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Ca_v2-Type Calcium Channels Encoded by *cac* Regulate AP-Independent Neurotransmitter Release at Cholinergic Synapses in Adult *Drosophila* Brain

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Gu H, Jiang SA, Campusano JM, Iniguez J, Su H, Hoang AA, Lavian M, Sun X, and O'Dowd DK. Ca_v2-type calcium channels encoded by *cac* regulate AP-independent neurotransmitter release at cholinergic synapses in adult *Drosophila* brain. *J Neurophysiol* 101: 42–53, 2009. First published November 12, 2008; doi:10.1152/jn.91103.2008. Voltage-gated calcium channels containing $\alpha1$ subunits encoded by Ca_v2 family genes are critical in regulating release of neurotransmitter at chemical synapses. In *Drosophila*, *cac* is the only Ca_v2-type gene. Cacophony (CAC) channels are localized in motor neuron terminals where they have been shown to mediate evoked, but not AP-independent, release of glutamate at the larval neuromuscular junction (NMJ). Cultured embryonic neurons also express CAC channels, but there is no information about the properties of CAC-mediated currents in adult brain nor how these channels regulate transmission in central neural circuits where fast excitatory synaptic transmission is predominantly cholinergic. Here we report that wild-type neurons cultured from late stage pupal brains and antennal lobe projection neurons (PNs) examined in adult brains, express calcium currents with two components: a slow-inactivating current sensitive to the spider toxin Plectreurys toxin II (PLTXII) and a fast-inactivating PLTXII-resistant component. CAC channels are the major contributors to the slow-inactivating PLTXII-sensitive current based on selective reduction of this component in hypomorphic *cac* mutants (*NT27* and *TS3*). Another characteristic of *cac* mutant neurons both in culture and in whole brain recordings is a reduced cholinergic miniature excitatory postsynaptic current frequency that is mimicked in wild-type neurons by acute application of PLTXII. These data demonstrate that *cac* encoded Ca_v2-type calcium channels regulate action potential (AP)-independent release of neurotransmitter at excitatory cholinergic synapses in the adult brain, a function not predicted from studies at the larval NMJ.

INTRODUCTION

Voltage-gated calcium channels located in the plasma membrane of nerve terminals are essential for neuronal communication. They mediate depolarization-induced calcium influx that drives release of neurotransmitter. Presynaptic calcium channels are composed of several different subunits. Among them, the $\alpha1$ subunit forms the ion conducting pore that defines many of the functional properties characteristic of distinct channel subtypes (Catterall 2000; Catterall et al. 2005). In vertebrates, the $\alpha1$ subunit genes are grouped into three families, Ca_v1, Ca_v2, and Ca_v3. Within the Ca_v2 family there are three genes (*$Ca_v2.1$, 2.2,* and *2.3*) that encode distinct presynaptic calcium channels (Ertel et al. 2000). Multiple genes, evidence of functional redundancy, and compensatory regulation in nulls (Ino et al. 2001; Jun et al. 1999; Urbano et al. 2003; Wilson et al. 2000) makes it a challenging task to sort out the functional role of different presynaptic calcium channel subtypes in regulating synaptic activity in central neural circuits in mammals.

In *Drosophila*, there is only one $\alpha1$ subunit gene in each of the Ca_v1, 2, and 3 families (King 2007; Littleton and Ganetzky 2000). The *cac* gene (also known as *Dmca1A*) encodes the Ca_v2-type $\alpha1$ subunit and null alleles cause embryonic lethality demonstrating its essential function (Smith et al. 1996, 1998). Although cacophony (CAC)-mediated currents have not been directly examined in motor neuron terminals at the neuromuscular junction (NMJ), analysis of synaptic currents in *cac* mutants indicate that CAC calcium channels regulate evoked release of neurotransmitter at these peripheral glutamatergic synapses (Kawasaki et al. 2000, 2002, 2004; Rieckhof et al. 2003; Wu et al. 2005). The absence of changes in miniature excitatory junction potential (mEJP) frequencies in *cac* mutants (Macleod et al. 2006; Rieckhof et al. 2003) further suggests that action potential (AP)-independent release of neurotransmitter at the larval NMJ does not depend on activity of CAC Ca_v2-type channels.

In addition to their important role at the NMJ, courtship and seizure phenotypes associated with some *cac* mutant alleles indicate CAC channels may also be involved in neurotransmission at central synapses in the adult fly (Chan et al. 2002; Rieckhof et al. 2003; Smith et al. 1998). Analysis of barium currents in embryonic neurons cultured from *cac* mutant animals provided the first insights into the properties of currents mediated by CAC channels (Peng and Wu 2007). However, there is no information regarding the functional properties of CAC channels in the adult brain nor how they regulate transmission at excitatory cholinergic synapses. This is essential for understanding the role these channels play in regulating activity in the adult CNS.

To address these questions, we examined calcium currents and excitatory synaptic currents in *Drosophila* neurons in wild-type and viable *cac* mutants, *NT27* and *TS3*. Our results demonstrate that *cac*-encoded Ca_v2-type channels mediate slow-inactivating Plectreurys toxin II (PLTXII)-sensitive calcium currents in pupal neurons in culture and in antennal lobe PNs in adult *Drosophila* brain. We also provide direct evident that PLTXII-sensitive CAC channels regulate miniature excitatory postsynaptic current (mEPSC) frequency at excitatory

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cholinergic synapses in these neurons, both in vitro and in situ. This indicates that CAC channels are important in regulating synaptic activity associated with AP-independent neurotransmitter release in central circuits in the adult brain.

METHODS

Fly strains and GFP labeling of neuronal subpopulations

The two homozygous hypomorphic mutant alleles of the *cac* gene used were cac^{NT27}(*NT27*) and cac^{TS3} (*TS3*) (Rieckhof et al. 2003). Wild-type (Wt) stock used was Canton-S, the background strain in which the CAC mutants were generated. Projection neurons (PNs) were identified as green fluorescent protein (GFP)-positive neurons in the antennal lobes of adult flies in Wt (+/+;GH146-Gal4/UAS-GFP) and *NT27* mutant (*NT27/NT27; GH146-Gal4/UAS-GFP*) backgrounds. GH146-GAL4 line was obtained from the Liqun Luo lab.

Climbing assay

Adult flies were collected on the day of eclosion and kept in a 12-h light-12-h dark incubator for 3 days. Single 3-day-old flies were transferred by aspiration to an empty test vial and kept in the vial 1–2 min prior to testing. Flies were tapped to the bottom of the test vial, and the distance climbed, measured from the bottom of vial, was recorded 10 s after the last tap (Agrawal et al. 2005). All climbing assays were performed at room temperature between 5 and 8.5 h after lights on.

Electrophysiological recordings in culture

Primary neuronal cultures were prepared as reported previously (Jiang et al. 2005; Sicaeros et al. 2007). All brains were obtained from animals 55–78 h after pupation and neurons from a single brain were plated on a glass coverslip coated with ConA-laminin. Cultures were maintained in a 22°C humidified 5% CO_2 incubator, and recordings were made from neurons in cultures between 2 and 7 days in vitro (DIV). Whole cell calcium currents and cholinergic mEPSCs were recorded with standard whole cell pipettes of 4–6 mΩ. Isolated calcium currents were recorded with pipette solution containing (in mM) 120 D-gluconic acid, 120 cesium hydroxide, 20 NaCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1.1 EGTA, 10 HEPES, and 4 ATP, pH 7.2, 285 mosM. External solution contained (in mM) 140 NaCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 5.4 KCl, and 5 HEPES, pH 7.2, 290 mosM. TTX was added to the external solution to block voltage-gated sodium currents, and synaptic currents were blocked by curare and picrotoxin. Cholinergic mEPSCs were recorded using the same internal solution with external solution that contained TTX/picrotoxin but no curare.

Drugs were applied in the external bathing solutions of specific experiments at the following concentrations: TTX (1 μM, Alomone Labs), D-tubocurarine (curare, 1 μM, Sigma), picrotoxin (10 μM, Sigma), verapamil (10 μM, Sigma), nifedipine (1 μM, Sigma), ω-conotoxin GVIA (2 μM, Sigma), $CoCl_2$ (2 mM), and PLTXII (20–100 nM, Alomone Labs). TEA (2.5 mM) and 4-aminopyridine (4-AP, 1 mM) were added in some experiments to block residual outward currents. Data were acquired with a List EPC7 amplifier, a Digidata 1320-D-A converter (Axon instruments), a Dell computer (Dimension 4100), and pClamp8 software (Axon instruments). Recordings were made at room temperature. Current records were filtered at 2 kHz and digitized at 10 kHz. All data shown were corrected for the 5-mV liquid junction potential generated in these solutions.

