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Developmental Consequences of Neuromuscular Junctions With Reduced Presynaptic Calcium Channel Function

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ABSTRACT Evoked neurotransmitter release at the *Drosophila* neuromuscular junction (NMJ) is regulated by the amount of calcium influx at the presynaptic nerve terminal, as for most chemical synapses. Calcium entry occurs via voltage-gated calcium channels. The temperature-sensitive *Drosophila* mutant, cac^{TS2}, has a reduced amount of calcium entry during evoked stimulation. We have used this mutation to examine homeostatic regulatory mechanisms during development of the NMJ on muscle 6 within the developing larva. The amplitude of the excitatory postsynaptic potentials are reduced for both the Ib and Is motor neurons in 3rd instar larvae which have been raised at 33°C from the 1st instar stage. Larvae raised at 25°C and larvae pulsed at 33°C from the late 2nd instar for various lengths of time show a reduced synaptic efficacy as a 3rd instar. The results indicate that the nerve terminal cannot fully compensate physiologically in the regulation of synaptic transmission during larval life for a reduced amount of evoked calcium entry. Morphological comparisons of Ib and Is terminals in relation to length and numbers of varicosities are significantly reduced in cac^{TS2}, which also suggests a lack in homeostatic ability. These findings are relevant since many deficits in synaptic transmission in various systems are compensated for either physiologically or structural over development, but not in this case for reduced calcium entry during evoked transmission. **Synapse 57:132–147, 2005.**

INTRODUCTION

For proper function of chemical synapses, the efficacy of communication is tightly regulated while remaining plastic enough to respond to changing circumstances and requirements. If synaptic transmission is too strong or weak, an inappropriate signal will be relayed and thus an altered behavioral response can be manifested. Fine tuning in connections within neural circuits is most dramatically represented during development and maturation (Burrone and Murthy, 2003; Davis and Murphey, 1994; Hubel and Wiesel, 1963a,b, 1968, 1970). For example, as the size of postsynaptic cells increase during development, a matched increase of neurotransmitter release occurs to maintain effective activation of target cells. Studies of this phenomenon are well documented at the *Drosophila* neuromuscular junction (NMJ) during larval development (Li and Cooper, 2001; Li et al., 2002; Lnenicka et al., 2002). With the rapidity in larval development and genetic manipulability, the *Drosophila* NMJ is advantageous for investigation of homeostatic regulation of development, plasticity, and maturation of synapses (Marek and Davis, 2003; Nudell and Grinnell, 1983; Wilkinson and Lunin, 1994; Wilkinson et al., 1992).

The degree of synaptic strength is dependent on the amount of transmitter released from the presynaptic neuron as well as the responsiveness of the postsynaptic target. Thus, both pre- and postsynaptic factors influence synaptic strength (Bhattacharya et al., 2002; Saitoe et al., 2001). During NMJ development, the morphology and ultrastructure of both pre- and postsynaptic components change dramatically. Anatomical visualization of NMJs in larval *Drosophila* (Cash et al., 1992), rodent (Balice-Gordon et al., 1990; Ogata and Yamasaki, 1985), and frog (Bennett and Pettigrew,

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1975; Herrera et al., 1985; Kuno et al., 1971; Nudell and Grinnell, 1983) have shown that growth occurs by a correlative enlargement of both pre- and postsynaptic structures. As for the developing NMJs of *Drosophila* larvae, as the nerve terminals grow, new varicosities are added (Sink and Whitington, 1991; Keshishian et al., 1993). The evidence as a whole implies that there are mechanisms of muscle–nerve communication that continuously monitor the efficacy of synaptic transmission to provide some order of synaptic homeostasis in both structure and function (Herrera et al., 1991; Nudell and Grinnell, 1985).

With manipulation of synaptic transmission of adult vertebrate motor nerve terminals, it is known that they maintain a high degree of plasticity. A classic example is the increased branching and growth of motor nerve terminals when the terminal is exposed to botulinum toxins that block synaptic transmission (Shupliakov et al., 2002). Possibly the same regulating factors in synaptic maintenance in adult animals are utilized during developmental stages, so understanding how synapses develop will likely provide a key to mechanisms underlying plasticity, at mature NMJs. It is of interest to understand why in some cases, motor nerve terminals are so dynamic in maintaining synaptic communication while in other situations there is a lack in the ability. For example, in humans the Lambert–Eaton myasthenic syndrome results in reduced neurotransmission from the motor nerve terminals because of autoimmune dysfunction from the production of antibodies against ones' own calcium channels. There are conflicting reports for this disease as to if there is compensation of the nerve terminal by an enhancement of terminal growth to overcome the reduced amount of transmitter release (Giovannini et al., 2002; Protti et al., 1996). There are several other human diseases associated with altered calcium channel function, which have an effect on the anatomical and physiological properties of both the pre- and postsynaptic cells (Lehmann-Horn and Jurkat-Rott, 2001). Because the fundamental basics of synaptic transmission in model systems is directly relevant to all chemical synapses, mimicking the reduced calcium entry in motor nerve terminals in accessible model preparations will lead to a better general understanding of regulatory synaptic mechanisms.

The mechanisms by which animals maintain synaptic strength under diverse developmental, genetic, and environmental conditions are still unclear. There is evidence that terminal morphology can regulate synaptic strength (Atwood and Cooper, 1996a,b; Govind and Chiang, 1979; Kuno et al., 1971; Stewart et al., 1996; Walrond et al., 1993); however, overall nerve terminal length and varicosity numbers are not sufficient to predict synaptic strength because structure has been shown to be dynamic (Cooper et al., 1996a; Davis et al., 1998; Schuster, et al., 1996a,b). Therefore, physiological measures of synaptic transmission of the terminals need to accompany morphological analysis.

The purpose of this study was to address whether synaptic homeostasis can be maintained over the long-term at *Drosophila* NMJs in the presence of a mutation that decreases the function of the calcium channels, resulting in an acute reduction in evoked synaptic transmission. To address this problem, both morphological and physiological parameters were measured. We choose to use the most commonly studied *Drosophila* NMJs, which are the most prominent ventral longitudinal abdominal larval muscle fibers 6 and 7 (Crossley, 1978). These muscles exhibit the simplest innervation pattern among *Drosophila* body wall muscles, since electrophysiological and morphological studies imply that each of these two muscles is innervated by only two axons (Atwood et al., 1993; Jan and Jan, 1976a,b; Kurdyak et al., 1994; Sink and Whitington, 1991). The development of the two motor nerve terminals (i.e., Is and Ib) has also been shown to demonstrate a "homeostatic" relationship with the amount of presynaptic calcium influx. It has been recently shown that if this influx is reduced, a resulting decrease in bouton number and nerve terminal branch complexity occurs, as compared with those in wild-type flies (Rieckhof et al., 2003).

We made use of a mutation in the cacophony gene which encodes a voltage-gated calcium channel used for evoked neurotransmitter release. The mutated gene, cac^{TS2}, is a temperature-sensitive paralytic allele of a *Drosophila* calcium channel α_1-subunit gene (Brooks et al., 2003; Catterall, 1998; Kawasaki et al., 2000, 2002; Wheeler et al., 1995). It has recently been demonstrated that expression of *cac*-encoded α_1-subunit fused with a green fluorescent protein localized to motor nerve terminals (Kawasaki et al., 2004) and that there is a reduction in exocytosis for the cac^{TS2} strain (Kuromi et al., 2004). Using the cac^{TS2} mutant allowed us to investigate the role a pathological influx of Ca^{2+} in the mechanisms of how synaptic efficacy and synaptic homeostasis may be maintained. Since the mutation exists in the channel there is the possibility that even at permissive temperatures there could be an effect on function. Thus, studies were also conducted at 25°C, in addition to higher temperatures (30°C and 33°C), to address physiological and anatomical differences between wild-type Canton-S (CS) and cac^{TS2} strains.

Extracellular calcium levels are critical for cellular functions, so it is not surprising that low extracellular calcium causes neurons to become hyperexcitable (Haas and Jefferys, 1984; Undem et al., 2003) and, over time, can lead to degradation of synaptic structure (Zefirov et al., 2003). On the other hand, high extracellular concentrations of calcium can result in substantially more transmitter being released during an evoked response and lead to even more rapid synaptic depression. Functionally well-tuned calcium

dynamics is important for development of neurons in vertebrate central neurons (Brandt et al., 2003) as well as for NMJs in *Drosophila* (Rieckhof et al., 2003). Methods are clinically used to regulate calcium channel function as a means to control pathological conditions such as epilepsy and convulsions (Araújo et al., 2003). The outcome of long-term treatments of calcium channel blockers for regulation of such diseases needs to be addressed in terms of potential consequences in which homeostatic synaptic mechanisms may be compromised. Examining effects on the long-term reduction of calcium influx in defined model systems will allow insight for clinical application.

