pixels within puncta. The average fluorescence intensity of cacGFP puncta was diminished by 28% and the integrated fluorescence intensity by 60% *in* $d\alpha_2\delta$-3^{DD106}*/Df(2R)7128; UAScacGFP/elavGal4* synaptic terminals compared with wild-type.

cent intensity, which reflects the density of the GFP-tagged channels at these sites, and the integrated fluorescent intensity, which reflects changes in both the density and size of the fluorescent puncta and may more accurately reflect the number of channels present. Although cacGFP puncta were observed at synaptic terminals of the mutant neuromuscular junctions, the average fluorescence intensity of these puncta was diminished by 25% (wt = 33 ± 0.6, $n = 345$ puncta from 4 larvae; mutant = 25 ± 0.3, $n = 476$ puncta from 3 larvae; mean ± SEM; $p < 0.001$), and the integrated intensity was diminished by 40% (wt = $13.3 \times 10^3 \pm 0.9 \times 10^3$; mutant = $7.9 \times 10^3 \pm 0.4 \times 10^3$; $p < 0.001$), indicating an apparent reduction in the number of α_1 subunits present at these active zones. Furthermore, cacGFP puncta intensities were quantified for the rescued $d\alpha_2\delta$-3^{DD196}*/Df(2)7128* mutants that survived to third instar during neuronal overexpression of cacGFP. These mutants also exhibited cacGFP puncta at the neuromuscular junction, however the fluorescence intensity was even lower than for the $d\alpha_2\delta$-3^{k10814} mutant, consistent with this being a more severe allele. The average fluorescence intensity of cacGFP puncta was diminished by 28% (Fig. 5) (wt = 271 ± 7, $n = 304$ puncta from 3 larvae; mutant = 195 ± 4, $n = 472$ puncta from 4 larvae; $p < 0.001$), whereas the integrated intensity was diminished by 60% (Fig. 5) (wt = $130 \times 10^3 \pm 11 \times 10^3$; mutant = $53 \times 10^3 \pm 5 \times 10^3$; $p < 0.001$).

Discussion

Using an unbiased forward genetic approach, we have identified a calcium channel subunit essential for proper neurotransmission. This is the first description in any organism of an $\alpha_2\delta$-*3* mutant and its phenotype indicates that the $\alpha_2\delta$-*3* isoform of this subunit may preferentially be engaged in transmission. $\alpha_2\delta$-2 mutant mice did not show physiological defects at synapses beyond what could be attributed to the small size of the animals (Barclay et al., 2001). $\alpha_2\delta$-1 has not been studied genetically, but it is expressed in both neuronal and non-neuronal tissues and therefore is likely to have a more general function. Loss of the murine $\alpha_2\delta$-4 subunit (Cacna2d4) results in a phenotype that comes closest to that which we have observed for loss of $d\alpha_2\delta$-3: defects in the synaptic endings of photoreceptors, as revealed by electroretinograms and histology (Wycisk et al., 2006). The *Drosophila* genome also contains predicted isoforms of $\alpha_2\delta$-*1* and $\alpha_2\delta$-*2,* but they do not appear to be functionally redundant with $\alpha_2\delta$-3 as $\alpha_2\delta$-*3* null alleles are lethal and mutations in this isoform produce severe phenotypes in both photoreceptors and neuromuscular junctions. Thus, despite studies in heterologous expression systems that indicate that each $\alpha_2\delta$ isoform will promiscuously promote the surface expression of any α_1 subunit, their functions *in vivo* are sufficiently distinct that loss of a single subunit can cause a severe phenotype.

Similarly, studies of mammalian channels have not resolved whether each $\alpha_2\delta$ isoform is associated *in vivo* with a particular α_1 isoform, although there does appear to be some level of selective association. This pairing may derive primarily from the expression patterns of the $\alpha_2\delta$ and α_1 subunits. However, from studies on gabapentin and on *ducky* mice, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 appear to be the primary subunits in brain and preferentially associate with P- and N-type calcium channels (Klugbauer et al., 2003). In *Drosophila*, we find that loss of $d\alpha_2\delta$-*3* gives an electrophysiological phenotype similar to loss of the *cac*-encoded presynaptic α_1 subunit in the ERG and neuromuscular junction. cac is the only *Drosophila* member of the Ca_v2 family, homologous to N-, P- and Q-type channels of mammals. Indeed, the *cac* channel has been established by both electrophysiological studies and cytochemical localization to be the major calcium channel in active zones for driving vesicle release (Smith et al., 1998a; Dellinger et al., 2000; Kawasaki et al., 2000, 2002; Peng and Wu, 2007). The present data indicate that the $\alpha_2\delta$-3 subunit is its partner and necessary for its proper localization to the active zone. A similar pairing may occur in *C. elegans* in which *unc-36,* an $\alpha_2\delta$ subunit mutant, displays an identical phenotype to the α_1 subunit mutant *unc-2* (Schafer and Kenyon, 1995; Schafer et al., 1996). At murine photoreceptor synapses, L-type calcium channels mediate transmitter release and therefore, in a subset of mammalian synapses, $\alpha_2\delta$-4 may play a similar role to $\alpha_2\delta$-3, but partnering L-type rather than N-type channels (Wycisk et al., 2006), although this has not been investigated with direct recordings of synaptic properties. In the present study, transgenic rescue experiments demonstrated that the only essential function of $\alpha_2\delta$-*3* in *Drosophila* is in the nervous system. Other isoforms are thus likely to promote calcium channel expression in other cell types including muscle cells, which express the L-type α_1 subunit Dmca1D (Ren et al., 1998).