Whole cell voltage-clamp recordings from PNs in isolated whole brain

All brains were obtained from adult female flies between 1 and 3 days after eclosion. The entire brain, including optic lobes, was removed from the head, prepared for recordings as previously described, and mounted in the recording chamber with the anterior face of the brain up (Gu and O'Dowd 2007; Gu and O'Dowd 2006). Pipettes were targeted to GFP+ PNs in the dorsal neuron cluster in the antennal lobe.

Isolated calcium currents were recorded using a pipette solution containing (in mM) 102 D-gluconic acid, 102 CsOH, 0.085 $CaCl_2$, 1.7 MgCl, 17 NaCl, 0.94 EGTA, 8.5 HEPES, and 4 ATP. The osmolarity was adjusted to 235 mosM and pH to 7.2. The external solution contained (in mM) 101 NaCl, 1.8 CaC_{l2}, 0.8 $MgCl_2$, 5.4 KCl, 5 glucose, 1.25 $NaH2PO_4$, and 20.7 $NaHCO_3$, TTX (1 μM), D-tubocurarine (curare; 20 μM), and picrotoxin (PTX; 10 μM). The osmolarity was adjusted to 250 mosM and pH to 7.2. In some experiments, TEA (2.5 mM) and 4-AP (1 mM) were added to block residual outward currents. Cholinergic mEPSCs were recorded using the same internal solution as that used for calcium currents except that cesium gluconate was replaced by potassium gluconate, and there was no curare added to the external solution. Recordings were performed with oxygenated saline at room temperature. Data shown were corrected for the 5-mV liquid junction potential generated in these solutions.

Data were acquired with a Axonpatch 200B amplifier, a digidata 1322A D-A converter (Molecular Devices), a Dell computer (Dimension 8200), and pClamp9 software (Molecular Devices.).

Analysis of synaptic currents

mEPSCs were analyzed using Minianalysis software (Synaptosoft, Decatur, GA). Events were accepted for analysis only if they were asymmetrical with a rising phase faster than 3 ms. The threshold criterion for inclusion was 7 pA in the cultured neurons, where the currents were relatively large as previously reported (Su and O'Dowd 2003), and 3 pA in the adult brain neurons where the synaptic currents are typically much smaller (Gu and O'Dowd 2006).

Fluorescent immunocytochemistry

Cultures were fixed at 4 DIV and processed for staining as described previously (Oh et al. 2008). Bruchpilot staining was visualized using primary antibody Nc82 (mouse MAB, 1:1000, Developmental Studies Hybridoma Bank, DSHB) and a goat anti-mouse conjugated to Alexa Fluor 568 secondary antibody (1:2000; Invitrogen; Carlsbad, CA). Cultures were co-stained with fluorescein-conjugated anti-HRP antbodies (1:500; MP Biochemicals, LLC; Solon, OH) to visualize all neuron cell bodies and processes. All data collection and analyses were done blind with respect to genotype.

Electron microscopy

Cultures were processed as previously described (Oh et al. 2008). Briefly, cultures were fixed in 2% glutaraldehyde in 0.01 M PBS (Sigma), pH 7.0 at 4°C for 1 h, washed in PBS, and postfixed in 1% OsO_4 on ice for 1 h. Cells were contrasted with 1% uranyl acetate at RT for 1 h, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections (~60 nm) were cut on a Reichert Ultracut Ultramicrotome. Sections were stained again in 2% uranyl acetate for 2 min, followed by Reynold's lead citrate for 2 min. Sections were examined on a Philips CM10 transmission electron microscope and micrographs were taken with a Gatan Ultrascan US1000 digital camera. All data collection and analyses were done blind with respect to genotype.

Statistics

Comparisons between two genotypes or treatments were made with Student's *t*-test. All comparisons between more than two genotypes or treatments were made using an ANOVA and Bonferroni post hoc analysis for pair-wise comparisons.

RESULTS

Calcium currents in cultured pupal neurons have two kinetically distinct components

To examine the biophysical and pharmacological properties of calcium currents in brain neurons in detail, initial recordings were focused on neurons in cultures prepared from brains of Wt pupae in which isolated calcium currents could be recorded under good voltage-control. All recordings were made in external saline containing a physiological concentration of calcium (1.8 mM).

A series of depolarizing voltage steps from a holding potential of −80 mV elicited rapidly activating inward calcium currents in Wt neurons (Fig. 1*A*). All of the neurons had calcium currents with both a fast- and a slow-inactivating component but the relative contribution of the two components varied from cell to cell (Fig. 1*A, i–iii*). The amplitude of the slow-inactivating component, measured at 75–80 ms after onset of a step depolarization represented, on average, 57 ± 2% (means ± SE, $n = 30$) of the peak current amplitude. Because neurons varied in size, all currents were normalized to whole cell capacitance and are expressed in terms of density (pA/pF). The current density voltage relationships of the peak and slow-inactivating currents were similar: both activated at approximately −40 mV and the maximal current density occurred between –10 and 0 mV (Fig. 1*B*). However, the fast- and slow-inactivating components were differentially sensitive to steady-state inactivation. In these studies, the test pulse was set to –5 mV, a voltage that elicited maximal current amplitude determined from the current-voltage curves. The prepulse duration chosen (100 ms) reduced the magnitude of the fast-inactivating current at depolarized prepulse voltages without reducing the amplitude of the slow-inactivating current (Fig. 1*C*). The percentage of peak calcium current was plotted as a function of prepulse potential and fit with a Boltzmann curve (Fig. 1*D*, $V_{1/2} = -29$ mV). The fast-inactivating current represented 42% of the peak inward current. The amplitude of the slow-inactivating current, defined as the current unaltered by a 100-ms prepulse to 0 mV, represented on average 58 ± 2% ($n = 12$) of the peak inward current (Fig. 1*D*). These data suggest that there are at least two calcium channel subtypes that contribute to the total whole cell calcium currents in pupal brain neurons.

PLTXII-sensitive and -resistant calcium currents

To determine if the slow- and fast-inactivating currents could be distinguished pharmacologically, we utilized PLTXII, a spider neurotoxin previously shown to reduce calcium currents in cultured Kenyon cells (Jiang et al. 2005), embryonic