Preliminary results of this work have been presented in abstract form (Xing and Cooper, 2002; Long et al., 2004).

MATERIALS AND METHODS

Strains

Wild-type (CS) and cac^{TS2} strains of *Drosophila melanogaster* (Garen et al., 1977) were maintained at 18°C on a standard cornmeal-dextrose-agar-yeast medium. The cac^{TS2} strain was provided by Dr. Richard Ordway (The Pennsylvania State University). To obtain staged larvae, flies were maintained for a few days at 25°C. A 30-min prepulse of egg laying was performed to clear the females of stored eggs in the ovipositor. Subsequently, eggs from both strains were collected separately at 25°C for 2-h periods on apple juice agar.

Developmental assays

Eggs were allowed to develop at 25°C. Upon hatching, the 1st instars were either transferred to 30°C or 33°C for long-term developmental studies. For short-term developmental studies at 33°C, the 1st instars were maintained at 25°C until they reached an early 2nd instar stage, then they were placed at 33°C. Acute studies consisted of taking early wandering 3rd instars and placing them at 33°C for 4 h. In these experimental paradigms, the wandering 3rd instar stage was used for electrophysiological studies. A wild-type group was used as control in all experiments. The larval body lengths were used to obtain overall growth patterns among experimental conditions.

Behavioral assays

Early 3rd instar larvae were used for behavioral assays. Feeding and locomotory behavior was assessed in both species as described in Neckameyer (1996) and Li et al., (2001). In brief, single animals were placed on a 2% agar surface and the number of body wall contractions was counted for 2 min, after which an animal was placed in a 2% yeast solution overlaid on an agar plate. In this condition, *Drosophila* larvae immediately fed, initiating a pattern of repetitive mouth hook movements. The number of full mouth hook contractions in 2 min was counted (Sewell et al., 1975). The results of these behaviors are plotted as body wall contractions or mouth hook movements per minute.

Anatomy

A fluorescent anti-horseradish peroxidase (HRP-FITC) primary antibody (ICN Pharmaceuticals, CA, USA) was used to aid in identifying the motor nerve terminals on muscle 6 (m6) and muscle 7 (m7) (Johansen et al., 1989). The terminals are primarily type I endings from the two major axons (Is and Ib) (Atwood et al., 1993; Kurdyak et al., 1994). Fluorescent images of the nerve terminals were viewed with a Leica DM RE upright fluorescent microscope, using a 40× water immersion objective with appropriate illumination. The composite images of Z-series were collected with a Leica TCS NT/SP confocal microscope for illustration. The confocal images were quantified for varicosity number, terminal length, and muscle dimensions, with the Leica confocal or SCION Image software.

Electrophysiology

Four experimental paradigms (one at 25°C and three at 33°C, pulsed for 4, 16, and 72 h) were used to assess physiological characteristics associated with the cac^{TS2} phenotype. Equal treatments were performed with wild-type for comparisons. Only animals at the wandering 3rd instar stage were assessed physiologically and anatomically in relation to the NMJs. The larval dissections were performed as described in Cooper et al. (1995b). In brief, a longitudinal mid-dorsal incision was made and the edges pinned so that the preparation was spread out. Internal organs were carefully removed to expose the body wall muscles, particularly the ventral longitudinal muscles of segments 3 and 4. The recordings were obtained from the prominent longitudinal m6 muscle.

The physiological solution used is the same as previously described (Stewart et al., 1994). In brief, the physiological saline contains (in mM) 1.0 $CaCl_2{\cdot}2H_2O$, 20 $MgCl_2{\cdot}6H_2O$, 70 NaCl, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, and 5 BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid). All experiments were performed at room temperature (20–25°C). The electrophysiological values of interest were the resting membrane potential (R_p) and the EPSP amplitudes elicited by Is and Ib motor nerve terminals in segment 3 of m6. Short trains of 4 stimuli at 20 Hz were given to determine whether the EPSP amplitudes depressed or facilitated.

Intracellular recordings were made with microelectrodes filled with 3 M KCl (30–60 mΩ). The responses were recorded with a 1× LU head stage and an Axoclamp 2A amplifier to a VHS tape (Vetter, 400), as well as online to a PowerMac 9000 via a

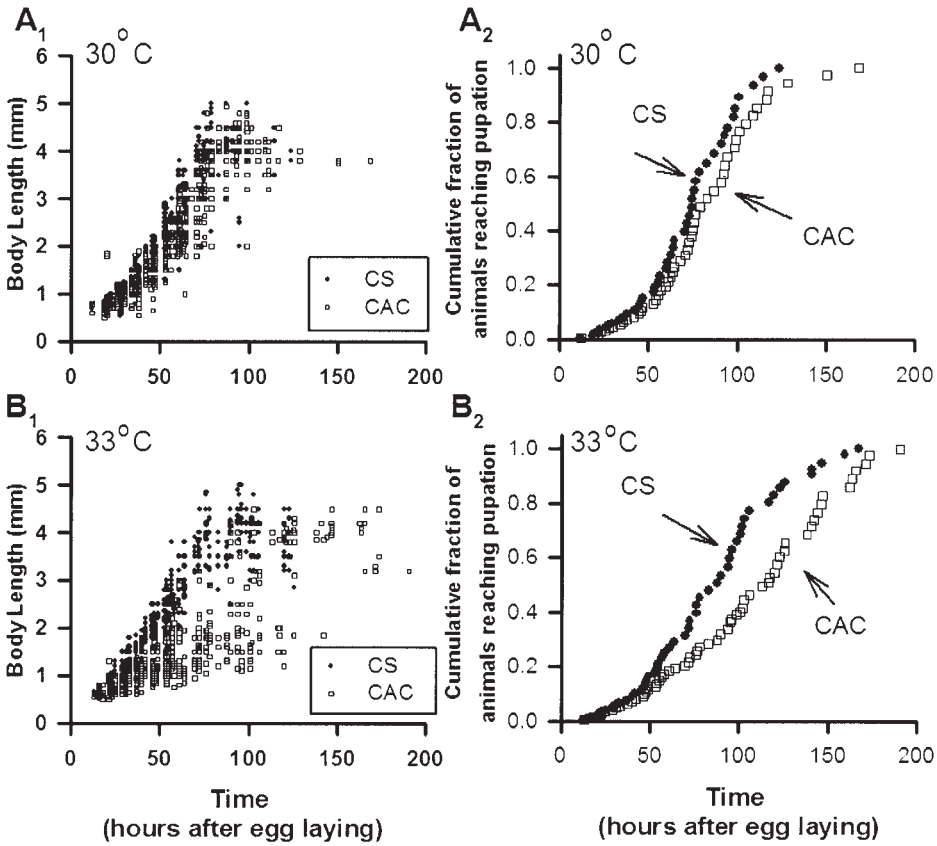

Fig. 1. Comparative growth curves of cac^{TS2} and CS at restrictive and permissive temperatures. By measuring the body length of larvae from 1st to the end of 3rd instar, the developmental rate of cac^{TS2} and CS can be assessed during the course of development, under permissive (A_1, A_2) or restrictive (B_1, B_2) temperatures. The body length over time (hours after egg laying) for the larvae at 30°C (**A_1**) or 33°C (**B_1**) only shows a shift in the distributions for the larvae raised at 33°C. The distinctions are better illustrated in the cumulative graphs for the fraction of animals reaching pupation over hours after egg laying at 30°C (**A_2**) or 33°C (**B_2**). When exposed to the permissive temperature of 30°C, no developmental differences between cac^{TS2} and CS can be noted; however, an exposure to the restrictive 33°C creates an evident difference in development.

MacLab/4s interface (ADInstruments, Mountain View, CA, USA). All events were measured and calibrated with the MacLab Scope software version 3.6.

Field excitatory postsynaptic potentials

Synaptic potentials were also measured with focal macropatch electrodes to determine the effect on presynaptic vesicular events of the *cac* ^{TS2} phenotype for the paradigm in which larvae were held for 72 h at 33°C. The varicosities on the living terminals were viewed with Nomarski optics. Field excitatory postsynaptic potentials (fEPSPs) were recorded in conjunction with a 0.1× LU head stage and an Axoclamp 2A amplifier in a bridge mode configuration. The fEPSPs were obtained using the loose patch technique, by lightly placing a 10–20 fire polished glass electrode directly over a spatially isolated varicosity along the nerve terminal that were viewed under a 40× water immersion lens (Nikon, NA 0.55). The macropatch electrode is specific for recordings within the region of the electrode lumen. Effort was taken to record from the most distal varicosity of the Is terminals, with the patch electrode. These varicosities are easier to identify since they are usually more centrally located on the fiber, away from the edge. Since the evoked release recorded at the *Drosophila* NMJ is multiquantal, the average area of mfEPSPs and the average area of fEPSPs were used to determine mean quantal content (m) (Cooper et al., 1995a,b, 1996a).

