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Spontaneous Acetylcholine Secretion from Developing Growth Cones of *Drosophila* Central Neurons in Culture: Effects of cAMP-Pathway Mutations

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We describe a novel bioassay system that uses *Xenopus* embryonic myocytes (myoballs) to detect the release of acetylcholine from *Drosophila* CNS neurons. When a voltage-clamped *Xenopus* myoball was manipulated into contact with cultured *Drosophila* "giant" neurons, spontaneous synaptic current-like events were registered. These events were observed within seconds after contact and were blocked by curare and α-bungarotoxin, but not by TTX and Cd^{2+}, suggesting that they are caused by the spontaneous quantal release of acetylcholine (ACh). The secretion occurred not only at the growth cone, but also along the neurite and at the soma, with significantly different release parameters among various regions. The amplitude of these currents displayed a skewed distribution. These features are distinct from synaptic transmission at more mature synapses or autapses formed in this culture system and are reminiscent of the transmitter release process during early development in other preparations. The usefulness of this co-culture system in studying presynaptic secretion mechanisms is illustrated by a series of studies on the cAMP pathway mutations, *dunce* (*dnc*) and *PKA-RI*, which disrupt a cAMP-specific phosphodiesterase and the regulatory subunit of cAMP-dependent protein kinase A, respectively. We found that these mutations affected the ACh current kinetics, but not the quantal ACh packet, and that the release frequency was greatly enhanced by repetitive neuronal activity in *dnc,* but not wild-type, growth cones. These results suggest that the cAMP pathway plays an important role in the activity-dependent regulation of transmitter release not only in mature synapses as previously shown, but also in developing nerve terminals before synaptogenesis.

Key words: Drosophila*; "giant" neuron culture; growth cone; neurotransmitter release; synaptogenesis;* Xenopus laevis*; myoball; cAMP;* dunce*; PKA*

Drosophila melanogaster has been successfully used for studying many cellular processes in the nervous system. The *Drosophila* neuromuscular junction has been used extensively as a model in studying mechanisms underlying synaptic development, function, and plasticity (Jan and Jan, 1976; Jan et al., 1977; Ganetzky and Wu, 1983; Zhong and Wu, 1991; Broadie and Bate, 1993; Wang et al., 1994; Zhong 1995; Davis et al., 1996; Schuster et al., 1996)(for review, see Keshishian et al., 1996). However, *in vivo* electrophysiological studies of synaptic transmission in *Drosophila* central neurons have been limited by technical difficulties in the experimental manipulations required for probing basic synaptic properties (Ikeda and Kaplan, 1970; Tanouye et al., 1981; Thomas and Wyman, 1984; Pavlidis and Tanouye, 1995; Engel and Wu, 1996; Trimarchi and Murphey, 1997; Baines and Bate, 1998).

To facilitate the study of central neurons, several primary culture systems have been developed to grow nerve tissue from embryos (Seecof et al., 1971; Wu et al., 1990; Saito and Wu, 1991; O'Dowd, 1995) and larvae (Wu et al., 1983), including the giant neuron culture derived from cytokinesis-arrested embryonic neuroblasts. Cells in the latter system develop a variety of neuronal properties, including characteristic arborization patterns (Wu et al., 1990), action potentials (Saito and Wu, 1991), and neurotransmitter production (Huff et al., 1989), suggesting that a significant degree of cell autonomous neuronal differentiation can occur. The increased size of the multinucleated neuronal somata and neuritic processes in this culture system have facilitated studies of ionic currents and firing patterns in these neurons (Saito and Wu, 1991, 1993; Yao and Wu, 1995; Zhao et al., 1995; Zhao and Wu, 1997, 1999). The nerve terminals of giant neurons display filopodia- and lamellipodia-like structures that form giant growth cones. More importantly, functional connections among neurons and between neurons and myocytes are established in these cultures, as indicated by the appearance of frequent postsynaptic potentials and myocyte contractions in the networks formed in older cultures (M. Saito, M.-L. Zhao, W.-D. Yao, P. Taft, and C.-F. Wu, unpublished results).

In this study, we describe a novel approach to study neurotransmitter secretion in developing giant neurons. Taking advantage of the fact that the major neurotransmitter in the *Drosophila* CNS is acetylcholine (ACh) (Hall and Greenspan, 1979; Salvaterra et al.,

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1987; Burrows, 1996), we used a vertebrate myocyte as a sensitive probe to detect the release of ACh from *Drosophila* central neurons. The use of myocytes from *Xenopus* embryos for the detection of ACh release has been well established for *Xenopus* nerve–muscle cocultures (Chow and Poo, 1985; Xie and Poo, 1986; Evers et al., 1989). We show that spontaneous ACh currents could be readily recorded from myoballs manipulated into contact with different regions of *Drosophila* neurons. In this system, in which a homogeneous population of *Xenopus* myocytes acts as the "postsynaptic" detector, the presynaptic mechanisms altered by different *Drosophila* mutations can be determined in the absence of the influence from postsynaptic cells. We applied this assay to cAMP cascade mutants and demonstrate that the cAMP pathway participates in the regulation of specific aspects of the release process in developing neurons and provide a first description of the cAMP regulation in transmitter secretion before synaptogenesis.