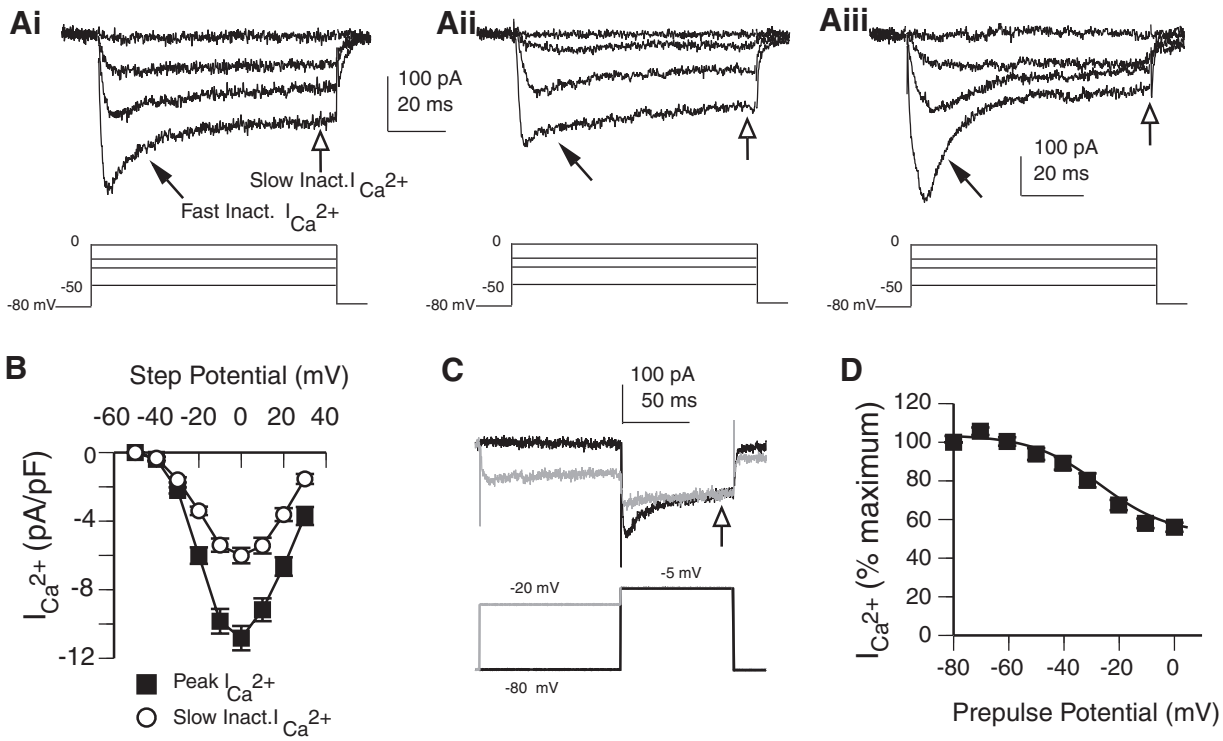

FIG. 1. Calcium currents in neurons cultured from brains of late stage wild-type pupae have fast- and slow-inactivating components. *Ai*: typical calcium currents recorded in wild-type neurons in 1.8 mM calcium have both fast-inactivating (black arrows) and slow-inactivating (white arrows) components. *Aii* and *Aiii*: the relative magnitude of the 2 components varied between cells. Current traces evoked by voltage steps indicated. *B*: mean current density-voltage curves generated for the peak and slow-inactivating current. The slow-inactivating component represents 57 ± 2% of the peak current. Each point represents mean current density (pA/pF), bars indicate SE, $n = 30$ wild-type neurons. *C*: comparison of currents elicited in a single cell by a test step to –5 mV when preceded by 100-ms prepulses to different potentials. Following a prepulse to –20 mV (gray trace), no transient current is evoked by the subsequent test step to –5 mV, consistent with inactivation of the transient current by the prepulse. No current is activated by a prepulse to –80 mV (black trace), and, as expected, the subsequent test step to –5 mV evokes both a fast- and slow-inactivating current. *D*: steady-state inactivation curve with peak calcium current, elicited by a test step to –5 mV at each prepulse potential between –80 and 0 mV, expressed as a percentage of peak calcium current without a prepulse. Data fit with a Boltzmann distribution ($v_{1/2} = -29$ mV). Each data point represents means ± SE; $n = 12$ neurons. Whole cell calcium currents in this and all other figures were recorded in 1.8 mM calcium, TTX to block sodium channels, and internal cesium to block potassium channels.

neurons (Leung et al. 1989), and at the neuromuscular junction (Branton et al. 1987). Because PLTXII is an irreversible calcium channel antagonist, calcium currents generated by depolarizing voltage steps were recorded from one to two neurons in each culture before PLTXII was bath applied and one to six additional neurons were examined in presence of the blocker.

In contrast to typical calcium currents in control solutions, currents recorded in the presence of 50 nM PLTXII generally had only a small slow-inactivating current with a fast-inactivating component remaining (Fig. 2, *A* and *B*). The slow-inactivating current density was reduced by 78–80% in 50 nM PLTXII ($P < 0.001$) and 100 nM PLTXII ($P < 0.001$) compared with control saline (Fig. 2*C*). These data indicate that PLTXII-sensitive channels underlie the majority of the slow-inactivating calcium current. The peak current density was also reduced by 66% in 50 nM PLTXII ($P < 0.001$) compared with control saline (Fig. 2*D*). This level of blockade is consistent with PLTXII blocking the slow inactivating component that accounts for 55% of the current measured at the peak. The current remaining in 50 nM PLTXII is defined as the PLTXII-resistant current (Fig. 2*B*) .

The inorganic calcium channel antagonist cobalt (2 mM) blocked all calcium currents ($P < 0.001$). This confirmed that both the PLTXII-sensitive and -resistant components were mediated by voltage-gated calcium channels (Fig. 2, *E* and *F*). However, neither the slow-inactivating nor peak currents were significantly reduced by three other calcium channel antagonists (verapamil, nifedipine, or ω-conotoxin GVIA) targeted to different calcium channel subtypes in vertebrates (Fig. 2, *E* and *F*).

These data demonstrate there are at least two different calcium channel subtypes in the pupal neurons: PLTXII-sensitive channels that underlie the majority of the slow-inactivating current and PLTXII-resistant channels that contribute predominantly to the fast-inactivating current.

cac mutants specifically reduce PLTXII-sensitive, slow-inactivating currents

Previous studies have shown that the *cac* gene is expressed in the *Drosophila* CNS (Smith et al. 1996). In addition, electrophysiology studies have shown that PLTXII is able to block evoked release of neurotransmitter (glutamate) at the neuromuscular junction (Branton et al. 1987), an effect that is mimicked by a mutation in the *cac* calcium channel gene (Kawasaki et al. 2000). Thus *cac* was a good candidate for a gene encoding the channels underlying the PLTXII-sensitive calcium current expressed in neurons from the *Drosophila* brain. Because *cac* nulls are late embryonic lethal mutants, and the cultures are prepared from late stage pupae, two homozy-

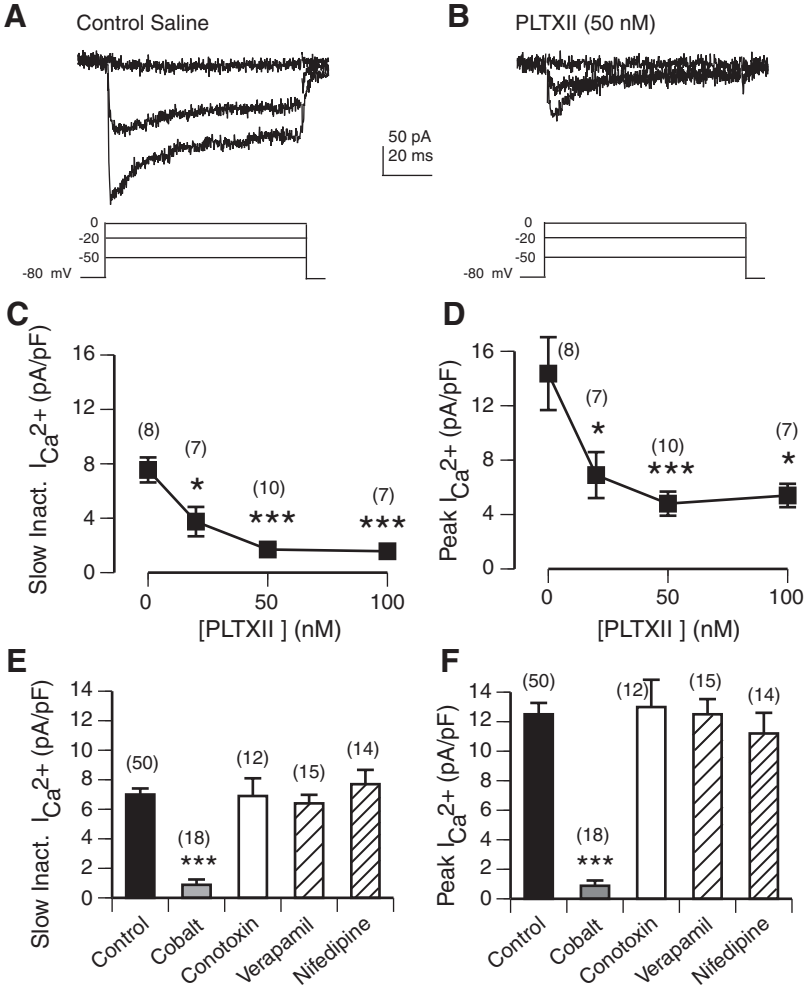

FIG. 2. Calcium currents in cultured wild-type pupal neurons have Plectreurys toxin II (PLTXII)-sensitive and -resistant components. *A*: typical calcium current family recorded from a wild-type neuron in control saline. *B*: calcium current family in a wild-type neuron in 50 nM PLTXII. *C*: incubation of cultures in PLTXII significantly reduced the slow-inactivating calcium currents. ANOVA, Bonferroni post hoc test, $*P < 0.05$, $***P < 0.001$. *D*: peak current was also significantly reduced by PLTXII (20–100 nM). Current remaining in saturating concentration of PLTXII (50–100 nM) is termed the PLTXII-resistant current. ANOVA, Bonferroni post hoc test, $*P < 0.05$, $***P < 0.001$. *E* and *F*: calcium currents were blocked by cobalt, confirming that both components are mediated by voltage-gated calcium channels. ANOVA, Bonferroni post hoc test, $***P < 0.001$. Neither slow-inactivating nor peak currents were significantly reduced by the other vertebrate calcium channel antagonists tested. Cobalt (2 mM) ω-conotoxin (2 μM), verapamil (10 μM), and nifedepine (1 μM). Each point/bar in figure represents means ± SE, from number of neurons indicated (*n*).

gous viable hypomorphic mutant alleles, cac^{NT27} (*NT27*) and cac^{TS3} (*TS3*) were used in this study (Rieckhof et al. 2003).

