Voltage-gated calcium channels couple changes in membrane potential to neuronal functions regulated by calcium, including neurotransmitter release. Here we report that presynaptic N-type calcium channels not only control neurotransmitter release but also regulate synaptic growth at Drosophila neuromuscular junctions. In a screen for behavioral mutants that disrupt synaptic transmission, an allele of the N-type calcium channel locus (Dmca1A) was identified that caused synaptic defects. The underlying molecular defect was identified as a neutralization of a charged residue in the third S4 voltage sensor. RNA interference reduction of N-type calcium channel expression also reduced synaptic growth. Hypomorphic mutations in syntaxin-1A or n-synaptobrevin, which also disrupt neurotransmitter release, did not affect synapse proliferation at the neuromuscular junction, suggesting calcium entry through presynaptic N-type calcium channels, not neurotransmitter release per se, is important for synaptic growth. The reduced synapse proliferation in Dmca1A mutants is not due to increased synapse retraction but instead reflects a role for calcium influx in synaptic growth mechanisms. These results suggest N-type calcium channels participate in synaptic growth through signaling pathways that are distinct from those that mediate neurotransmitter release. Linking presynaptic voltage-gated calcium entry to downstream calcium-sensitive synaptic growth regulators provides an efficient activity-dependent mechanism for modifying synaptic strength.

Axonal sprouting and synaptic rewiring are key regulators of neuronal plasticity in the developing and adult brain (1, 2). Both synapse formation (3) and synapse elimination (4) have been suggested to underlie structural changes in the wiring of the nervous system important for long term modification of brain function. Modulation of synapse formation in Drosophila has been implicated in olfactory learning (5). Likewise, long term habituation and sensitization in Aplysia are associated with short term alterations in synaptic connections between sensory and motor neurons (6). Long term potentiation in the hippocampus is accompanied by changes in the number, size, and structure of synapses within the affected circuit (7, 8). One signaling pathway required for these structural changes is the cyclic AMP cascade, which participates in hippocampal long term potentiation (9), long term facilitation in Aplysia (10), and activity-dependent plasticity in Drosophila (11). Notably, the synaptic structure at neuromuscular junctions (NMJs) is dramatically altered in Drosophila mutants defective in cAMP metabolism (12). In addition to cAMP, one synaptic signaling molecule that may function in synaptic development is calcium, which has numerous downstream effectors implicated in behavioral and synaptic plasticity.

Voltage-gated calcium channels are a major source of calcium entry into synapses and are multimeric proteins containing a pore-forming α-subunit and accessory β- and α2-subunits. A large family of these channels has been identified with isoforms classified according to their gating kinetics, single channel conductances, and pharmacological profiles (13). Five high threshold subtypes (L, N, P, Q, and R), together with the low threshold T-type, form the major calcium entry pathways in mammalian excitable cells. Genome sequencing has revealed a conserved set of L-, T-, and N-type calcium channels throughout vertebrate and invertebrate evolution (14, 15). Localized elevation of calcium concentration during presynaptic depolarization triggers the fusion of synaptic vesicles and subsequent neurotransmitter release. However, other roles for presynaptic calcium entry during synaptic transmission are poorly defined.

The Drosophila NMJ provides a useful model system for determining the role of N-type calcium channels in synapse formation. Shortly after motor neurons leave the central nervous system and contact their muscle target, there is a well defined morphological transition from growth cone to pre-varicosity to varicosity that occurs over several hours (16). Following initial synapse formation, there is a second phase of synaptic development that results in a 10-fold increase in varicosity number on the well studied ventral muscle fibers 6 and 7 during the period between embryogenesis to the third larval instar phase. This growth process is characterized by the addition of new varicosities and additional synaptic branches (17). For new synaptic connections to form, changes in existing terminals must allow for directed synaptic growth and sprouting. These structural changes require temporally controlled and spatially regulated modifications to the nerve terminal cytoskeleton and trans-synaptic cell adhesion complexes. Several calcium-dependent proteins have been implicated in synaptic growth, but the source of calcium and its regulation are unknown. Here we demonstrate that presynaptic N-type calcium channels contribute to the regulation of synaptic growth. The requirement of N-type channels for synaptic growth is not

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Gabrielle E. Rieckhof, Motojiro Yoshihara, Zhuo Guan, and J. Troy Littleton‡

From the Picower Center for Learning and Memory, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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‡ Alfred P. Sloan Research Fellow. To whom correspondence should be addressed: Picower Center for Learning and Memory, Massachusetts Institute of Technology, E18-672, 50 Ames St., Cambridge, MA 02139. Tel.: 617-452-2605; Fax: 617-452-2249. E-mail: troy@mit.edu.