Quantal release over time was also monitored by examining the area of the evoked potential. The time of the peak in evoked events varies because of latency jitter when multiple events occur; thus, measures of peak amplitude are not as reliable as the area measure (Cooper et al., 1995b). The motor nerve was stimulated at 0.5 Hz in order not to facilitate the responses between trials.

Input resistance

The amplitude of the receptor potential is related to the amount of net current and the cell's input resistance (R_{in}), both of which could possibly influence the

membrane potential. To examine if there was a difference in the R_{in} pulses of current (200–300 ms in duration) ranging from –2.0 to +2.0 nA in steps of 0.2 nA were injected through an intracellular electrode and measures of the plateaued potentials were recorded through the same electrode. The slope of the current–voltage plots was used to determine R_{in}.

Statistical analysis

When the basic assumption of parametric Student's *t*-test was valid, it was used; otherwise, the nonparametric Wilcoxon rank sum test was used.

RESULTS

In cac^{TS2}, larval development is slowed at the restrictive temperature of 33°C

Development was assayed by whole body length. Larvae as groups were taken at various times throughout their development to be measured at 30°C (Fig. $1A_1$) and 33°C (Fig. $1B_1$); however, discrete individuals were not monitored over time. For comparisons between the groups, the cumulative sum is shown as a relative cumulative frequency for all the larvae over time. The cumulative fraction of animals reaching pupation over hours after egg laying were plotted by averaging the body length for each hour and adding the value to the previous value for the preceding hour. The maximum values were divided into each running average value, to obtain a fraction of the total. Exposing 1st instar wild-type and cac^{TS2} larvae to 30°C did not result in any strong shift in the developmental curve to the time of pupation (Fig. $1A_2$). The exposure to 33°C, however, did cause a few (~2%) to die before pupation was reached and a slowing of development could be observed as early as in the early 2nd instar between the controls and cac^{TS2} (Fig. $1B_2$). The retarded development was maintained throughout the remaining larval stages of development to the onset of pupation. We did not monitor the duration of pupation and adult development for this present study. Attempts to raise 1st instar cac^{TS2} larvae, but not wild-type, at 36°C resulted in high larval mortality; therefore, electrophysiological analyses were not performed at this temperature (data not reported). In addition since no significant change could be observed for the larvae raised at 30°C, we did not conduct the growth curves at 25°C.

Locomotion and feeding behaviors are slowed for cac^{TS2} as compared with those for controls

To better characterize the developmental differences between cac^{TS2} and wild-type larvae at 25°C and 33°C, standard behavioral indices were examined. Standard locomotion and feeding assays were used to assess 3rd instar larvae at their wandering stage

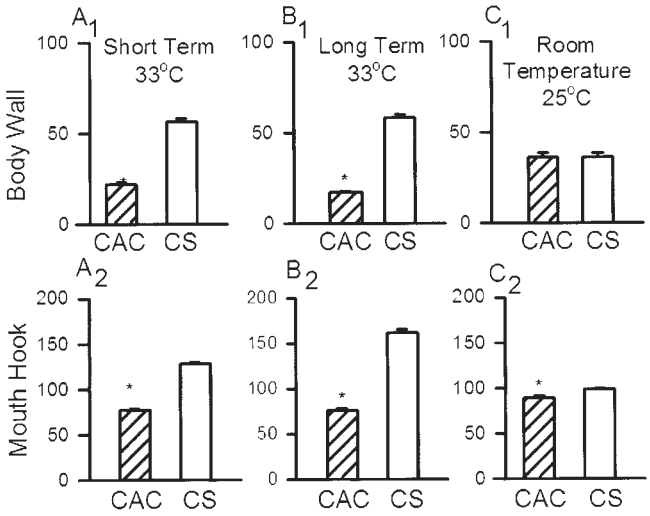

Fig. 2. Comparative analyses of behavioral movements at restrictive and permissive temperatures. When behavioral assays of locomotion and feeding were carried out using cac^{TS2} and CS, it was determined that the contractions of the body wall and movements of the mouth hooks are reduced at the restrictive 33°C, both during short-term (**A_1**, **A_2**) and long-term (B_1, B_2) exposure periods. Third instar larvae that were grown at the cac^{TS2} restrictive temperature of 33°C for 4 h showed affected behavior selectively in the cac^{TS2} strain. Long-term exposure to 33°C of larvae from 1st instar to earlier 3rd instar (72 h) also caused a reduction in body wall contractions (**B_1**) and mouth hook movements (**B_2**) for cac^{TS2} as compared with that for wild-type. Both strains showed a greater reduction in the behavioral indices for the long-term exposure as compared with the short-term period. Compare A_1 with B_1 and A_2 with B_2 for a given strain. (**C_1**, **C_2**) When behavioral movements were assessed at room temperature (25°C), there were no differences that could be seen between cac^{TS2} and CS for body wall movements but there is a significant difference in mouth hook movements. For purposes of quantification, averages of movement within a 2-min period were used. Asterisks indicate a significant difference between cac^{TS2} and wild-type ($P < 0.05$, Student's *t*-test). In each parameter, $n = 15$.

while exposed to 25°C and 33°C. The average number of body wall contractions within a 2-min period was used for quantification. The animals were provided with a 1-min time to adapt to the experimental dish prior to the onset of monitoring the body wall contractions. There is a significant alteration in number of body wall contractions for the cac^{TS2} strains for the short- and long-term exposure at 33°C ($P < 0.05$, Student's *t*-test; see Fig. 2). The average rate of mouth hook movements between wild-type and cac^{TS2} was also significantly different for the two exposure periods at 33°C (Fig. 2; $P < 0.05$, Student's *t*-test). For these behavioral indices, there is a prominent effect for both wild-type and cac^{TS2}, with exposure to 33°C. This suggests that the restrictive temperature of 33°C has an effect on not only cac^{TS2} but also wild-type, in a time-dependent manner. The larvae that were exposed to 33°C from 1st instar (72 h) were more severely effected than the ones that were exposed from early 2nd instar (16 h) when comparing cac^{TS2} with cac^{TS2} or wild-type with wild-type for the two exposure times ($P < 0.05$, Student's *t*-test; compare

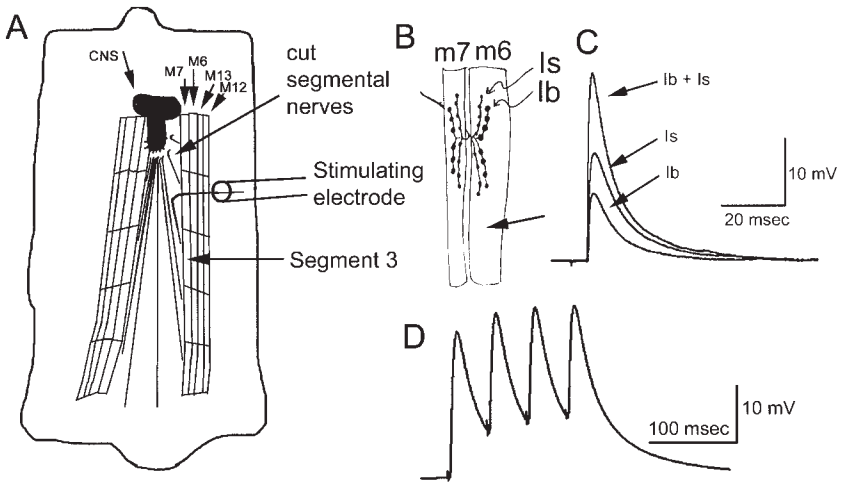

Fig. 3. Diagrammatic scheme of *Drosophila* larval dissection. (**A**) The preparation is opened and pinned at four corners to allow the preparation to remain extended and tight. The prominent m6 (segment 3) was used for experimentation, where the third segmental nerve is cut away from the CNS, and pulled into a stimulatory suction electrode. (**B**) Schematic depiction of motor nerve terminals Ib and Is, which innervate m6 and m7. (**C**) Intracellular electrophysiological recordings can be made from m6 by the stimulation of Ib and Is, which can be stimulated singularly, or together, which results in a compound "Ib + Is" EPSP. (**D**) To examine facilitation, the nerves were stimulated at 20 Hz for 4 pulses. The 1st and 4th EPSP amplitudes of summated Ib and Is responses are used as an index of facilitation.

Fig. $2A_1$ with $2B_1$ and $2A_2$ with $2B_2$). Third instar of wild-type and cac^{TS2} at 25°C showed no significant difference in body wall contractions (Fig. $2C_1$). There is a slight reduction, albeit significant ($P < 0.05$, Student's *t*-test; Fig. $2C_2$), in the number of mouth hook movements for cac^{TS2} as compared with that for wild-type. The behavioral data is averaged to convey movements per minute over the 2-min observation period.