Although both *NT27* and *TS3* alleles were initially isolated as temperature-sensitive paralytics, there is evidence that the *NT27* mutation causes constitutive defects because the mutant flies exhibit locomotory deficits at room temperature when placed in *trans* to a null allele (Rieckhof et al. 2003). In our standard growth conditions, the *NT27* homozygous mutant flies appeared less active than Wt flies even at room temperature. Activity differences were quantified using a simple negative geotaxis assay. Individual flies were placed in vials and tapped to the bottom of the vial, and the distance climbed after 10 s was scored. The mean distance climbed was reduced by 14% in *TS3* ($P < 0.01$) and 51% in *NT27* ($P < 0.001$) mutants compared with Wt (Fig. 3*A*). Moreover, *NT27* was significantly reduced compared with TS3 ($P < 0.001$).

These data suggest that when homozygous, both *NT27* and *TS3* mutant alleles result in a defect in calcium channels that is manifest at room temperature. To test this hypothesis and determine if these encode neuronal calcium channels in the CNS, calcium currents were recorded from cultured neurons prepared from brains of *NT27* and *TS3* mutants. All recordings were done at room temperature (22–25°C), blind with respect to genotype, and each neuron with a stable baseline was included in analysis.

Similar to Wt neurons, the majority of neurons in cultures made from the two *cac* mutants express calcium currents (Fig. 3*B*). However, whole cell calcium currents in typical *TS3* and *NT27* mutant neurons had smaller slow-inactivating components when compared with Wt (Fig. 3*C*). Quantitative comparison demonstrated that the slow-inactivating current density was reduced by 47% in *TS3* ($P < 0.001$) and 75% in *NT27* ($P < 0.001$) compared with Wt neurons (Fig. 3*D,* dark bars). This decrease in density is not due to a shift in voltage dependence because the current density-voltage curves and the normalized conductance-voltage curves were similar in all three genotypes (Fig. 3*F*).

Although PLTXII caused a large reduction in the slow-inactivating current density in Wt neurons, it did not further reduce the density of these currents in the *NT27* mutant neurons (Fig. 3*D,* white bars). This is consistent with *cac* encoding the channels that underlie the PLTXII-sensitive current (Fig. 3*D*). PLTXII decreased the slow-inactivating current in *TS3* ($P < 0.05$) mutants to *NT27* levels (Fig. 3*D*), consistent with *TS3* being a less severe hypomorphic allele of the *cac* gene. These results also demonstrate that both mutant alleles, when homozygous, result in constitutive decrease in density of slow-inactivating calcium currents at room temperature.

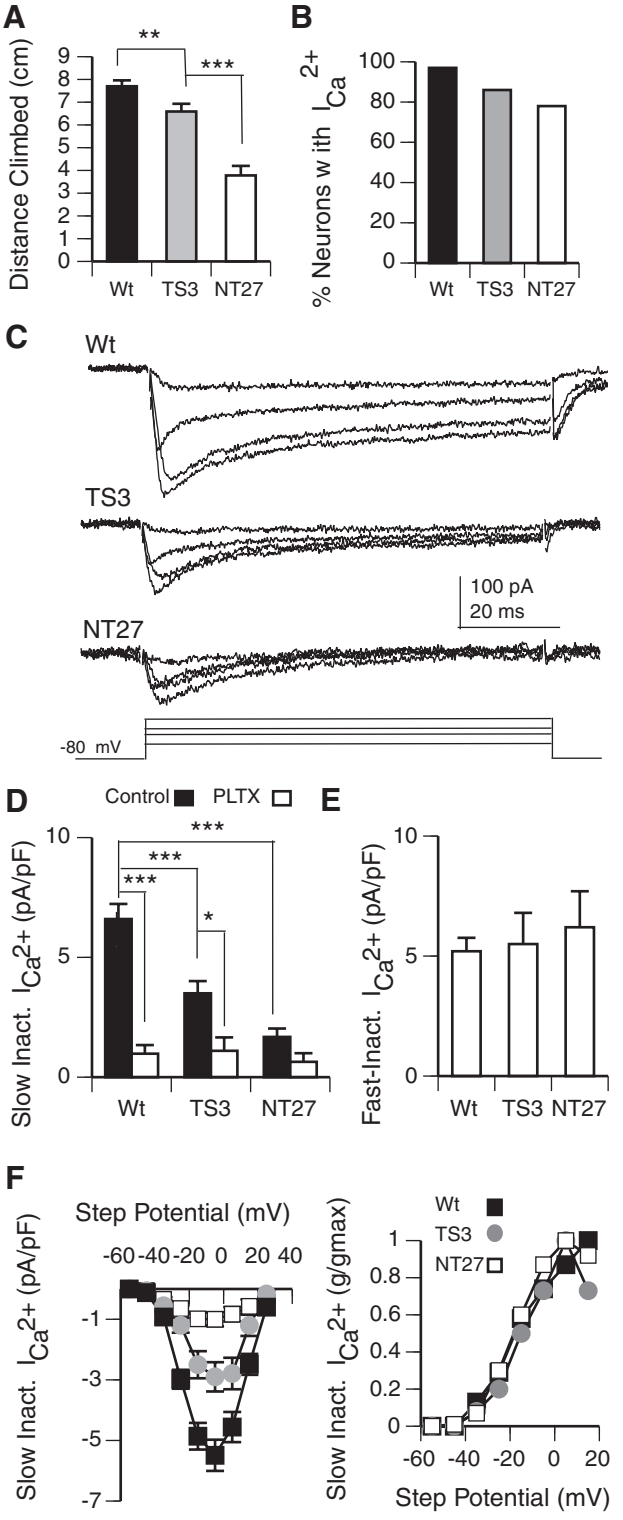

FIG. 3. Mutations in the *cac* gene specifically reduce slow-inactivating, PLTXII-sensitive calcium currents. *A*: flies homozygous for mutations in the *cac* gene, (*TS3, NT27*) exhibit significantly reduced activity compared with wild-type (Wt) at room temperature in a negative geotaxis assay. ANOVA, Bonferroni post hoc test, $^{**}P < 0.01$, $^{***}P < 0.001$. Bars represent distance climbed up the vial in 10 s determined from individual flies (means ± SE, Wt, $n = 67$; *TS3*, $n = 81$, *NT27*, $n = 40$). *B*: the percentage of neurons expressing calcium currents in *TS3* ($n = 23$) and *NT27* ($n = 46$) and Wt ($n = 38$) cultures. *C*: whole cell calcium currents elicited by voltage steps to –35, −15, 5, and 15 mV from a holding potential of –80 mV in a Wt, *TS3,* and *NT27* neuron. *D*: the magnitude of the slow-inactivating calcium current in control saline was significantly reduced in *TS3* ($n = 23$) and *NT27* ($n = 46$) compared with Wt ($n = 38$) (ANOVA, Bonferroni post hoc test, $^{***}P < 0.001$). PLTXII (50 nM), results in a significant reduction in the slow inactivating current in Wt ($n = 10$) and *TS3* ($n = 7$) but no additional reduction in *NT27* ($n = 14$; *t*-test, $^{*}P < 0.05$, $^{***}P < 0.001$). *E*: density of the fast-inactivating current, measured at peak of the transient current present in 50 nM PLTXII, is similar in Wt, *TS3*, or *NT27*. *F*: comparison of the $I_{density}$-*V* curves for the slow-inactivating current demonstrates a clear reduction in the magnitude in *NT27* ($n = 46$) and *TS3* ($n = 23$) neurons compared with Wt ($n = 38$) but no obvious shift in the voltage dependence. The same data plotted in normalized conductance-voltage curves (g/g_{max}) confirms the similarity in the voltage-dependence of activation of the slow-inactivating current in the Wt and *cac* mutants.