The abbreviations used are: NMJs, neuromuscular junctions; EMS, ethyl methanesulfonate; DLMs, dorsal longitudinal flight muscles; CS, Canton S; HRP, horseradish peroxidase; dsRNA, double-stranded RNA; SNARE, SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TS, temperature-sensitive; ORF, open reading frame; CaMKII, calcium/calmodulin-dependent protein kinase II.
Calcium Channels Regulate Synaptic Growth

dependent upon its role in triggering neurotransmitter release, but rather reflects a requirement for calcium influx. These findings indicate N-type calcium channels provide an important source of calcium that regulates synaptic growth.

EXPERIMENTAL PROCEDURES

Drosophila Genotypes—Drosophila melanogaster was cultured on standard medium at 22 °C. Temperature-sensitive behavioral mutants on the X chromosome were generated by feeding isogenized Canton S males with ethyl methanesulfonate (EMS) for 12 h and mating to C (1DX females via standard techniques (55). Recombination mapping was performed with a y ch v f marked strain. Rescue experiments were performed using a duplication (Dp(1;2)65b) covering the Dmca1A locus (19). The Dmca1A\textsuperscript{−/−} mutant has also been referred to as l(1)1L132\textsuperscript{−/−} and l(1)1L13A\textsuperscript{19,42}.

Electrophysiology and Morphological Analysis—Electrophysiological analysis of wandering third stage larva larva was performed in Drosophila saline (NaCl, 70 mM; KCl, 5 mM; MgCl\textsubscript{2}, 4 mM; NaHCO\textsubscript{3}, 10 mM; Tris(hydroxymethyl)aminomethane in HL3 for 45 min. Immunostaining on filleted embryos was performed as described previously (16). Embryos were dissected at 21–24 h (25 °C) after fertilization. Fluorescein isothiocyanate-conjugated IgG against HRP, which labels neuronal cell membranes (57), was purchased from Cappel and used at 1:1000. DSYT2 against synaptotagmin (58) was used at 1:100. Anti-CSP (59) was used at 1:100 and anti-DLG at 1:1000. Immunoreactive proteins were visualized on a Zeiss Pascal Confocal using fluorescent secondary antibodies (Molecular Probes, Chemicon, The Jackson Laboratory). All error measurements are S.E. unless indicated.

dsRNA Interference—Wild-type embryos were collected at stage 5, dechorionated, mounted on coverslips with double-stick tape, and allowed to desiccate for 6 min at 18 °C. The embryos were covered with halocarbon oil and injected with ~34 picoliters of a 3 μM solution of Dmca1A dsRNA in injection buffer or with injection buffer alone. dsRNA was isolated by PCR amplification of a 725-bp region corresponding to Dmca1A nucleotides 50–772 (following the initiating ATG) using two unperturbed PCR primers with the T7 RNA polymerase promoter sequence attached to the 5′ end: primer 1, 5′-gaattaatacgactcactatagggagacgata-tttactaaccgag-3′; and primer 2, 5′-gaattaatacgactcactatagggagacattg-ttactaaccgag-3′.

Two μg of PCR product was used directly in an \textit{in vitro} RNA transcription reaction with T7 RNA polymerase using the Megascript Kit (Ambion). dsRNA was evaluated by gel electrophoresis prior to use. Following injection, embryos were incubated at 18 °C until hatching, at which time they were transferred to grape juice plates with yeast paste. Larvae were allowed to develop into third instar larvae at 25 °C and processed for immunocytochemistry as described above.

Sequencing—Dmca1A\textsuperscript{NT277} and CS cDNA was used as template to PCR-amplify the complete ORF in two segments. High fidelity Taq (Takara) was used for these reactions to minimize PCR-introduced errors. Sequencing primers spanning the full ORF were used to sequence in duplicate from either of these two templates in both the forward and reverse direction. Sequences were aligned and compared with the predicted ORF using commercially available software.

RESULTS

To identify neuronal mechanisms that underlie synaptic function, we conducted large scale behavioral screens in Drosophila for EMS-induced temperature-sensitive (TS) paralytic mutations that result in motor abnormalities at 38 °C. Mutagenized homozygous viable lines were screened for behavioral defects by observing each strain individually in preheated vials in a 38 °C water bath for 5 min. Any strain showing behavioral abnormalities such as paralysis or abnormal seizure-like movements (Fig. 1, A and B) was maintained as a laboratory stock and subjected to two additional screening procedures to detect electrophysiological and morphological defects. First, the mutants were analyzed for temperature-induced seizure activity by screening for recurrent spontaneous neural activity in the adult giant fiber system at the nonpermissive temperature (Fig. 1, C–F). The giant fiber flight circuit is important in escape responses and flight initiation and includes both chemical and electrical synapses. The pathway can be activated by the stimulation of the brain, and extracellular recordings can be made from the dorsal longitudinal flight muscles (DLMs). Wild-type Drosophila (Canton S) display little to no spontaneous activity when the temperature is raised to 38 °C (Fig. 1C). In contrast, many of the TS mutants resulted in robust spontaneous spiking in the DLM flight muscles when exposed to the nonpermissive temperature (Fig. 1, D–F). The activation of the DLM flight muscles reflects the firing rate of the innervating motor neurons. To date, 37 seizure-inducing mutations that define 12 complementation groups on the X chromosome have been identified in large scale screens of homozygous viable lines generated in the lab.