The significance of the $d\alpha_2\delta$-3 calcium channel subunit to synaptic function

How does the $d\alpha_2\delta$-3 subunit contribute to presynaptic function? The leading hypothesis from mammalian work and studies in heterologous systems is that $\alpha_2\delta$ subunits promote robust plasma membrane expression of the α_1 subunit (Gurnett et al., 1996; Wiser et al., 1996; Felix et al., 1997; Gurnett et al., 1997), at least in part by stabilizing them at the plasma membrane (Bern-

stein and Jones, 2007). The phenotype of $d\alpha_2\delta$-3^{k10814} is consistent with the hypothesis that $d\alpha_2\delta$-*3* is similarly required for proper synaptic expression of the *cacophony* α_1 subunit. Although we cannot record directly from these synaptic boutons to determine the amplitude of calcium currents, the reduction in quantal content per active zone and the decreased amplitude of the EJP are consistent with a decrease in calcium influx at the terminals attributable to decreased channel density. Because of the fourth-order dependence of release on calcium influx (Dodge and Rahamimoff, 1967), even a 33% reduction in channel density could account for the ~5-fold observed reduction in vesicles released per active zone in the $d\alpha_2\delta$-3^{k10814} allele. By fluorescent imaging of the cacGFP transgene, we observed a 25–60% reduction in the level of the cac α_1 subunit in 2 different allellic combinations that, because they are not completely null, survive to the third-instar stage. This degree of reduction in α_1 subunits at the active zone is consistent with the physiological findings and the hypothesis that the synaptic role of $d\alpha_2\delta$-*3* is to promote the expression, localization, or retention of the cac α_1 subunit at active zones. Similarly, the ability of *cac* overexpression to extend the lifespan of $d\alpha_2\delta$-3^{DD196}/*Df(2)7128* mutants suggests that the $d\alpha_2\delta$-*3* phenotype arises from an insufficiency of synaptic *cac* channels. In the context of the P-element insertion allele $d\alpha_2\delta$-3^{k10814}, however, in which substantial amounts of the α_1 subunit likely were already present in the terminals, this overexpression was not adequate to significantly increase synaptic transmission.

The 20% decrease in active zones observed per neuromuscular junction, suggested by the decrease in nc82-immunoreactive puncta, is likely to account for a significant component of the 37% decrease in mEJP frequency, but a portion of the decrease may also arise from a change in calcium channel density. Similar decreases in mini frequency arise when neuromuscular junctions are placed in calcium-free salines (Sweeney et al., 1995; Okamoto et al., 2005), indicating that mEJPs are at least partially dependent on the entry of extracellular calcium. One possibility is that the ambient, resting calcium concentration in the synaptic cytosol depends on the amount of calcium that enters through spontaneous openings of calcium channels even at hyperpolarized potentials. Alternatively, sporadic, spontaneous calcium channel openings at the active zones of unstimulated terminals may cause brief, local increases in cytosolic calcium and trigger a portion of the observed minis.

Altered synaptic morphology in $d\alpha_2\delta$-*3*

An unexpected feature of the $d\alpha_2\delta$-*3* mutants was their anatomical phenotype at the neuromuscular junction. In particular, mutants had slightly more boutons per junction, although the muscles themselves were smaller, and these boutons had a lower density of active zones, as scored by detectable puncta of nc82 immunoreactivity. This led to an overall 20% decrease in active zones per muscle.

Changes in the size and morphology of the *Drosophila* neuromuscular junction have been shown to occur as a result of perturbations in both presynaptic activity (Lnenicka et al., 2003; Mosca et al., 2005) and regulatory signaling (Packard et al., 2002; Keshishian and Kim, 2004; Ruiz-Canada and Budnik, 2006). However, many other perturbations of synaptic function do not have these effects, including viable mutations in synaptotagmin, syntaxin and SNAP-25 (DiAntonio and Schwarz, 1994; Schulze et al., 1995; Vilinsky et al., 2002). Mutations in the calcium channel α_1 subunit, *cac*, did not cause an overgrowth of boutons in our studies, and others have reported fewer than normal boutons in *cac* alleles (Rieckhof et al., 2003; Xing et al., 2005). The changes in bouton number and active zone density in $d\alpha_2\delta$-*3* mutants therefore merit additional study. At present, these phenotypes may be direct consequences of the loss of this subunit or may include indirect consequences, possibly compensatory, in response to changes in the activation of the terminals, calcium influx, or transmitter release.

In summary, although much attention has been paid to the pore-forming α_1 subunits, the calcium channel is a multi-subunit complex whose other subunits can also serve essential functions. The profound synaptic consequences of the loss of the $d\alpha_2\delta$-*3* subunit highlights the need to understand these subunits in their normal cellular milieu in which both physiological and developmental phenotypes may emerge that could not be appreciated in heterologous systems. These *in vivo* phenotypes should ultimately refine our understanding of the calcium channel complex in neuronal development, function, and disease.

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