To evaluate the effect of the mutants on the fast-inactivating current, the peak of the transient current observed in the presence of 50 nM PLTXII was measured (Fig. 3*E*). Neither mutant reduced the density of the fast-inactivating PLTXII-resistant current compared with Wt (Fig. 3*E*). Taken together these data indicate that the majority of the slow-inactivating PLTXII-sensitive calcium current is mediated by *cac* encoded Ca_v2-type calcium channels. PLTXII-resistant fast-inactivating current is mediated by a different channel subtype/s that is likely to be encoded by another calcium channel gene/s.

cac channels regulate mEPSC frequency at cholinergic synapses in culture

Most cultured pupal neurons do not fire spontaneous APs, making it difficult to directly examine the role of CAC channels in evoked synaptic transmission in this system. However, they do form functional synaptic connections at which physiological concentrations of calcium support AP-independent release of neurotransmitter at excitatory synapses (Su and O'Dowd 2003). In addition, because the mEPSC frequency at cholinergic synapses in embryonic *Drosophila* neurons is highly dependent on calcium flux through voltage-gated channels (Lee and O'Dowd 1999), it seemed likely that CAC channels are involved in regulating spontaneous release at central excitatory synapses in adult brain. To test this hypothesis, we asked if there were alterations in the frequency of cholinergic mEPSCs at synapses formed between neurons in cultures prepared from the *cac* mutants. Recordings were done blind with respect to genotype.

All neurons in which a stable whole cell recording was established were scored for presence of cholinergic mEPSCs by recording for a minimum of 1 min in the presence of TTX to block sodium channels and picrotoxin to block GABAergic synaptic transmission. Previous studies have shown that the mEPSCs recorded under these conditions are blocked by curare and are not affected by glutamate receptor antagonists, identifying them as cholinergic (Su and O'Dowd 2003).

Synaptic currents (Fig. 4*A*) were recorded in a similar percentage of neurons in cultures from the three genotypes: wild -type (55%, $n = 20$ cultures), *NT27* (53%, $n = 10$ cultures), and *TS3* (62%, $n = 7$ cultures). However, there was a large and significant reduction in mean mEPSC frequency in both *TS3* ($P < 0.01$) and *NT27* ($P < 0.01$) cultures compared with Wt (Fig. 4*B*). This result is consistent with the hypothesis that AP-independent release of neurotransmitter at cholinergic synapses is directly regulated by the activity of CAC channels. However, the calcium currents in the mutants are constitutively reduced, and it was possible that the reduction in mEPSC frequency in *cac* mutant cultures could be due to developmental changes not directly related to channel function at the synapse. Therefore PLTXII was applied to Wt neurons. The frequency of the mEPSCs in Wt was reduced 86% by acute exposure to PLTXII ($P < 0.05$), confirming the role of calcium influx through CAC channels in regulation of AP-independent neurotransmitter release at these central synapses (Fig. 4*B*). PLTXII does not further reduce the frequency of mEPSCs in *NT27* or *TS3* (Fig. 4*B*). In addition, there is no significant difference in the mEPSC amplitude between mutant and Wt neurons (Fig. 4*C*). These data suggest that the reduction in frequency in the mutants is due to a decrease in CAC calcium

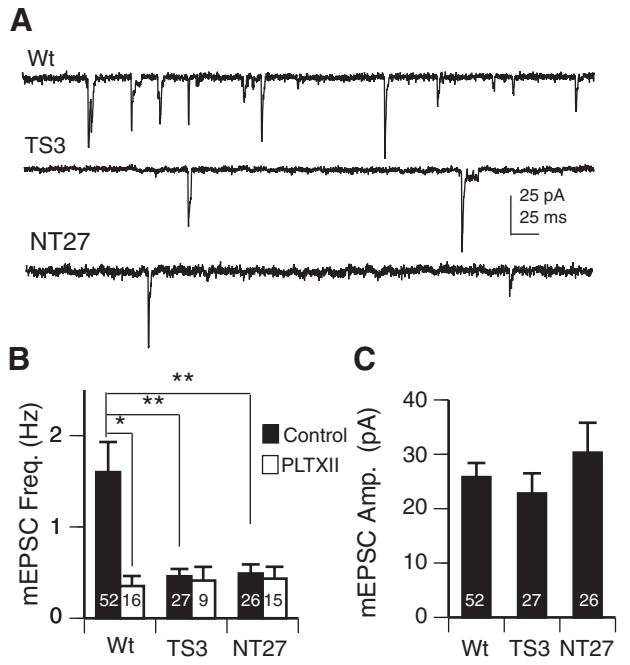

FIG. 4. Frequency of cholinergic miniature excitatory postsynaptic currents (mEPSCs) is reduced in *cac* mutant neurons and by acute application of PLTXII in wild-type neurons. *A*: spontaneously occurring cholinergic mEPSCs recorded in neurons from Wt, *NT27,* and *TS3* cultures. *B*: the mEPSC frequency, recorded in control saline (■) was significantly reduced in *NT27* and *TS3* when compared with Wt. ANOVA, Bonferroni post hoc test, (**$P <$ 0.01). In addition, PLTXII (□) significantly reduced the mEPSC frequency in Wt neurons (*$P < 0.05$, *t*-test). PLTXII does not further reduce the mEPSC frequency in either *TS3* or *NT27*. *C*: the mEPSC amplitude, recorded in control saline, is similar in Wt, *TS3*, and *NT27*. Each bar indicates the means ± SE from indicated number of neurons (*n*). mEPSCs recorded at a holding potential of −75 mV. Control saline included TTX to block sodium channels and picrotoxin to block GABA receptors.

channel number and/or function and not to changes in postsynaptic receptors or downstream signaling pathways.

The ability of acutely applied PLTXII to reduce the mEPSC frequency in Wt cultures to *NT27* levels confirms that CAC channel activity regulates AP-independent release of neurotransmitter at central cholinergic synapses. However, given that a previous study reported a reduction in terminal growth and varicosity density at the NMJ of *NT27* mutants (Rieckhof et al. 2003), it was possible that there were also changes in neurite outgrowth or morphology of central synapses in *NT27* mutant cultures. To evaluate this possibility, a set of cultures from Wt and *NT27* mutant brains were fixed at 4 DIV. All cultures were coded, and subsequent processing and data analysis was done blind with respect to genotype.

For analysis at the light level, cultures were double-stained with an anti-HRP antibody (Green) to visualize neuronal morphology and the Nc82 antibody (Red) against the presynaptic protein Bruchpilot. Neurons in both Wt and *NT27* cultures extended branching processes that form overlapping neuritic plexuses and showed a similar pattern of punctate staining for Nc82 in both genotypes (Fig. 5, *A* and *B*). Assessment of puncta density in fields selected on the basis of cell and neurite outgrowth revealed no significant difference in Wt and *NT27* mutant cultures (Fig. 5*E*). For electron microscopy (EM) analysis, ultrathin sections were scanned at low magnification to locate regions of high neurite density. At high magnification,