To examine the link between neuronal activity and synaptic development, we performed a morphological analysis of the seizure mutant collection by visualizing axonal arboreations and terminal morphology with anti-HRP (a general neuronal marker) and anti-synaptotagmin (an antibody against an abundant synaptic vesicle protein) immunocytochemistry at muscle fibers 6 and 7 of third instar larvae. This larval synapse is more accessible to morphological studies than the DLM pathway described above. Abnormal overproliferation of pre-synaptic varicosities was observed in several of the seizure mutants,\textsuperscript{2} similar to the excessive synaptic overgrowth seen in Shaker, ether-a-go-go mutants secondary to increased nerve activity (18). Notably in our screen, a single complementation group defined by four EMS alleles resulted in a decrease in axonal terminal branching and synaptic varicosity number (see below), suggesting the mutated gene product defined by this complementation group is likely to function in the control of synaptic growth. The synaptic undergrowth in the mutant, as opposed to the increased synaptic overproliferation observed in several hyperexcitable mutants, prompted us to clone the mutated gene product to characterize further its role in synaptic structure and function.

To identify the gene product defined by this complementation group, we mapped the Ts phenotype to 33 centimorgans on the X chromosome via recombination. Subsequent deficiency mapping placed the complementation group near the Dmca1A locus. Complementation analysis with null mutants of Dmca1A (cacophony/nightblind/\textit{L}11\textit{L3}) demonstrated that the four mutations we isolated (NT27, TS3, TS4, TS5) represented new Dmca1A alleles that disrupt the Drosophila homolog of the N-type calcium channel. Upon exposure to 38 °C, the Dmca1A TS alleles, unlike wild-type CS controls, showed a rapid onset of uncoordination, seizure-like activity, and loss of motor control (Fig. 1A). All of the alleles except one showed a rapid recovery when returned to room temperature, regardless of the length of exposure to 38 °C (Fig. 1B). Dmca1A\textsuperscript{NT277} was the most severe allele isolated, requiring progressively longer recovery times with increased length of exposure to 38 °C. The temperature-sensitive phenotype of each allele was rescued with a small chromosomal duplication (Dp(1;2)65b) encompassing the wild-type Dmca1A locus (19), indicating the behavioral defects result from disruption of the N-type calcium channel. Behavioral analysis of the TS mutants as well as complementation testing with other Dmca1A alleles suggest an allelic series of decreasing severity, NT27 > TS3 > TS4 > TS5.

\textsuperscript{2} Z. Guan and J. T. Littleton, unpublished observations.
The most severe allele, \textit{Dmca1AN27}, also shows behavioral defects at room temperature when placed in trans to a null mutant (\textit{Dmca1A13}) (19). These flies are flightless, exhibit an uncoordinated gait, and have reduced viability and life span. Thus, \textit{Dmca1AN27} mutants disrupt N-type calcium channel function constitutively, with the defect becoming worse at the elevated temperature. Further reduction in channel function at 38 °C triggers the onset of seizure-like activity in the giant fiber circuit (Fig. 1, \textit{E} and \textit{F}), accounting for the rapid onset of behavioral dysfunction.
To determine the cause of the behavioral and physiological defects observed in \textit{Dmca1A}\textsuperscript{NT27}, we sequenced the calcium channel ORF in the mutant. A single bp change (CGT to TGT) at position 2626 of the ORF was detected that converted a charged arginine residue at site 876 to a neutral cysteine residue (Fig. 2A). This amino acid substitution was confirmed by sequencing an independent isolate of the \textit{Dmca1A}\textsuperscript{ANT27} cDNA. Arginine 876 is a universally conserved residue in all voltage-gated ion channels (Fig. 2B) and corresponds to the third charged residue of the S4 voltage sensor in the third set of six transmembrane domains. We were unable to test directly the effect of the R876C change on the current-voltage relationship of the mutant channel, as \textit{Drosophila} presynaptic terminals are inaccessible for direct voltage clamp analysis, and experimental attempts to express recombinant \textit{Drosophila} calcium channels in heterologous systems have not succeeded (20). However, the identical charged arginine residue altered in \textit{Dmca1A}\textsuperscript{ANT27} has been mutated to a cysteine in recombinant \textit{Shaker} potassium channels and mammalian L-type calcium channels and expressed in oocytes, mimicking the R876C defect found in \textit{Dmca1A}\textsuperscript{ANT27} mutants (21, 22). In both cases, the R876C charge neutralization reduces the sensitivity of the channel to voltage, shifting the conductance-voltage relationship and requiring more depolarization for channel activation.