The temperature effects on behaviors for the wild-type strain stresses the point that parallel controls are needed for assaying effects on the cac^{TS2} strain. There are several possible reasons for the effect noted with the wild-type strain, such as the impact of constitutively active heat shock proteins since the animals are maintained at 33°C. This relative high temperature may also have developmental consequences in muscle function and possible function of the nervous system as a whole (i.e., sensory, CNS, and motor) that we have not been able to ascertain in the boundaries of this study.

The function of the NMJ is compromised by the cac^{TS2} mutation

To monitor the effects of the calcium channel mutation in synaptic function, the common NMJ model of m6 in segment 3 of the 3rd instar larva was used. The layout of the dorsal filleted 3rd instar is depicted in Figure 3A. While pinning the preparation at the four corners, the longitudinal segmental muscles are stretched, which allows physiological measures to be obtained across the muscle membrane while stimulating the selective segmental nerves. Both m6 and m7 are innervated by the Ib and Is nerve terminals, as schematically shown (Fig. 3B). It is documented in *Drosophila* (Kurdyak et al., 1994; Stewart et al., 1994) and other species of fly (Harrison and Cooper, 2003) that the anatomical Ib type of terminals give rise to the smaller excitatory postsynaptic potentials (EPSPs) as compared with those by the Is terminals. The axons that give rise to the Ib and the Is terminals can be recruited together to produce a compound EPSP (Ib + Is) (Fig. 3C). Unless all three responses are obtained within a recording, we did not utilize the data for distinguishing the individual EPSPs of the Ib and Is responses. Since the degree of short-term facilitation (STF) is dependent on the concentration of the extracellular calcium in *Drosophila* (Stewart et al., 1994) as well as for NMJs of other species (Atwood and Wojtowicz, 1986; Dodge and Rahamimoff, 1967; Katz and Miledi, 1968), we utilized a facilitation index (FI) in our studies, which depends on the relative amplitude of 4th and 1st EPSPs within a 20 Hz-4 pulse train (Fig. 3D). For the FI measures, the composite Ib and Is response is used since it is relatively easy to consistently recruit both axons as compared with one or the other axons.

Synaptic transmission is reduced in cac^{TS2} larvae over time and is not compensated

The three exposure paradigms, 4, 16, and 72 h, at 33°C were used to assay the impact of the cac^{TS2} mutation on synaptic transmission. All preparations

were examined during the 3rd wandering stage after the exposure periods were completed. The indices used were the resting membrane potential, the amplitude of the EPSPs for the discrete Ib and Is responses as well, as the composite EPSP for the Ib and Is terminals. Facilitation was also substantially altered over the time for the 33°C-exposure in the cac^{TS2} line.

The early 3rd instars exposed to 33°C for 4 h and electrophysiologically examined at room temperature (25°C) show only a slight difference in overall synaptic transmission as compared with wild-type. The amplitude of the EPSPs for the Ib terminal of the cac^{TS2} has a significant reduction as compared with that of the wild-type controls ($P < 0.05$, Student's t-test); however, no differences are observed for the discrete EPSP amplitudes for the Is, and the combined Is and Ib measures (Fig. $4A_1$). In addition, there is no difference for the FI between cac^{TS2} and wild-type groups (Fig. $4B_1$). When late 2nd instars are exposed to 33°C for 16 h and monitored for the same physiological measures, there are significant differences in the amplitudes of the EPSPs for the Ib, Is, and Ib+Is responses as well as for FI ($P < 0.05$, Student's t-test; Fig. $4A_2$ and $4B_2$). Given that the resting membrane potentials indicate healthy muscle fibers and that no significant differences exist between cac^{TS2} and wild-type, the most probable explanation is that synaptic transmission itself is compromised. Since there is a drastic change in the degree of facilitation for the cac^{TS2} at 16 h and 4 h, as well as comparing it with the wild-type controls, it would indicate that the handling of calcium during STF is substantially altered. The negative facilitation is expected for wild-type, given that the bathing saline (HL3) contains 1.0 mM $CaCl_2$ (Ball et al., 2003; Stewart et al., 1994). The degree of positive facilitation, as observed for the cac^{TS2} strain at this temperature and exposure, is more like what is observed when the extracellular calcium concentration is reduced to 0.5 mM (Stewart et al., 1994). Thus, the results suggest less calcium entering the presynaptic nerve terminals during the evoked depolarization of the terminals. The same trends and degree of significant change is present for the larvae exposed to 33°C for 72 h as for 16 h (Fig. $4A_3$ and $4B_3$). In addition to the alterations between the cac^{TS2} and the wild-type for the 16-h and 72-h exposure, there is a significant difference over time for the cac^{TS2} groups from 4 h to the 16 h ($P < 0.05$, Student's t-test; Fig. $4A_1$ and $4A_2$) and for 4–72 h ($P < 0.05$, Student's t-test; Fig. $4A_1$ and $4A_3$). The FI measures also mimic the significant changes between the cac^{TS2} groupings ($P < 0.05$, Student's t-test, Fig. $4B_1$–$4B_3$). However, there are no substantial changes among the indices for the 16-h and 72-h exposures. What is of particular interest is that the cac^{TS2} at 25°C shows a significant reduction in the Ib, Is, and the Ib+Is EPSP amplitudes as compared to wild-type ($P < 0.05$, Student's t-test; Fig. 5A), but with a 4-h pulse at 33°C the differences between wild-type and cac^{TS2} are negligible except for the Ib EPSP amplitudes. Comparing the cac^{TS2} at 25°C and 33°C, the responses are very similar. However, the differences in facilitation at 25°C mimic the responses observed for 72-h pulse at 33°C (Fig. 5B). In both, there is a significant difference between cac^{TS2} and CS ($P < 0.05$, Student's t-test; Fig. 5B).

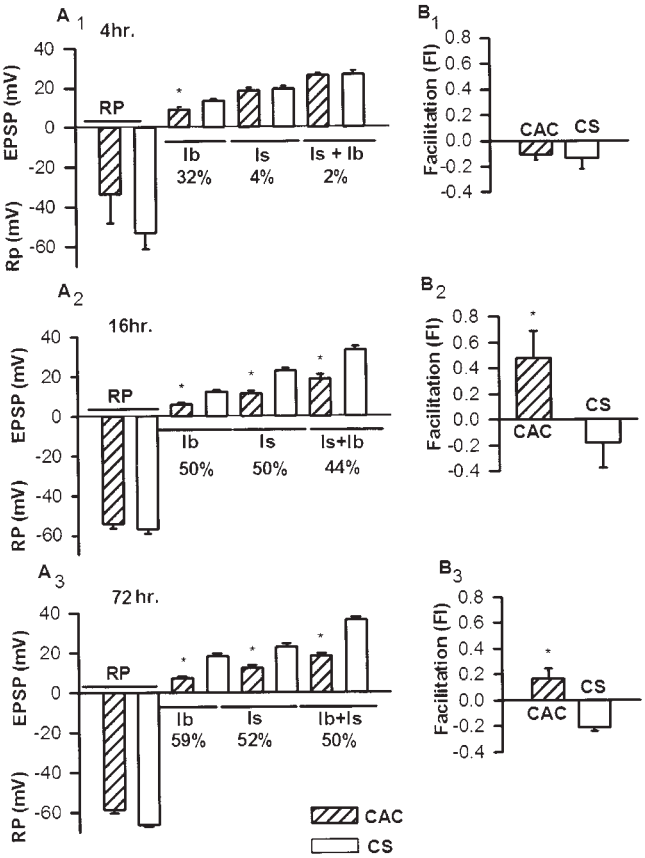

Fig. 4. Comparative electrophysiological analyses of cac^{TS2} and CS at a restrictive temperature. The cac^{TS2} and CS larvae were exposed to one of three experimental parameters: (**A_1**, **B_1**) Eggs were laid and maintained at 25°C until early 3rd instar and were then placed into restrictive 33°C for 4 h; (**A_2**, **B_2**) Larvae were removed from 25°C as 2nd instars and were placed into 33°C for 16 h, and examined as 3rd instars; (**A_3**, **B_3**) First instars were removed from 25°C and placed into 33°C for 72 h, and were examined as 3rd instars. For both controls (wild-type, CS) and cac^{TS2}, a minimum of 15 larvae were used. In each paradigm, the resting potential of m6, EPSP responses from Ib, Is, and Ib+Is were noted, as well as the FI (B_1, B_2, B_3) from 4 pulse trains at 20 Hz for cac^{TS2} and CS. The percentage differences in EPSP amplitude for Ib, Is, and Ib + Is responses are noted for cac^{TS2} and CS. Prolonged exposure to the restrictive temperature resulted in decreases in EPSP amplitude and alterations in the FI, which can be observed most prominently in cac^{TS2} ($P < 0.05$, Student's t-test). There are significant differences between the cac^{TS2} and wild-type for 16-h and 72-h exposures. In the 4-h exposure, the only significant difference was for the amplitudes of the EPSPs for the Ib nerve terminal. Asterisks indicate a significant difference between cac^{TS2} and wild-type ($P < 0.05$, Student's t-test). For the 4-h condition, $n = 10$ for cac^{TS2} and $n = 7$ for CS; for the 16-h condition, $n = 17$ for cac^{TS2}, $n = 16$ for CS; for the 72-h condition, $n = 15$ for cac^{TS2} and $n = 16$ for CS.