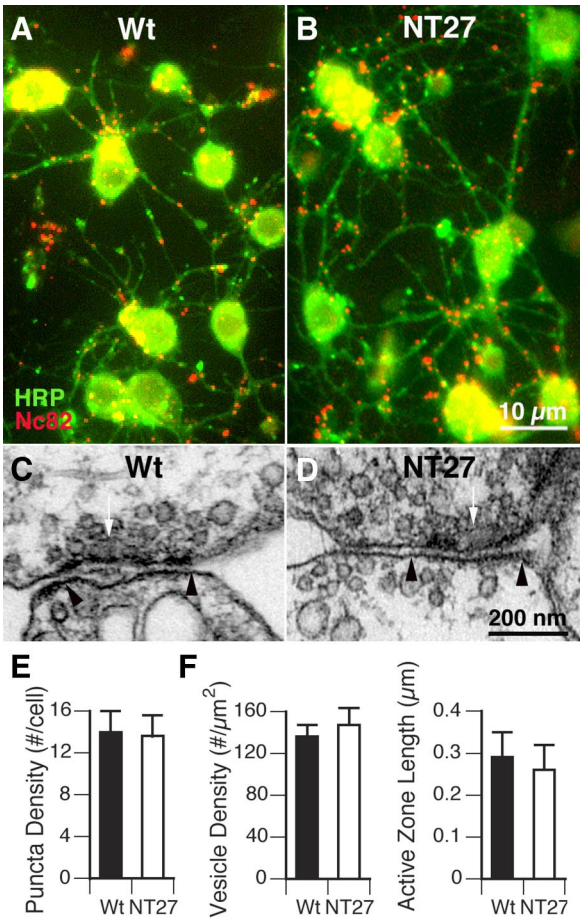

FIG. 5. Neurite elaboration, expression of Bruchpilot, and synapse ultrastructure were similar in *NT27* and wild-type cultures. *A* and *B*: Bruchpilot was expressed in a punctate pattern in Wt and *NT27* neurons. Cultures fixed at 4 days in vitro (DIV), double-labeled with fluorescein-conjugated anti-HRP antibody (green channel) to visualize neuronal somata and processes, and a primary antibody (Nc82) with an Alexa Fluor 546-conjugated secondary (red channel) to visualize Bruchpilot. *C* and *D*: examples of active zone regions characterized by presence of vesicle clusters, presynaptic T-bars/densities (white arrows), synaptic cleft, and region of postsynaptic density (bounded by black arrowheads) in Wt and *NT27* mutant cultures. *E*: mean density of Nc82 puncta/neuron was not significantly different in Wt and *NT27* cultures ($P > 0.05$, Student's *t*-test, $n = 7$ cultures for each genotype). *F*: mean density of vesicles within 200 nm of the active zone presynaptic membrane and mean active zone length were similar in Wt and NT27 cultures ($P > 0.05$, Student's *t*-test). All images were captured and analyzed blind with respect to genotype ($n = 13$ synapses, 4 *NT27* cultures; $n = 20$ synapses, 3 Wt cultures).

classic synaptic profiles were located, photographed, and analyzed blind with respect to genotype in both Wt and *NT27* cultures (Fig. 5, *C* and *D*). There was no significant difference in active zone length nor density of vesicles within 200 nm of the active zone between Wt and *NT27* mutants (Fig. 5*F*). Thus constitutive disruption of the CAC channels in *NT27* mutant cultures does not appear to affect the ability of neurons to elaborate branching processes, form Bruchpilot positive presynaptic specializations or form synapses with standard vesicle density and active zone lengths.

cac encodes PLTXII-sensitive calcium currents in antennal lobe PNs in adult brain

To determine if calcium influx through CAC calcium channels is involved in regulating spontaneous release of neurotransmitter at excitatory cholinergic synapses in vivo, as in cultured neurons, we monitored calcium currents and synaptic currents in antennal lobe projection neurons (PNs) in whole brains isolated from adult flies. PNs were chosen for analysis because they are both cholinergic and cholinoceptive. PNs were labeled in the Wt and *NT27* mutant background with GFP using the *GH146-Gal4* driver. All recordings were from PNs in the dorsal antennal lobe clusters in whole brains isolated from adult flies 1–3 days after eclosion. Similar to the conditions used in culture, all PN calcium current recordings were made in physiological concentrations of external calcium (1.8 mM).

In the Wt background, calcium currents were recorded in all PNs examined. In a small percentage of PNs, the currents were well-clamped and had distinct fast- and slow-inactivating components similar to those seen in the cultured neurons (Fig. 6*Ai*). However, in the majority of PNs the calcium currents were not well space-clamped (Fig. 6*Aii*), consistent with localization of the underlying channels in compartments electrotonically distal to the soma. This could include dendrites in antennal lobe glomeruli and/or the axon projecting to the mushroom bodies in PNs in the intact brain. However, similar to the results in culture, addition of PLTXII to the recording saline reduced the magnitude of the calcium currents in Wt neurons (Fig. 6*B*). The slow-inactivating calcium current density, estimated by measuring the maximal current at the end of an 80-ms depolarizing current step (Fig. 6*A, i* and *ii,* and *B,* white arrow), was reduced by 68% in 50 nM PLTXII ($P < 0.001$) compared with control saline (Fig. 6*E*). The fast-inactivating PLTXII-resistant current density in Wt neurons was calculated from the peak transient current measured in 50 nM PLTXII (Fig. 6, *B,* black arrowhead, and *E*). These data demonstrate that Wt PNs in the adult brain express two calcium currents: PLTXII-sensitive slow-inactivating and PLTXII-resistant fast-inactivating currents.

As predicted from the studies in culture, *NT27* PNs also express calcium currents in the adult brain (Fig. 6*C, i* and *ii*). The slow-inactivating PLTXII-sensitive currents were reduced by 57% in *NT27* PNs ($P < 0.01$) compared with Wt PNs (Fig. 6*E*). The fast-inactivating, PLTXII-resistant calcium current density in *NT27* neurons (Fig. 6*D,* black arrowhead) was similar in magnitude to Wt (Fig. 6, *B,* black arrowhead, and *F*). These data demonstrate that slow-inactivating PLTXII-sensitive calcium current is predominantly mediated by *cac* encoded Ca_v2-type calcium channels in cholinergic antennal lobe PNs in the adult brain. The PLTXII-resistant fast inactivating current is mediated by a functionally distinct channel subtype/s that is likely to be encoded by a different gene/s.

CAC channels regulate AP-independent neurotransmitter release at cholinergic synapses onto PNs in adult brain

PNs receive cholinergic synaptic input from olfactory receptor neurons as well as potentially from lateral excitatory connections recently described in the antennal lobe (Kazama and Wilson 2008; Olsen et al. 2007; Shang et al. 2007). To determine if CAC channels regulate release of synaptic vesicles at excitatory cholinergic synapses onto PNs, the frequency and properties of cholinergic mEPSCs in PNs from Wt and *NT27* brains were compared. Recordings were performed at room temperature in the presence of picrotoxin to block GABA receptors and TTX to block sodium channels.