Indeed, the third arginine in the S4 voltage domain has been shown to contribute more than any other S4 charged residue in

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**Fig. 2. Mutations in the N-type calcium channel disrupt the S4 voltage sensor.** A, domain diagram of the \textit{Drosophila} N-type channel showing the localization of known mutations in \textit{Dmca1A}, including the R876C alteration in the S4 voltage sensor found in \textit{Dmca1A}\textsuperscript{ANT27}, the F1029I change found in \textit{cacS}\textsuperscript{I929F} (19), and the P1385S change found in \textit{Dmca1A}\textsuperscript{ATS2} (62). B, the Arg-876 amino acid (circled) is conserved in all voltage-gated ion channels through evolution and forms the third charged residue in the S4 voltage sensor transmembrane helix. Recent crystallization of a bacterial voltage-gated channel suggests the S4 domain forms a paddle within the lipid bilayer that senses voltage by transversing the membrane from inside to outside when the channel opens (63). The R876C change would lead to the loss of a core charge on the voltage-sensor paddle that could alter gating movements required for channel opening. A mutation in the mammalian homolog of \textit{Dmca1A} has also been mapped to the third transmembrane helix (boxed residue) in the ataxic mouse mutant rolling mouse Nagoya (64). Abbreviations: \textit{Na}, sodium channel; \textit{Ca}, calcium channel; \textit{H}, human; \textit{D}, \textit{Drosophila}. The roman numerals in parenthesis refer to the specific set of six transmembrane domains (from 1 to 4) found in sodium and calcium channels. C, dendrogram of the calcium channel subtypes from humans (\textit{H}), rodents (\textit{R}), \textit{Caenorhabditis elegans} (\textit{Ce}), and \textit{Drosophila} (\textit{Dm}). Analysis of the \textit{Drosophila} genome identifies a single family member of the L-type, N-type, and T-type voltage-gated calcium channels.
determining total gating charge for voltage-dependent channels (23).

The *Drosophila* genome encodes three calcium channel α-subunit genes (15), including homologs of the mammalian T-type (*Dmca1D*), and N-type (*Dmca1A*) channels (Fig. 2C). Previous physiological analysis in *Dmca1A* mutants has confirmed that N-type calcium channels are the primary source of extracellular calcium entry into *Drosophila* presynaptic nerve terminals (24). To determine whether *Dmca1A<sup>NT27</sup>* disrupts calcium channel function at the permissive temperature where we observe the morphological defects in synapse formation, we examined neurotransmitter release at 22 °C. Calcium influx through voltage-dependent calcium channels triggers presynaptic vesicle fusion, allowing us to test the *in vivo* effects of the R876C change in *Dmca1A*. Electrophysiological recordings at the neuromuscular junction of third instar *Dmca1A<sup>NT27</sup>* larvae demonstrated a dramatic defect in evoked neurotransmitter release compared with control animals (Fig. 3, A–C). At low extracellular calcium levels, evoked release was undetectable in the mutants upon nerve stimulation, whereas control lines showed substantial synaptic transmission. At higher calcium levels, evoked release was partially restored, but with a greatly reduced quantal content compared with controls. Mini amplitude was unchanged in the mutant (CS, 0.87 ± 0.05 mV; *Dmca1A<sup>NT27</sup>/Dmca1A<sup>13</sup>, 0.89 ± 0.04 mV), indicating post-synaptic sensitivity was not affected, and the observed defects in evoked release represent presynaptic dysfunction. Likewise, mini frequency was in the normal range in *Dmca1A<sup>NT27</sup>*, indicating spontaneous release was largely unaffected. The electrophysiological analysis of neurotransmitter release in *Dmca1A<sup>NT27</sup>* mutants confirms that the R876C change disrupts calcium channel function, calcium entry, and subsequent vesicle fusion.