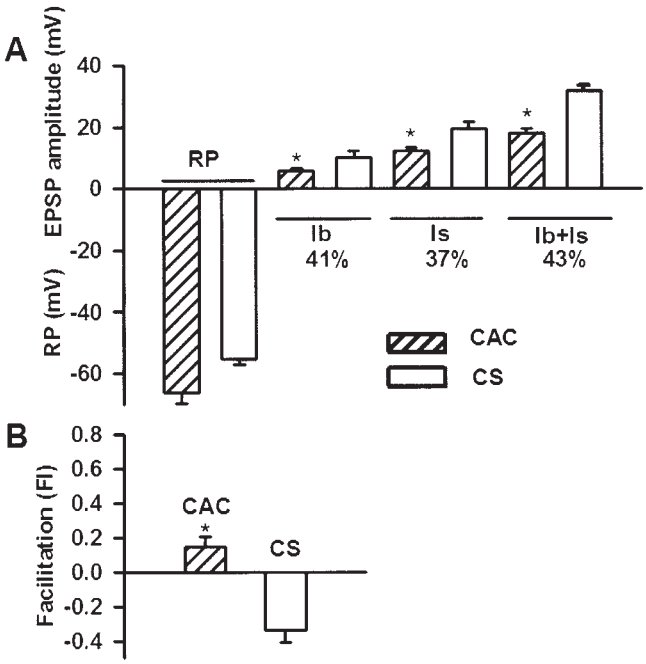

Fig. 5. Comparative electrophysiological analyses of cac^{TS2} and CS at room temperature. The cac^{TS2} and CS larvae raised at room temperature (25°C) were subjected to electrophysiological analyses. The resting potential of m6 and EPSP responses from Ib, Is, and Ib+Is are noted (**A**), as well as the FI (**B**), from a 4 pulse train at 20 Hz. The percentage differences in EPSP amplitude for Ib, Is, and Ib+Is responses are listed for cac^{TS2} and CS (A). Exposure to room temperature resulted in the cac^{TS2} to have a lower resting membrane potential and reduced amplitudes in the EPSPs for Ib, Is, and the combined Ib+Is response ($P < 0.05$, Student's *t*-test). The FI is also significantly different ($P < 0.05$, Student's *t*-test).

This may indicate either that effects of reduced cac^{TS2} activity on physiology are present even at 25°C and maximal by 16 h and that there may be a mechanism to partly compensate for reduced synaptic transmission that is active by 72 h. One potential compensatory mechanism may involve obvious structural changes, such as the length of the nerve terminals and the number of varicosity along the length of the nerve terminals.

Pronounced reduction in motor nerve terminal length and number of varicosities for the cac^{TS2} strain

Since the physiological differences are pronounced during the development of the cac^{TS2} mutant, one might expect a parallel alteration in the overall innervation pattern of the motor nerve terminals on m6. We therefore measured common indices of motor nerve terminals in larval NMJ preparations, which are the total length of the Ib and Is terminals and the number of varicosities on the terminals (Li et al., 2002; Lnenicka and Keshishian, 2000; Schuster et al., 1996a,b; Stewart et al., 1996). The terminal type is based on morphological measures as typically revealed visualized with fluorescently tagged anti-HRP antibody (Atwood et al., 1993; Johansen et al., 1989). Representative images for NMJs of a cac^{TS2} and a wild-type 3rd instar that were exposed for 72 h at 33°C are shown in Figure 6A and 6B, respectively. Note the reduced amount of terminal structure and fewer varicosity numbers on m6 for the cac^{TS2} as compared with that for the wild-type larva.

Measures in the total terminal length of the discrete Ib and Is terminals as well as the combined total length revealed a significant reduction in the cac^{TS2} strain for both short- (16 h, Fig. $7A_1$) and long-term (72 h, Fig. $7A_2$) exposure to 33°C as compared with that in the wild-type controls ($P < 0.05$, Student's *t*-test). The only difference between the 16-h group and the 72-h group is for the Ib distributions between the cac^{TS2} strains. There are no differences in measures within the wild-type control groups between the different exposure periods. The total number of varicosities paralleled the reductions observed in the terminal length for the cac^{TS2} with a slightly greater reduction for the longer 72-h exposure time ($P < 0.5$ Student's *t*-test; Fig. 7 B_1 and $7B_2$). In examining differences in the extent of innervation for the entire muscle, the dorsal surface area of m6 was used as an index. The dorsal surface area (length×width) was measured. The ratio in the total terminal length per muscle surface area is substantially less for the cac^{TS2} for both exposure periods, as compared with those for the wild-type ($P < 0.5$, Student's *t*-test; Fig. $7C_1$ and $7C_2$) and the ratio for the number of varicosities per muscle surface area is also reduced for the cac^{TS2} strain ($P < 0.5$, Student's *t*-test, Fig. $7D_1$ and $7D_2$).

Examining if the differences between the cac^{TS2} and wild-type strains at 33°C are also present at a lower temperature, larvae raised at 25°C were compared. The Ib nerve terminals are significantly longer for the cac^{TS2} as compared with that for the controls ($P < 0.5$, Student's *t*-test; Fig. 8A). No other differences in lengths among the nerve terminals for cac^{TS2} and to controls were noted. The overall length for the combined measures of Ib and Is did not reveal any difference. The total number of varicosities for the Ib and Is terminals is significantly lower for the cac^{TS2} larvae as compared with that for the controls ($P < 0.5$, Student's *t*-test; Fig. 8B). The ratio of the combined Is and Ib nerve terminal length to the dorsal surface area of m6 revealed a significantly larger value for the cac^{TS2} larvae as compared with that for the controls ($P < 0.5$, Student's *t*-test; Fig. 8C). This is indicative since the cac^{TS2} larvae have a tendency to be slightly retarded in total body length, which is manifested by short segmental muscles. However, no significant difference is present in the ratio of the number of varicosities to the dorsal surface area of m6 (Fig. 8D).

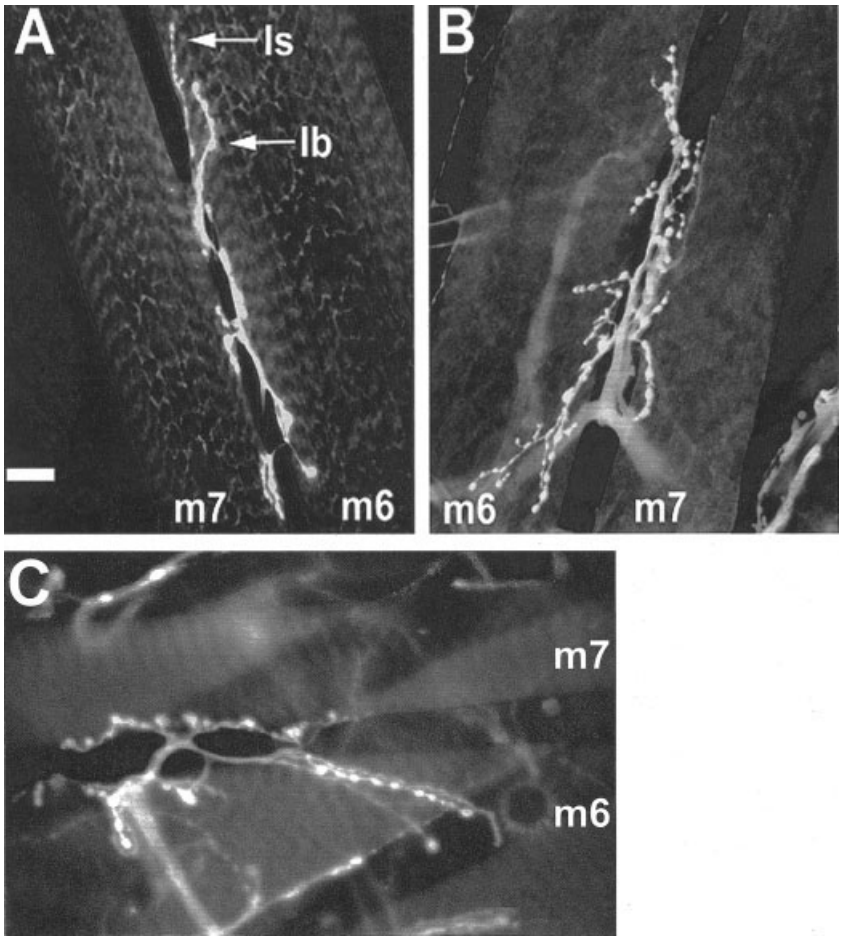

Fig. 6. The morphology of nerve terminals on m6, segment 3, was observed on 3rd instar cac^{TS2} and CS larvae, using light micrographs from confocal microscope images. Representative cac^{TS2} nerve terminals can be seen for larvae raised at (**A**) 33°C, (**B**) 30°C, and (**C**) 25°C. In each figure, the nerve terminals of Ib and Is are preferentially stained using an HRP antibody tagged with fluorescein. Scale bar A, B, and C = 20 μm.