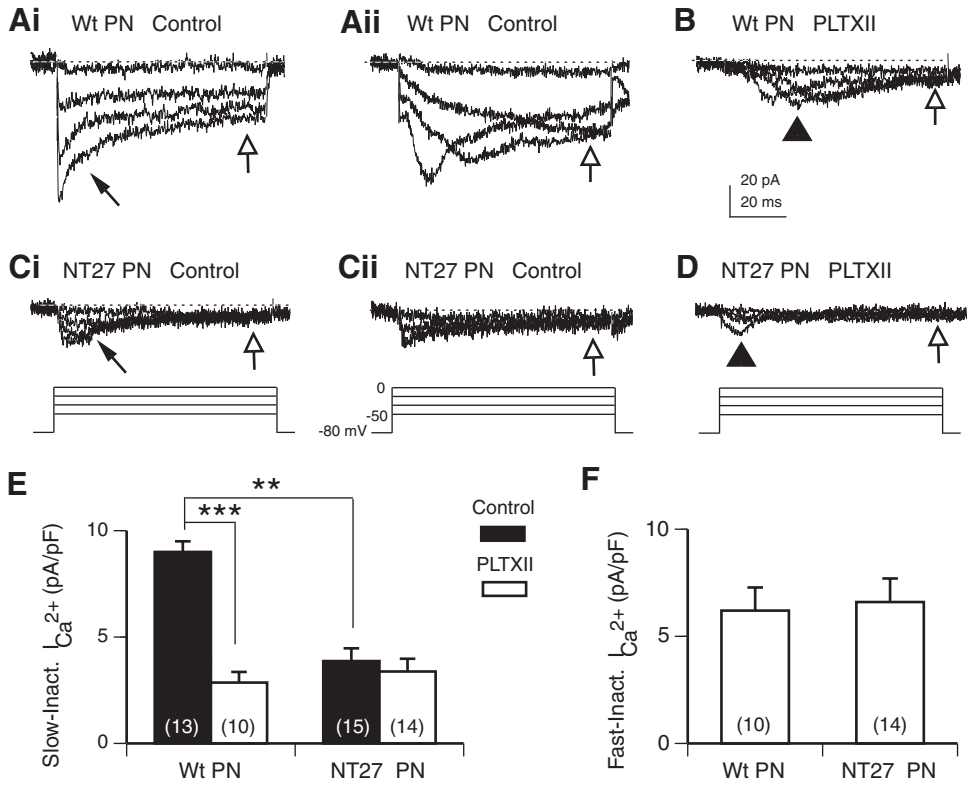

FIG. 6. *cac* encodes PLTXII-sensitive calcium currents in antennal lobe projection neurons (PNs) in the adult brain. All recordings made from PNs in brains isolated from adult flies 1–3 days old. *Ai*: calcium currents, under good voltage control, demonstrate the presence of fast-inactivating (black arrow) and slow-inactivating (white arrow) components in a Wt PN. *Aii*: calcium currents not well space-clamped were more typical of recordings from Wt PNs. Magnitude of the slow-inactivating component in all records was estimated from the current amplitude at the end of an 80-ms depolarizing voltage-step (white arrow). *B*: current traces recorded from Wt PN in 50 nM PLTXII. Fast-inactivating current density estimated from peak transient current (black arrowhead). *C, i* and *ii*: calcium currents recorded from *NT27* PNs in control saline. *D*: current traces recorded from an *NT27* PN in 50 nM PLTXII. Fast-inactivating current was estimated from peak transient current (black arrowhead). *E*: in Wt neurons, the slow-inactivating current density was significantly reduced by PLTXII compared with control saline (*t*-test, $P < 0.001$). In addition, the density of the slow-inactivating current in PNs recorded in control saline was significantly lower in *NT27* compared with Wt brains (*t*-test, $^{**}P < 0.01$). PLTXII did not further reduce the slow-inactivating current density in *NT27* PNs. *F*: density of the fast-inactivating current, peak transient recorded in 50 PLTXII, was not significantly different in Wt and *NT27* neurons (*t*-test, $P > 0.05$). Bars represent means ± SE from number of neurons indicated (*n*).

Spontaneous synaptic currents were recorded in all PNs examined in Wt ($n = 16$) and *NT27* mutant ($n = 18$) brains. Their blockade by curare ($n = 3$ in each genotype) confirmed that the majority of the mEPSCs recorded under these conditions were cholinergic (Fig. 7, *A* and *B*). Similar to the results in culture, mEPSCs frequency was reduced by 73% ($P < 0.001$) in *NT27* compared with Wt PNs (Fig. 7, *C–E*). There is no significant difference in the mEPSC amplitudes in the two genotypes (Fig. 7*F*). This suggests that CAC channels are important in regulating mEPSC frequency. The ability of acute application of PLTXII to reduce the mEPSC frequency in Wt neurons by 62% ($P < 0.01$) confirms that the activity of CAC channels regulate AP-independent release of neurotransmitter in the adult brain (Fig. 7, *C* and *E*).

Synaptic currents at low frequency are still seen in 50 nM PLTXII in both Wt and *NT27* PNs (Fig. 7, *C–E*). These data suggest that the PLTXII-resistant calcium channels also contribute to regulation of a small component of AP-independent release of neurotransmitter in the adult brain.

DISCUSSION

The data presented here demonstrate that the Ca_v2-type α-1 subunit gene *cac* encodes voltage-gated calcium channels that mediate PLTXII-sensitive, slow-inactivating calcium currents in pupal neurons and adult antennal lobe PNs. In addition, we show that the activity of presynaptic CAC channels regulates spontaneous, AP-independent release of excitatory neurotransmitter at central cholinergic synapses both in pupal cultures and in the adult brain. In previous studies, recordings from hypomorphic mutants have demonstrated that CAC channels regulate evoked release of neurotransmitter at the larval NMJ. However, the absence of changes in mEJPs in NT27 and cac^{ts2} mutants suggests that CAC channels do not regulate AP-independent release of neurotransmitter at these peripheral glutamatergic synapses (Macleod et al. 2006; Rieckhof et al. 2003). Thus our data identify a function for CAC channels at central synapses in the adult brain that was not predicted from studies at the larval NMJ.

Similar to the mammalian CNS, our data support the hypothesis that there are cell- and developmental-specific patterns of expression of different calcium channel subtypes in *Drosophila* neurons. The pupal and adult brain neurons express voltage-gated calcium currents at much higher density than reported in embryonic neurons (Baines and Bate 1998; Byerly and Leung 1988; O'Dowd 1995; Peng and Wu 2007; Schmidt et al. 2000) consistent with developmental upregulation of

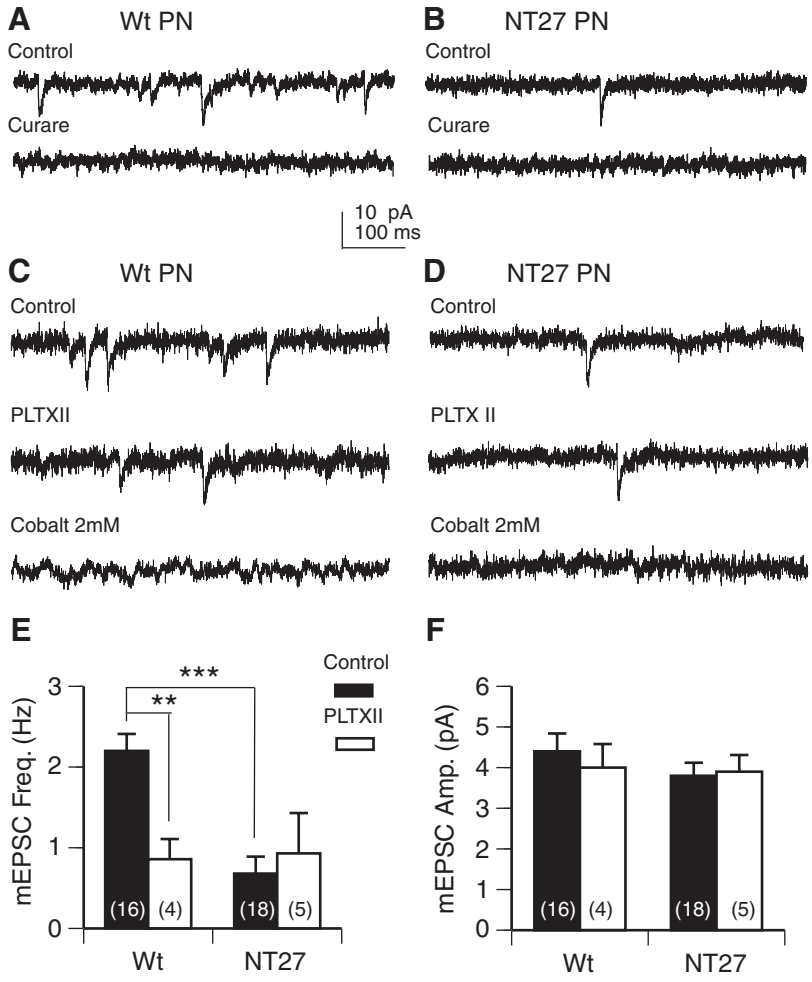

FIG. 7. CAC calcium channels regulate cholinergic mEPSC frequency in PNs in the adult fly brain. *A* and *B*: sodium action potential (AP)-independent mEPSCs recorded from single Wt and *NT27* PNs in control saline are blocked by addition of 10 μM curare to the bath indicating they are cholinergic. *C*: in a Wt PN, the cholinergic mEPSCs frequency is reduced by bath application of PLTXII (50 nM) and is blocked by addition of cobalt (2 mM). *D*: the low mEPSC frequency characteristic of *NT27* PNs in control saline is not further reduced by PLTXII but is blocked by addition of cobalt (2 mM). *E*: the mean mEPSC frequency in control saline is significantly reduced in *NT27* PNs compared with Wt PNs (*t*-test, $***P < 0.001$). In addition, the mean mEPSC frequency in Wt PNs is significantly reduced by PLTXII compared with control saline (*t*-test, $**P < 0.01$). The mEPSC frequency in *NT27* is not further reduced by addition of PLTXII (50 nM). *F*: amplitude of the mEPSCs in Wt and *NT27*. Each bar indicates the mean ± SE from indicated number of neurons (*n*). All synaptic currents recorded at a holding potential of –75 mV.