Our initial observations of abnormal synaptic development in the *Dmca1A<sup>NT27</sup>* mutant suggested that presynaptic calcium entry might not only control neurotransmitter release but also modulate synaptic growth. To explore this possibility, we performed a quantitative analysis of synaptic growth in the mutants by visualizing anti-synaptotagmin staining as a presynaptic marker (Fig. 4, A and B). In *Drosophila* larvae, each abdominal hemisegment (A2–A7) has a stereotyped muscula-
ture with a precise motor neuron innervation pattern. In wild-type third instar larvae, an average of 65 ± 2.5 (S.E.) varicosities are present on muscle fibers 6/7 and 7/8 (average of segments A3 to A5). In contrast, both Dmca1A\textsuperscript{NT27} homozygotes and Dmca1A\textsuperscript{NT27}/Dmca1A\textsuperscript{13} larvae showed a significant reduction in varicosity number. Dmca1A\textsuperscript{NT27}/Dmca1A\textsuperscript{13} third instar larvae had on average 42 ± 2.4 (S.E.) varicosities, a 35% reduction in synaptic growth (p < 1 × 10\textsuperscript{-5}, Student’s t-test). This defect was also manifested at other NMJ synapses, including those at muscle fiber 4, where we observed a 34% reduction in varicosity number in Dmca1A\textsuperscript{NT27}/Dmca1A\textsuperscript{13} third instar larvae compared with controls. In addition, Dmca1A\textsuperscript{NT27} mutants exhibited reduced axonal terminal branching and a smaller synaptic innervation domain (Fig. 4B). Because NMJ growth varies in proportion with muscle size (25), we assayed in detail NMJ 6/7s at segment A2 in control and Dmca1A\textsuperscript{NT27} mutants and normalized measurements to muscle surface area. Normalized varicosity number was also significantly (p < 0.05) reduced in mutant animals (Fig. 4F).

To confirm further that calcium channel dysfunction results
Calcium Channels Regulate Synaptic Growth

in altered synaptic growth, we used RNA interference to generate loss-of-function animals for morphological characterization. We injected double-stranded RNA (dsRNA) prepared from Dmca1A or buffer alone into stage 5 CS embryos. 85% of embryos injected with Dmca1A dsRNA resulted in late stage embryonic lethality (compared with only 28% lethality for buffer-only injections), mimicking the embryonic lethal phase of Dmca1A null mutants. The remaining 15% of animals were able to hatch and proceed through development, suggesting RNA interference generated partial loss-of-function mutants in a small number of cases. We used these animals to quantify the number of varicosities at muscle 6/7 (segments A3 to A5) and normalized measurements to muscle surface area in both buffer and Dmca1A dsRNA-injected third instar larvae. Similar to observations in Dmca1A\textsuperscript{NT27} mutants, normalized varicosity number in Dmca1A dsRNA-injected animals was reduced by 32% (p < 0.001) compared with buffer-only-injected animals (Fig. 4G).

These observations suggest that either presynaptic calcium entry or synaptic transmission is an important modulator of synaptic growth during development. To differentiate between these two possibilities, we examined synaptic development in viable hypomorphic mutations of \textit{n}-synaptobrevin and syntaxin-1A that disrupt SNARE-mediated vesicle fusion and result in more severe defects in neurotransmitter release than observed in Dmca1A\textsuperscript{NT27} mutants (26). If defects in neurotransmitter release were the cause of the abnormal synaptic proliferation, we would expect a similar, if not more severe, reduction in synaptic growth in the two SNARE mutants. However, this was not observed. Mutations in syntaxin-1A or \textit{n}-synaptobrevin did not affect synaptic proliferation at the third instar NMJ (Fig. 4, C and D) and showed varicosity numbers identical to controls (Fig. 4E). These observations are in agreement with previous analysis of synaptic growth in synaptotagmin (27) and SNARE mutants (26). Thus, our data suggest that calcium entry through presynaptic N-type calcium channels, not neurotransmitter release \textit{per se}, is important for normal synaptic growth.

To address whether N-type calcium channels are required for the initial formation of the synapse during embryogenesis, or instead function in the second phase of developmental synaptic proliferation at the NMJ, we analyzed synapse formation in Dmca1A null mutant embryos. Previous genetic analysis of Dmca1A revealed several lethal mutations that map to the locus (19). Out of five embryonic lethal alleles of Dmca1A, we examined Dmca1A\textsuperscript{6}, Dmca1A\textsuperscript{10}, Dmca1A\textsuperscript{12}, Dmca1A\textsuperscript{20}, and Dmca1A\textsuperscript{22}; Dmca1A\textsuperscript{13} behaves similar to a deficiency in complementation testing with our TS mutants and has been reported previously (19) to act as a null mutant in complementation testing with other \textit{cacoophany} and \textit{nightblind} alleles of the Dmca1A locus. Therefore, we performed immunocytochemistry on Dmca1A\textsuperscript{13} embryos in \textit{trans} to a small deficiency (Df(1)RC29) that removes the Dmca1A locus. Morphological analysis with anti-HRP and anti-synaptotagmin revealed that Dmca1A null mutants and Dmca1A\textsuperscript{NT27} hypomorphic mutants do not affect neuronal pathfinding or synaptic morphology at muscle fiber 6/7 in fully matured embryos (Fig. 5, A–C). These data rule out a role for presynaptic calcium entry through N-type calcium channels in the initial stages of growth cone guidance and synapse formation. Given the evidence for a role for calcium in axonal pathfinding and neurite extension in neuronal culture systems, we considered the possibility that L-type channels may function in this regard. Mutations in the L-type channel have been generated previously and cause embryonic lethality secondary to a loss of muscle contractions (28).