The cac^{TS2} mutation results in an overall presynaptic reduction of synaptic transmission

By measuring both the fEPSPs and the spontaneous or miniature field excitatory postsynaptic potentials (fmEPSPs), it is possible to obtain a relative measure of mean quantal content (m). In addition, the shape and duration of the fEPSPs reveal information about evoked release from the presynaptic terminal.

The placement of the focal macropatch electrode directly over the distal-most varicosity along the Is terminals is a bit tenuous, but attempts were made to check that the most distal varicosities were recorded by moving the electrode lumen even more distally along the direction of the visualized terminal to ensure that signals could not be recorded. In fact, when placing the lumen of the electrode off the visualized varicosity, the signals (i.e., fEPSPs) reversed and were greatly attenuated in size. So, we are fairly confident that the most distal varicosity along a Is terminal was recorded for the fEPSP measures. While measuring the evoked field potentials occasionally, a spontaneous release could be detected. In some preparations, their occurrence was sparse (i.e., 3–5 over 2,000 s). An observation window of 500 ms after each stimulus to evoke a response was recorded digitally for analysis. The stimulation frequency used to evoke fEPSPs was 0.5 Hz so as not to facilitate or to cause pronounced muscle contractions that could displace the seal of the focal recording electrode.

A representative recording of a fEPSP and fmEPSPs obtained by a focal patch electrode is shown in Figure 9A. The area in the deflection of the signals from baseline is used as a measure of the fEPSP and fmEPSPs. The distribution in the area of the evoked fEPSP for 1,000 trials is depicted in Figure 9B. The area measure is also used for determining the distribution of the single quantal events (fmEPSPs). The mean quantal content, as determined by the ratio of the mean area calculated for fEPSP and fmEPSPs, is shown in Table I. Recordings were obtained from five

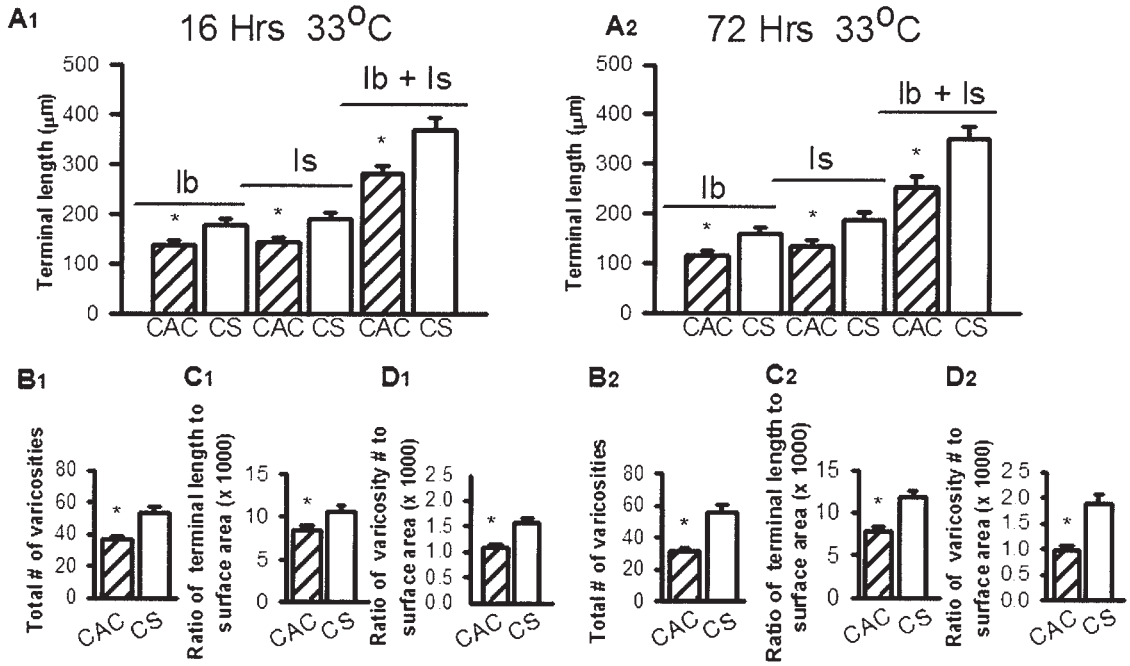

Fig. 7. Comparative anatomical analyses of the nerve terminal for cac^{TS2} and CS at a restrictive temperature. Morphometric analyses of m6 motor nerve terminals were made of cac^{TS2} and CS larvae from one of two exposure paradigms: (1) larvae were exposed to 33°C from 2nd to 3rd instar (16 h) (A_1, B_1, C_1, D_1); (2) larvae were exposed to 33°C from 1st to 3rd instar (72 h) (A_2, B_2, C_2, D_2). (**A_1**, **A_2**) The length of the motor nerve terminal is shorter for cac^{TS2} as compared with that of CS. (**B_1**, **B_2**) The number of varicosities present along the Ib and Is terminals is reduced for cac^{TS2} as compared with that of CS. (**C_1**, **C_2**) Cac^{TS2} has a smaller ratio of nerve terminal length per dorsal surface area of m6 than CS. (**D_1**, **D_2**) cac^{TS2} has fewer varicosities per dorsal muscle surface area compared to that of CS. All measurements were made on m6, within segment 4. Note that there is a slight trend for the effects to be more severe for the long-term exposure of the cac^{TS2} line. Larvae raised from 2nd to 3rd instar wild-type ($n = 12$) and cac^{TS2} ($n = 18$). Larvae were raised from 1st to 3rd instar wild-(n = 9 for wild-type and n = 12 for cac^{TS2}). Asterisks indicate a significant difference between cac^{TS2} and wild-type ($P < 0.05$, Student's t-test).

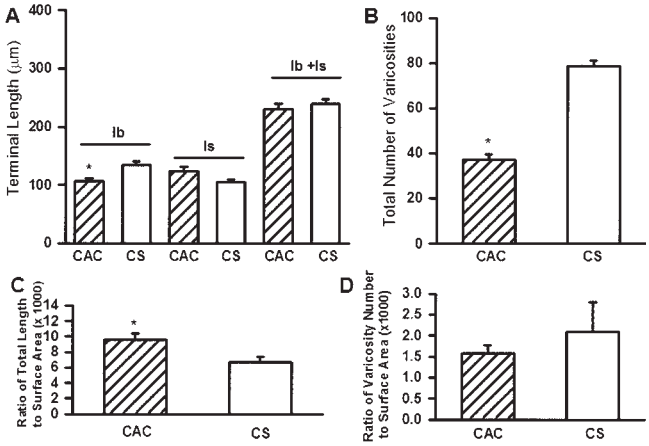

Fig. 8. Morphometric analysis of m6 motor nerve terminals were made of cac^{TS2} and CS larvae at room temperature. The cac^{TS2} and CS larvae were subjected to a room temperature condition, where eggs were laid and larvae maintained at 25°C, and examined at 3rd instar. The total length of the motor nerve Ib+Is terminals is similar for cac^{TS2} and CS, but the individual length of the Ib terminals was shown to be significantly shorter (**A**). (**B**) The total number of varicosities present along the Ib and Is terminals is significantly reduced for cac^{TS2} as compared with that for CS. (**C**) cac^{TS2} has a larger ratio of nerve terminal length per dorsal surface area of m6 as compared with CS. (**D**) The cac^{TS2} did not show any significant difference to CS in the numbers of varicosities per dorsal muscle surface area. Asterisks indicate a significant difference between cac^{TS2} and wild-type ($P < 0.05$, Student's t-test; CS, $n = 9$; cac^{TS2}, $n = 17$).

wild-type and five cac^{TS2} larvae. The mean value of the fEPSPs was taken only from stable recordings in which the variation was consistent throughout a period containing at least 400–1,000 trials. The fmEPSPs were also recorded within this time period to determine the quantal nature of the evoked signals. In two preparations of the cac^{TS2} group, a single recorded fmEPSP was considered to be a giant mini since the area was greater than two times the average fmEPSP area within the distribution. Mean quantal content (m) was calculated without the inclusion of the two large minis, as shown in Table I.