CAC mRNA in late stage pupae (Smith et al. 1996). In the presence of physiological concentrations of calcium (1.8 mM), pupal neurons in culture and adult PNs in situ have calcium currents with two kinetically and pharmacologically distinct components. The slow-inactivating, PLTXII-sensitive current is mediated predominantly by CAC channels based on selective reduction of this component in *cac* mutants, *NT27* and *TS3*. A recent report indicates that calcium currents in cell-division arrested "giant" neurons in culture are virtually eliminated in a *cac null (H129*), suggesting that all neuronal calcium currents are mediated by CAC channels at this early developmental stage (Peng and Wu 2007). Although we were not able to examine pupal and adult neuronal calcium currents in the *cac null* because these are embryonic lethal, the fast-inactivating, PLTXII-resistant currents characteristic of Wt neurons were not significantly reduced in either hypomorphic *cac* mutant tested. This suggests that the calcium channels underlying this kinetically and pharmacologically distinct current in adult PNs are encoded by one of the other two calcium channel α1 subunit genes, Ca_v1-type (*Dmca1D*) or Ca_v3-type (*Ca-α1T).*

Expression of multiple calcium channels in a single-cell type was reported in a recent study of larval motor neurons in situ. These neurons, in contrast to embryonic "giant neurons" in culture, express currents mediated by both CAC and Dmca1D (Cav1-type) channels (Worrell and Levine 2008). The CAC channels are localized in the nerve terminals, whereas the Dmca1D channels are primarily in the somatodendritic compartment. The Dmca1D channel-mediated current in the motor neurons is PLTXII-resistant so the *Dmca1D* gene is a potential candidate for encoding the PLTXII-resistant channels in adult brain neurons. However, in the absence of information about the functional properties of *Ca-α1T* encoded Ca_v3-type calcium channels, additional studies will be necessary to clarify the contribution of these and/or Dmca1D calcium channels in pupal neurons in culture and adult PNs in situ. The cell-specific patterns of expression illustrate the importance of assessing calcium current properties in identified neuronal subpopulations to understand the contribution of different channel subtypes to activity and synaptic transmission in specific circuits.

The major effect of both *TS3* and *NT27* mutations on the slow-inactivating calcium currents was a decrease in current density. This is somewhat unexpected because although the site of the *TS3* mutation has not been reported, molecular analysis of the *NT27* mutant reveals a single missense mutation in the S4 voltage-sensor region of the channel (Rieckhof et al. 2003). A mammalian calcium channel with an identical mutation causes a 10-mV shift in the voltage dependence in a heterologous expression system (Garcia et al. 1997). The decrease in the density of the PLTXII-sensitive current in *NT27* neurons in our study is not accounted for by a simple shift in the voltage dependence

because the current density was decreased between –70 and +30 mV. The *NT27* mutation could potentially decrease the number of CAC channels localized in presynaptic zones by altering interactions with accessory proteins such as $\alpha 2\delta$ or Fus1, that have been shown to be important in regulating CAC channel function/localization at the NMJ (Dickman et al. 2008; Long et al. 2008; Ly et al. 2008). It is also possible that *NT27* mutant CAC channels have a reduced single-channel conductance and/or open time that contribute to the mutant phenotype.

Our analysis of mutants indicate that CAC channels are critical for regulation of AP-independent neurotransmitter release at central cholinergic synapses both in vitro and in PNs in the adult *Drosophila* brain. The importance of calcium influx through CAC channels in regulating spontaneous neurotransmitter release is confirmed by the ability of acutely applied PLTXII to reduce cholinergic mEPSC frequency in Wt neurons. In a previous study, we also demonstrated that PLTXII reduced basal calcium levels, assessed by Fura-2 imaging, in cultured pupal neurons (Jiang et al. 2005). These data thus indicate that CAC channels, in physiological concentrations of calcium (1.8 mM), actively mediate influx of calcium into cholinergic neurons influencing the probability of AP-independent release of neurotransmitter at central cholinergic synapses. In contrast, the absence of changes in mEJP frequency at the larval NMJ in *NT27* and cac^{ts2} mutants suggests that CAC channels do not play a major role in regulating AP-independent synaptic release at this peripheral synapse (Macleod et al. 2006; Rieckhof et al. 2003). The ability of PLTXII to block evoked transmission at the larval NMJ in Wt animals without significantly affecting the mEJP amplitude or frequency is consistent with this interpretation (Branton et al. 1987). Although both of these studies were done in low external calcium, the mEJP frequency at the larval NMJ is similar at calcium concentrations between 0.4 and 1.8 mM in the absence of elevated potassium (Littleton et al. 1994). This suggests that the probability of CAC channel opening in the absence of stimulation is lower at the larval NMJ when compared with central cholinergic synapses.

AP-independent release of vesicles at the NMJ appears to be calcium-dependent and regulated by calcium channels in some conditions. For example the mEJC frequency recorded in TTX and 1.8 mM calcium is significantly reduced when calcium is removed from the external solution (Sweeney et al. 1995). In addition, AP-independent exocytosis of synaptic vesicles induced by high potassium is regulated by external calcium levels, is sensitive to blockade by PLTXII, and depends on flux through voltage-gated calcium channels (Kuromi et al. 2004; Okamoto et al. 2005; Yoshihara et al. 1999). A recent study at the embryonic NMJ reported a twofold decrease in mEPSC frequency in *cac* nulls compared with Wt, suggesting that CAC channels may be important in regulation of spontaneous release at these immature peripheral synapses (Hou et al. 2008).

Activity of CAC channels is also important in regulating growth at peripheral synapses based on a reduction in terminal branching and a 30% decrease in varicosity density at the larval NMJ in *NT27* mutants (Rieckhof et al. 2003). In contrast, while the possibility of subtle changes in synaptic morphology in the *NT27* mutant cannot be ruled out, there was no evidence of morphological changes at central synapses in our studies. Neurons in cultures from *NT27* mutants extended branching neurites, exhibited focal expression of the preysnaptic protein Bruchpilot, and formed synapses with ultrastructural features that were not significantly different from those seen in Wt neurons. The ability of acute application of PLTXII to reduce Wt mEPSC frequency to *NT27* levels, both in vitro and in the brain, confirms that CAC channel activity regulates AP-independent release of neurotransmitter at central synapses. Taken together these data support the hypothesis that the reduction in cholinergic mEPSC frequency in *NT27* and *TS3* mutant neurons is predominantly due to a direct reduction in function of CAC channels as opposed to major reductions in synaptic growth.

While the role of mEPSCs in the *Drosophila* CNS remains to be explored, they could be involved in synaptic stability and/or plasticity as they are at some synapses in the mammalian CNS (McKinney et al. 1999; Sutton et al. 2006; Yamasaki et al. 2006). Single quantal events have also been shown to influence firing of cerebellar interneurons, cells that have a small size and high-input resistance (Carter and Regehr 2002), characteristics common to most neurons in the adult *Drosophila* brain. A recent study indicates that CAC channels are involved in regulating rapid synaptic homeostasis in the absence of evoked neurotransmission at the NMJ (Frank et al. 2006), another potential role for CAC regulated mEPSCs at central synapses. Additional experiments will be important in determining how calcium influx through CAC channels regulates evoked as well as AP-independent vesicular release of transmitter and possibly aspects of plasticity that have been recently described at synapses onto cholinergic PNs in vivo (Kazama and Wilson 2008).

The *cac* alleles used in this study, *NT27* and *TS3*, were first isolated as conditional mutants, exhibiting paralysis and seizures at elevated temperature. The *NT27* allele showed behavioral and physiological deficits at room temperature when placed in *trans* to a null mutant (Rieckhof et al. 2003). Our data confirm the constitutive nature of the *NT27* mutation. In our study, both *NT27* and *TS3* homozygous mutants showed significant deficits in climbing behavior when assayed at room temperature with the phenotype more pronounced in *NT27*. This was consistent with our electrophysiological results demonstrating a larger reduction in CAC channel-mediated currents assayed at room temperature in *NT27* compared with *TS3*. These data suggest that a constitutive decrease in CAC channel function in motorneurons could contribute to the locomotory behavioral deficits at permissive temperatures, where severity is correlated with magnitude in reduction of the calcium current and the temperature-dependent effects result from a lower threshold for disrupting transmission at the NMJ. Our findings also indicate that it will be important to determine if deficits in centrally generated behaviors in *cac* mutants, including courtship and seizure phenotypes (Chan et al. 2002; Rieckhof et al. 2003; Smith et al. 1998), involve disruption of spontaneous as well as evoked transmitter release. This points out that while the NMJ is an excellent model for studying cellular mechanisms of synaptic transmission and plasticity, the role of specific channels in regulating centrally generated behaviors also requires exploration of their role at synapses in central neural circuits.

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