Therefore, we generated double mutants disrupting both N-type and L-type channels. Immunocytochemical analysis of double mutants removing both channels reveals normal axonal pathfinding and initial synapse formation (Fig. 5D). One possibility is that intracellular calcium release channels or presynaptic ligand-gated channels provide calcium signals subserving neurite outgrowth. However, our data indicate that presynaptic calcium entry through voltage-gated calcium channels is not required during embryonic development for neurite outgrowth, axonal pathfinding, or initial synapse formation \textit{in vivo}. Following synapse formation, presynaptic calcium entry through N-type channels regulates a second phase of activity-dependent synaptic growth during synapse maturation.

Our results suggest that calcium entry through N-type calcium channels contributes to the formation of new varicosities. However, recent data (29) indicate that newly formed varicosities at \textit{Drosophila} NMJs must be stabilized to prevent synapptic retraction. Thus, calcium entry might be required to stabilize newly formed synapses. We tested these two alternative hypotheses using a recently developed assay for synaptic retraction. We co-stained third instar larvae with antisera against the presynaptic vesicle protein synaptotagmin and the post-synaptic marker DLG. Presynaptic retraction precedes disassembly of the postsynaptic apparatus, leaving behind a long lived postsynaptic footprint (29). This is manifested im-

Fig. 5. Defects in initial synapse formation do not account for reduced proliferation in Dmca1A\textsuperscript{NT27}. A–D, immunohistochemical analysis of embryonic muscle fiber 6/7 NMJs stained with anti-HRP and anti-synaptotagmin antibodies at hatching stage: A, CS (n = 25); B, Dmca1A\textsuperscript{NT27} (n = 23); C, Dmca1A\textsuperscript{10} (n = 13); and D, Dmca1A\textsuperscript{12}; Dmca1D\textsuperscript{16} (n = 7) double mutants. No defect in varicosity number or innervation pattern was observed, indicating presynaptic calcium channels are not required for axonal pathfinding, target recognition, or the initial stages of synapse formation. Scale bar is 5 μm. E, quantification of varicosity number at NMJ 6/7 of embryonic segments A2 to A7 of CS, Dmca1A\textsuperscript{NT27}, Dmca1A\textsuperscript{13}; Dmca1DX10 and Dmca1A\textsuperscript{13}; Dmca1D. Error bars are S.E.
Calcium Channels Regulate Synaptic Growth

The regulation of synaptic connectivity is a dynamic process, with synapse formation, synapse retraction, and synaptic remodeling occurring throughout life. This dynamic plasticity underlies the activity-dependent refinement of developing neuronal circuits and is thought to be essential for plasticity in the adult brain as well. Due to its accessibility and stereotypic development, the Drosophila glutamatergic neuromuscular junction has become a widely studied model system for investigating the molecular mechanisms that underlie synaptic growth (30). The NMJ forms during late embryogenesis and requires outgrowth of the motor neuron growth cone from the central nervous system, recognition, and contact with its target muscle fiber, and formation of enlargements of the growth cone or "prevaricosities" upon contact with the target muscle. Synaptic varicosities develop as a result of constrictions of larger prevaricosities (16). Following the formation of the initial synaptic field, a second phase of synaptic growth occurs that results in a 10-fold increase in varicosity number throughout larval development. This growth process has been shown to be modulated by activity-dependent mechanisms (12, 18, 31). Mutations that lead to increased cAMP levels (dunce) or increased nerve activity (Shaker and ether-a-go-go) result in a developmental increase in varicosity number and terminal branches at the NMJ. Many of these activity-dependent growth mechanisms impinge on the regulation of cell adhesion molecules like FASII and integrins (25, 32). For example, activated CaMKII has been demonstrated to regulate FAS2 localization and modulate synaptic growth (33). Recently, members of the transforming growth factor-β superfamily of ligands and receptors have also been found at the NMJ and shown to modulate synaptic number (34, 35). Downstream of these signaling pathways, modulation of the actin and tubulin cytoskeleton is important in synapse maturation (36, 37). Finally, both local protein synthesis and ubiquitin-dependent degradation have been implicated in synaptic growth in Drosophila (38–40). Although a relatively large number of growth pathways have been found, including several that are calcium-dependent, it is largely unknown how they function together and what signals regulate their activity.