There is considerable variation in the m among the preparations for both the wild-type and cac^{TS2} groups. Since the range in values greatly overlaps, a trend is indistinguishable between the groups; however, only one preparation with the cac^{TS2} group (no. 5, Table I) is extremely low with all four of the remaining preparations depicting a substantial m value. This indicates that the varicosities of the mutant cac^{TS2} strain are able to produce a noteworthy amount of synaptic transmission. In fact, considering that the terminals of the cac^{TS2} larva exposed for 72 h have fewer varicosities than the wild-type controls and that the average m for all five preparations of the wild-type group

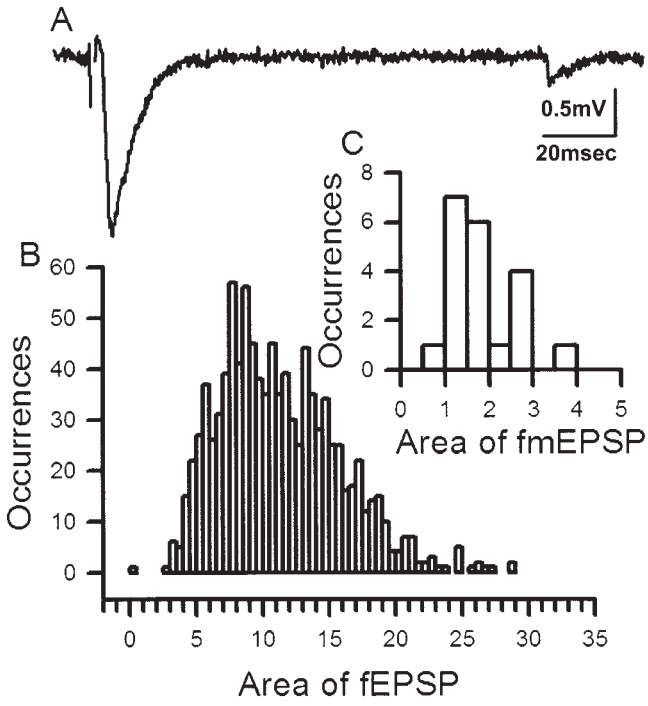

Fig. 9. Measures of the fEPSP and fmEPSPs with a focal patch recording. Evoked fEPSP and spontaneous quantal events (fmEPSPs) over distal Is varicosities were measured. A typical recording is depicted in **A**. A measure in the area of the fEPSPs from baseline was used for 500 trials and plotted as a bar chart for measures binned in 0.5 relative units versus the number of occurrences (**B**). The same approach was used for the fmEPSPs for the 20 responses recorded (**C**). The average fEPSP and fmEPSP was used to determine the mean quantal content for each preparation. The distribution of the fmEPSPs mostly revealed a right-hand shoulder, since a few large (i.e. giant) minis were observed. Analysis of mean quantal content was determined without the outlier large minis.

TABLE I. The mean quantal content for five preparations[a] of CS and cac^{TS2} larvae

	m[b]	
Preparation	CS	cac^{TS2}
1	4.76	5.18
2	4.06	16.12
3	5.20	8.02
4	4.66	8.04
5	12.19	1.08

[a]Five preparations for wild-type and five for cac^{TS2} larvae were used that had been exposed to 33°C from 1st instar to 3rd instar.
[b]Mean quantal content (m) was determined for each preparation and when large minis were observed, the analysis of m was made without including these outliers since they skewed the mean of the fmEPSPs to larger values. The m was determined by the ratio of the mean values of fEPSP and fmEPSP.

is 6.17, and 5.78 for cac^{TS2}, the average m per varicosity is higher for cac^{TS2} than for wild-type. However, it should be noted that this is a gross over-simplification since only distal Is varicosities were monitored and a large variation on m exists between the groups. The quantifying exercise in the average m per varicosity does imply that possibly there could be some compensatory modification in synaptic efficacy in the cac^{TS2} larva to enhance transmission per varicosity.

To determine whether the input resistance of the postsynaptic muscle fiber membrane might contribute to the reduced amplitude in the EPSPs, the input resistance was measured. The values of input resistances for wild-type and cac^{TS2} larvae raised under the same conditions of 72 h were not significantly different (data not shown).

DISCUSSION

In this investigation, the larval cac^{TS2} strain of *Drosophila* was used, which exhibits delayed developmental characteristics at a restrictive temperature of 33°C. A factor that maybe responsible for the reduced body growth is the reduced presynaptic influx of Ca^{2+} and, consequently, a decrease in evoked neurotransmitter release. During a long-term, 72-h exposure at 33°C, larvae experienced both a delay in growth, and an overall smaller body length. When assessing the nerve terminal anatomy of cac^{TS2} larvae that were subjected to the long term paradigm, it was evident that there was an overall decrease in varicosity number and nerve terminal length, eliminating the possibility of nerve terminal branching and up-regulation of varicosities as a means for homeostatic regulation of synaptic efficacy. Similar, but less dramatic results could be seen in a 16-h exposure study. A similar trend of decreasing function over long-term periods could also be observed in behavioral and electrophysiological assays, where longer periods of calcium channel impairment always led to a more dramatic decrease in function. On average, the overall amount of neurotransmitter released from the cac^{TS2} presynaptic nerve terminal was less than that released from wild-type CS nerve terminals, as was determined by quantal measures of synaptic transmission.

From these results, we report a phenomenon whereby the NMJs of *Drosophila* larvae appear to lack the ability to undergo synaptic modifications to compensate this reduction in synaptic transmission. Since the cac^{TS2} is a mutation in the genome, the effects of this mutation were also assessed at a nonrestrictive temperature of 25°C. Even though only slight developmental differences in overall body length were detectable between the CS and cac^{TS2} strains at 30°C, there were significant differences in behavior, synaptic responses of NMJs, and terminal morphology, at 25°C. If the synapses are developmentally crippled, they may simply be incapable of any form of modification such as synaptic homeostasis.

Consistency of neuromuscular transmission is dependent upon the organization and efficacy of the NMJ. *Drosophila melanogaster* provides a useful model for NMJ studies because its NMJ is relatively simple compared with that of vertebrates, and its well-characterized genome is easy to manipulate. These factors have contributed to an increased under-

standing of the progressive stages of synaptogenesis, which bear many similarities to vertebrate systems (Rose and Chiba, 2000). In *Drosophila*, NMJ systems have also been shown to exhibit characteristics of malleability, as well as alterations in synaptic strength that occur with activity changes (Kuromi and Kidokoro, 2003).

The importance of cytoplasmic Ca^{2+} to developmental processes has only recently been well-characterized in invertebrate models. In a recent study by Rieckoff et al. (2003), it was determined that when the influx of presynaptic calcium is decreased during development, a decrease in synaptic function also occurs, which is due to slowed development of the synapse. While it was known that, during development, path-finding in the tips of axons are guided by molecular cues, growth cones have been determined to have Ca^{2+} transients that control the rate of axon outgrowth in response to extracellular guidance factors (Ming et al., 2002; Spitzer, 2002). Calcium appears to be so important to the functioning of growth cones that, in certain invertebrate systems, growth cones showed evidence of compensating for decreases in Ca^{2+}, by restoring Ca^{2+} to baseline levels, after experimental elimination of Ca^{2+} influx into the nerve terminal (Rehder et al., 1991). The filipodia of growth cones have also been determined to rely on calcium transients to signal information needed for growth cone turning. Calcium has also been shown to induce the growth of filipodia along the neurite, and has been proven to be developmental-state dependent (Williams et al., 1995). In fact, developmental changes in the levels of Ca^{2+} and Ca^{2+} responsiveness have been shown to underlie shifts from plasticity to stability in neuronal growth and architecture in certain invertebrate systems (Williams et al., 1995). Likely, the ability to regulate calcium levels can have tremendous consequences upon the generation and degeneration of neuronal architecture (Mills and Kater, 1990). It has been recently shown that the Nervous Wreck (*nvw*) gene in *Drosophila* encodes an SH3 adaptor, which interacts with the *Drosophila* orthologue Wsp, and strongly regulates ARP 2/3-dependent actin polymerization, thought to be an underlying process of neurite extension. When temperature sensitive paralytic *nvk* mutants were made, an excessive growth of the NMJ was observed, with an increase in bouton number, and nerve terminal branching observed as well. These results implicate that *nvk* likely plays a role in contributing to the morphology of the neurite and the synapse, through the regulation of actin dynamics (Coyle et al., 2004), which require Ca^{2+} for normal activity (Henley and Poo, 2004).