Cell adhesion proteins and synaptic growth regulators reside in regions adjacent to the active zone where presynaptic calcium channels localize (40, 41). Here we demonstrate that presynaptic calcium channels regulate synaptic growth. These findings suggest calcium diffusion from N-type channels may regulate the properties of calcium-activated synaptic growth mechanisms at the Drosophila NMJ.

**DISCUSSION**

The regulation of synaptic connectivity is a dynamic process, with synapse formation, synapse retraction, and synaptic remodeling occurring throughout life. This dynamic plasticity underlies the activity-dependent refinement of developing neuronal circuits and is thought to be essential for plasticity in the adult brain as well. Due to its accessibility and stereotypic development, the Drosophila glutamatergic neuromuscular junction has become a widely studied model system for investigating the molecular mechanisms that underlie synaptic growth (30). The NMJ forms during late embryogenesis and requires outgrowth of the motor neuron growth cone from the central nervous system, recognition, and contact with its target muscle fiber, and formation of enlargements of the growth cone or "prevaricosities" upon contact with the target muscle. Synaptic varicosities develop as a result of constrictions of larger prevaricosities (16). Following the formation of the initial synaptic field, a second phase of synaptic growth occurs that results in a 10-fold increase in varicosity number throughout larval development. This growth process has been shown to be modulated by activity-dependent mechanisms (12, 18, 31). Mutations that lead to increased cAMP levels (dunce) or increased nerve activity (Shaker and ether-a-go-go) result in a developmental increase in varicosity number and terminal branches at the NMJ. Many of these activity-dependent growth mechanisms impinge on the regulation of cell adhesion molecules like FASII and integrins (25, 32). For example, activated CaMKII has been demonstrated to regulate FAS2 localization and modulate synaptic growth (33). Recently, members of the transforming growth factor-β superfamily of ligands and receptors have also been found at the NMJ and shown to modulate synaptic number (34, 35). Downstream of these signaling pathways, modulation of the actin and tubulin cytoskeleton is important in synapse maturation (36, 37). Finally, both local protein synthesis and ubiquitin-dependent degradation have been implicated in synaptic growth in Drosophila (38–40). Although a relatively large number of growth pathways have been found, including several that are calcium-dependent, it is largely unknown how they function together and what signals regulate their activity. Cell adhesion proteins and synaptic growth regulators reside in regions adjacent to the active zone where presynaptic calcium channels localize (40, 41). Here we demonstrate that presynaptic calcium channels regulate synaptic growth. These findings suggest calcium diffusion from N-type channels may regulate the properties of calcium-activated synaptic growth mechanisms at the Drosophila NMJ.

**Calcium Channels Regulate Synaptic Growth**

**Fig. 6. Synapse retraction does not account for presynaptic undergrowth of nerve terminals in Dmca1A<sup>NT27</sup>.** Co-staining with anti-synaptotagmin and anti-DLG at muscle fibers 6/7 in segment A3 of third instar larvae: A, CS; B, Dmca1A<sup>NT27</sup>; and C, GI<sup>+/−</sup>. In CS and Dmca1A<sup>NT27</sup>, DLG post-synaptic staining was co-localized with presynaptic synaptotagmin immunoreactivity. No DLG footprints suggestive of synaptic retraction were observed, indicating the reduced synapse number in Dmca1A<sup>NT27</sup> is not due to an increase in synapse retraction. In contrast, GI<sup>+/−</sup> mutants that disrupt dynactin function display DLG footprints without corresponding synaptotagmin staining (arrows), consistent with synaptic retraction as reported previously (29).
number compared with wild-type controls and hypomorphic alleles of syntaxin and synaptobrevin. The reduced synaptic proliferation is not secondary to defective synaptic transmission because syntaxin and synaptobrevin show more profound defects in transmitter release but have normal synaptic proliferation. Similar results have also been reported in synaptotagmin mutants as well as SNARE mutants (26, 27). Thus, mutations in Dmca1A affect calcium-regulated synaptic pathways separate from those that regulate transmitter release.

We found no evidence for a role of presynaptic calcium entry through either N- or L-type calcium channels in the early stages of synapse formation during late embryogenesis. We also determined that the morphological defects in Dmca1A mutants are not due to an increase in terminal retraction, suggesting active growth rather than synapse stability is defective. Therefore, the structural defects we observe in Dmca1A mutants occur between the establishment of the initial synaptic field and its final larval maturation. Previous work (17) has demonstrated that the overall shape and branching pattern at the Drosophila NMJ is established early in development. Subsequent growth largely requires the addition of new varicosities to previously formed terminal branches. It is within this second activity-dependent growth phase that we propose presynaptic calcium entry is required to promote synaptic maturation. The 35% reduction we observe in varicosity number in Dmca1AN1277 mutants at the end of larval development is likely an underestimate of the actual contribution of presynaptic calcium entry to synaptic growth regulation. First, the initial activity-independent elaboration of synapses during late embryogenesis does not require calcium channel function, allowing the establishment of the initial synaptic field. Second, the Dmca1AN1277 mutant is a hypomorphic allele, reducing calcium channel function but not eliminating it. Previously isolated alleles of the Dmca1A locus that are more severe than Dmca1AN1277 are embryonic lethal, preventing an analysis of the activity-dependent phase of synaptic growth in more severe alleles. Further studies with mosaic animals will be required to fully characterize the persistence of synaptic growth mechanisms in the complete absence of presynaptic calcium influx.

The opening of presynaptic N-type channels during robust synaptic activity may allow calcium to influence varicosity sprouting mechanisms to locally control synaptic remodeling. Changes in intracellular calcium have been shown to affect growth cone motility (43, 44) and neurite outgrowth (45, 46). Indeed, filopodial protrusions from neuronal growth cones are triggered by altered calcium concentrations (46). Synaptic activity results in calcium-dependent CaMKII activation via binding of calcium/calmodulin and subsequent auto-phosphorylation (47). Activated CaMKII phosphorylates the synaptic MAGUK protein DLG, causing release of FAS2 from its synaptic scaffold and subsequent modulation of synaptic growth in Drosophila (32, 33). In addition, CaMKII activation also regulates the activity of the ether-a-go-go ( eag) family of potassium channels in Drosophila, altering aspects of nerve excitability that could contribute to synaptic growth (48, 49). Intracellular calcium levels directly regulate cAMP signaling through the activation of adenylate cyclase by calmodulin (50), enhancing cAMP-dependent pathways implicated in synaptic growth (12). It is likely that disruptions in presynaptic calcium entry in Dmca1AN1277 mutants leads to alterations in several presynaptic signaling cascades that modulate growth. Further genetic analysis should begin to elucidate how the regulation of calcium entry modulates these activity-dependent synaptic growth pathways. Voltage-gated calcium channels consist of four repeated units (I–IV) containing six α-helical transmembrane segments (S1–S6). The fourth transmembrane segment (S4) of voltage-gated ion channels has been shown to function as a voltage sensor. It is thought that the S4 sensor, a transmembrane α-helix in which every third or fourth residue is basic and carries a positive charge, undergoes conformational changes during depolarization that result in channel opening. Sequence analysis of Dmca1AN1277 identified a charge-neutralizing mutation in a highly conserved arginine residue in the S4 voltage sensor, supporting an essential role for S4 helix movement during channel gating and subsequent calcium influx. The altered S4 charged amino acid likely explains the TS phenotype of the mutant, as channel gating requires conformational changes in the S4 helix that are temperature-dependent. Mutations in mammalian Dmca1A homologs have been linked to a variety of neuronal disorders, including episodic and spinocerebellar ataxias, hemiplegic migraine, blindness, hypokalemic periodic paralysis, and epilepsy (51). Although it seems paradoxical that a hyperexcitability phenotype like seizures could arise from a reduction in calcium channel function, the Drosophila giant fiber pathway is extremely sensitive to changes in both inhibition and excitation. The decrease in calcium channel function in the inhibitory pathways may bear more weight in the overall output of the circuit, even though the excitatory outputs have reduced release as well. Similar seizure defects are seen in mammalian N-type calcium channel mutants (tettering, lethargic, and rocker) where overall calcium channel function is also reduced (52). Similar to what we have observed in Drosophila N-type mutants, mutations in the mouse α1a calcium channel locus (rocker) that disrupt the pore region of the channel and reduce calcium influx cause a profound reduction in Purkinje cell dendritic arborization (53). In addition, pharmacological disruption of calcium channel function in salamander rod photoreceptors has been shown recently (54) to inhibit varicosity formation. The reduced varicosity number in calcium channel mutants suggests an important role for normal levels of calcium entry during synaptic activity for the proper modulation of synaptic growth, in addition to its well-established role in neurotransmitter release. Modulation of N-type calcium channel function via alterations in the rates of presynaptic action potential firing could provide an efficient mechanism for the regulation of activity-dependent synaptic growth.

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Calcium Channels Regulate Synaptic Growth

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