There are informational signaling pathways that can be triggered by Ca^{2+}, which could possibly provide cues to microtubules to signal plus-end tracking proteins. Such proteins have been shown to serve as targets for guidance cues needed to steer axons (Kalil and Dent, 2004). Decreased levels of cytoplasmic Ca^{2+} have been implicated in growth cone retraction and collapse, which are two mechanisms by which a growth cone may respond to a repulsive target (Fritz and VanBerkum, 2000). It has been demonstrated that, when cytoplasmic Ca^{2+} is decreased, an associated decrease in axonal membrane dynamics occurs, which leads to a decrease in growth cone activity, and consequently, a reduction in synaptic connectivity results (Henley and Poo, 2004). Blocking free intracellular Ca^{2+} has also been shown to stall the formation of growth cones from a differentiated axonal segment (Spira et al., 2001). It has also long since been determined that the establishment of "axonal trajectories" is ultimately determined by the integration of intracellular signaling pathways. The regulatory protein calmodulin is a major mediator of calcium-induced changes in cellular activity; however, little is known about its role in developmental and behavioral processes. In *Drosophila*, it has been shown that when a targeted disruption of Ca^{2+}/calmodulin (CAM) signaling in growth cones occurs, it leads to a "stalling" of axon extension and errors in axonal guidance (Van Berkum, 1995). When the function of CAM is selectively disrupted within a specific subset of growth cones in transgenic embryos (where a specific enhancer element drives the expression of the kinesin motor domain fused to a CAM antagonist peptide, which blocks CAM binding to target proteins), these embryos exhibit "dosage-dependent stalls" in axon extension, and axonal guidance errors, which include defects in fasciculation and abnormal crossings of the midline (Van Berkum, 1995). These results implicate an in vivo function for CAM signaling growth cone decisions, including when to defasciculate and whether to cross the midline. When a loss in either the calmodulin or Son of Sevenless signaling pathway occurred, a loss of axonal projections across the midline toward a postsynaptic target was noted, suggesting that these pathways function to interpret midline repulsive cues that prevent the axon from crossing the midline and ultimately reaching its target (Fritz and Van Berkum, 2000). Two other CAM target proteins, cAMP adenylyl cyclase and phosphodiesterase, regulate cAMP cellular concentrations and have the ability to alter a neuronal response to Netrin 1, and other guidance cues like Netrin 1. Activation of the Sos pathway can affect dynamics of the cytoskeleton by activating various GTPases known to regulate growth cone behavior and also the guidance of axons. Moreover, the cytoplasmic tail of the Robo protein, known to be essential for signaling, has a tyrosine residue that could recruit Sos via Drk or dreadlocks (dock), an adapter protein that affects the guidance of axons (Van Berkum, 1995).

It remains to be determined if there is an interrelationship of the cellular cascades (mentioned above) and Ca^{2+} within the motor nerve terminals examined in this study. However these cascades used in other cell types may provide a likely explanation for the lack of synaptic homeostasis and compensations observed during development at the cac^{TS2} NMJ. This lack in synaptic development may account for decreases in body growth, synaptic development, electrophysiological synaptic responses, and ultimately, altered behavioral responses. The reduction of neuromuscular activity (likely due to an incomplete or dramatically reduced development of the synapse) has also been shown to drastically slow the sprouting of axons within the muscles of similar vertebrate systems. This suggests that calcium influx into the presynaptic nerve terminal is critical for the sprouting of new axons (Tam and Gordon, 2003).

Neurotransmitters have also been shown to regulate neuronal development in *Drosophila,* and the decreased transmitter release evident in cac^{TS2} may also impair development. In studies concerning the regulation of synaptogenesis, it was found that when acetylcholine was depleted, there was an associated increase in the branching of retinal axons (Dearborn and Kunes, 2004). GABA has also been found to regulate synaptogenesis in GABA-ergic systems, where it was found to modulate the activity of the "developmental switch," which regulates excitatory and inhibitatory synaptic responses (Kuppers et al., 2003). The synaptic release of glutamate has also been shown to regulate the location of postsynaptic receptor fields of the NMJ in *Drosophila* (Featherstone et. al., 2000).

There are a number of documented cases where, under normal conditions, synaptic plasticity and NMJ remodeling are able to take place in *Drosophila*. In many cases, postsynaptic cells in *Drosophila* can "signal back" to the presynaptic neuron to regulate synaptic structure and function (Davis et al., 1998). Essentially, the *Drosophila* NMJ serves as an example of how retrograde signaling may account for the regulation of synaptic homeostasis. In the *Drosophila* embryo, motor neurons also appear to compensate for the growth of their innervated muscles, by making extra synaptic contacts (i.e., varicosities) and by increasing sites of transmitter release at each synapse. These events have been shown to rely on bone morphogenetic protein (BMP) signaling through the Wishful thinking (Wit) receptor located on the plasma membrane of the presynaptic terminal (Keshishian et al., 1994; Keshishian, 2002). When Wit receptor knockouts were made, they exhibited smaller NMJs with dramatically decreased function, and altered synaptic structure (Marques et. al., 2002), implicating Wit as an important component of synaptic development. Since Wit function is required in the presynaptic cell, it is speculated that Wit could transduce a retrograde signal from the muscle to the nerve cell that provides a means of coordinating synapse growth with muscle growth (Brody and Odenwald, 2002). Until 2003, the ligand associated with the Wit receptor was unknown, but McCabe et al. (2003, 2004) confirmed through knockout and in-vitro studies that the Glass Bottom Boat (*Gbb*) gene, which encodes a BMP homologue, was found to be associated with Wit. When *Gbb* was knocked out, reductions in NMJ growth, synaptic development, and function were evident, a phenotype identical to that of the Wit knockout. From this, McCabe et al. postulated that *Gbb* must act as the ligand for Wit, and also likely plays a role in the regulation of NMJ development. In a separate study carried out by McCabe et al. (2004), it was shown that when the postsynaptic activity of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) was reduced, a corresponding increase in neurotransmitter exocytosis from the presynaptic neuron occurred. When CaMKII levels were increased in the postsynaptic muscle cell, the level of synaptic transmission, as well as nerve terminal growth, was found to be reduced. McCabe et al. postulated that, based on these results, CaMKII may act as a type of calcium sensor, by monitoring levels of synaptic transmission.

In *Drosophila* mutants where the adhesion molecule Fasciclin II is reduced, it was determined that, similar to cac^{TS2} larvae, the length of the nerve terminals is decreased, and the number of varicosities is reduced (Schuster et al., 1996). However, in contrast to cac^{TS2}, EPSP were found to be comparable with that of wild-type flies, indicating that any initial decreases in synaptic transmission had been compensated for over time (Schuster et al., 1996). It is thought that these compensations occurred by an increase in synaptic complexity, where an increase in transmitter output sites occurred per each varicosity. Similar structural complexitiy occurs in other invertebrate synapses at the NMJ (Atwood and Cooper, 1995; Cooper et al., 1996b).

Similar to the cac^{TS2} phenotype, there are also well-documented studies where larvae are not able to recover from mutations that decrease synaptic transmission. When *Drosophila* mutants were made of the SNARE docking proteins syntaxin and synaptobrevin, both types of mutants were found to experience reduced EPSPs that only decreased over time, indicative of an accumulation of vesicles, and an absence of calcium-dependent vesicular fusion events at the plasma membrane (Rieckoff et al., 2003). Similar to the findings of cac^{TS2} larvae, in this study, it was also noted that in mutants of the *DMCA-1* gene (which encodes N-type calcium channels), there was a decrease in nerve terminal growth, as well as a combined reduction in varicosities on m6 and m7. In contrast to the Rieckoff et al. study, our study of cac^{TS2} refined the physiological measures involved in distin-

guishing the evoked EPSPs and quantal responses from individual varicosities.

Taken together, our results indicate that the larval cac^{TS2} mutants cannot compensate for reduced presynaptic calcium influx during long-term or acute exposure periods, because of impaired synaptic development. These findings may well have clinically implications since the ingestion of calcium channel blockers (such as *N*-salicyloyltryptamine to treat epilepsy) by pregnant women may affect the development of functional synapses in the human fetus. The cac^{TS2} phenotype also resembles that of Eaton–Lambert Syndrome in humans, where antibodies are mysteriously produced against voltage-gated calcium channels, resulting in their inactivity. This inactivity causes altered locomotion, and fatigued leg and hip muscles. The calcium channel antibodies are produced in response to an as-yet unexplained association with small-lung carcinomas. However, in contrast to the anatomical results seen with cac^{TS2} larvae, an elongation of the postsynaptic membrane is observed in humans with Eaton–Lambert Syndrome, as well as an upregulation of acetylcholine receptors, evidence of both anatomical and physiological compensations (Takamori, 1996).

Since this study on the cac^{TS2} strain has demonstrated that a decrease in presynaptic Ca^{2+} influx substantially decreases both neurotransmission and synaptic development, it is evident that this decreased influx causes a decrease in Ca^{2+}-dependent vesicular fusion events, and consequently, an overall decrease in the release of neurotransmitter. Although not entirely characterized, it is thought that N-type Ca^{2+} channels contribute to synaptic development and growth through signaling pathways that involve Ca^{2+} influx as a modulator of synaptic growth. It is believed that this pathway is independent of transmitter exocytosis (Rieckof et al., 2003).

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