MATERIALS AND METHODS

Animal stocks. The fruit fly *Drosophila melanogaster* and the African clawed frog *Xenopus laevis* were used. All fly stocks were maintained at 20–22°C on standard media. The wild-type strain Canton S (CS) and the mutant stocks, dnc^1 (Dudai et al., 1976), dnc^2 (Bellen and Kiger, 1988), and $PKA\text{-}RI^{715}$ (*PKA-RI;* Goodwin et al., 1997) were used in this study.

Cell culture. The procedure for culturing *Drosophila* giant neurons has been described previously (Wu et al., 1990; Saito and Wu, 1991). Briefly, embryos were collected on agar plates for 1 hr and incubated for 3–4 hr at 25°C. The embryos (at early gastrulation stages) were homogenized in modified Schneider medium (Life Technologies, Grand Island, NJ) containing 200 ng/ml insulin (Sigma, St. Louis, MO), 20% fetal bovine serum (FBS), 50 mg/ml streptomycin, and 50 U/ml penicillin. Cells were washed two times in the above medium and resuspended in medium containing 2 μg/ml cytochalasin B (Sigma), and then plated on glass coverslips. Alternatively, cultures were prepared from individual embryos (Seecof et al., 1971) by aspirating the contents of entire, EtOH-sterilized embryos with a small amount of culture medium into a micropipette, thereby dissociating the embryos into individual cells and plating two or three embryos per coverslip in 100 μl of the above culture medium containing cytochalasin B. Cultures were maintained in humidified chambers at room temperature (20–25°C) for 2–5 d before recording. *Xenopus* nerve–muscle cocultures were prepared according to previously reported methods (Spitzer and Lamborghini, 1976; Tabti and Poo, 1990). The neural tube and associated myotomal tissue of 1-d-old *Xenopus* embryos (stage 20–22; Nieuwkoop and Faber, 1967) were dissociated in Ca^{2+}- and Mg^{2+}-free Ringer's solution supplied with EDTA (in mM: 115 NaCl, 2.6 KCl, 0.4 EDTA, and 10 HEPES, pH 7.6) for 20–30 min. The cells were then plated on clean glass coverslips and were used for experiments after 20–24 hr of incubation at room temperature. The culture medium consisted of 50% (v/v) of Ringer's solution (in mM: 115 NaCl, 2 $CaCl_2$, 2.6 KCl, and 10 HEPES, pH 7.6), 49% of Leibovitz medium (L-15; Life Technologies) and 1% fetal bovine serum (Life Technologies).

Immunocytochemistry. Cultured cells were fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature, washed, permeabilized in PBS containing 1% Tween 80 (PBT), blocked in 10% BSA or 2.5% horse serum in PBT for 2 hr, and then incubated with the monoclonal mouse anti- choline acetyltransferase (ChAT) antibody 4B1(Takagawa and Salvaterra, 1996) in PBT + 1% BSA overnight at 4°C, at a dilution of 1:3000. The cells were then washed six times with PBT–BSA and incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA; 1:200) in PBT–BSA for 2–3 hr. After washing, the cultures were incubated with preformed avidin–biotin complexes (Vectastain peroxidase kit; Vector Laboratories) according to the manufacturer's instructions for 1 hr, washed four times, rinsed twice in 100 mM Tris, pH 7.3, and then stained using diaminobenzidine and H_2O_2 (Sigma Fast Tablets; Sigma). The staining reaction was stopped by transferring the cultures into PBT. The stained cultures were then mounted in glycerol and photographed using Nomarski optics.

Electrophysiology. In a typical experiment, a piece of a coverslip containing cultured *Xenopus* myocytes was placed in the recording dish, next to a coverslip containing *Drosophila* giant neurons. Individual spherical *Xenopus* myocytes (myoballs) were first loosened from the coverslip by pushing on the cells with a heat-polished micropipette. The loose cells were then patched with whole-cell electrodes and were lifted up from the substratum and transferred to the coverslip containing *Drosophila* cells. Contact was then made between the myoball and, unless otherwise specified, growth cones of *Drosophila* neurons. Gentle pressure was applied to the patch pipette to ensure a close apposition between the two cells. Methods of whole-cell voltage-clamp recording were described previously (Hamill et al., 1981; Xie and Poo, 1986; Saito and Wu, 1991). Recording electrodes were prepared from 75 μl glass micropipettes (VWR Scientific, Chicago, IL), with an input resistance of 3–5 MΩ in bath solution. The external bath solution contained (in mM): 128 NaCl, 2 KCl, 4 $MgCl_2$, 1.8 $CaCl_2$, and 35.5 sucrose, buffered with 5 HEPES at pH 7.1–7.2. Patch pipettes were filled with intracellular solution containing (in mM): 144 KCl, 1 $MgCl_2$, 0.5 $CaCl_2$, and 5 EGTA, buffered with 10 HEPES, pH 7.1–7.2. Recordings were performed on an Axopatch 1B or 200B patch clamp amplifier (Axon Instruments, Foster City, CA). Junction potentials were nulled before the establishment of the whole-cell configuration. All recordings were made at room temperature. Data were digitized at 10 kHz and analyzed with the WCP (whole-cell program) software (Strathclyde Electrophysiology Software, Glasgow, Scotland).

RESULTS

Spontaneous ACh release from growth cones of developing *Drosophila* central neurons

Previous studies have indicated that the major excitatory neurotransmitter in the *Drosophila* CNS is ACh (Hall and Greenspan, 1979; Salvaterra et al., 1987). To confirm that the prevalence of cholinergic neurons is preserved in the giant neuron culture system, we performed immunostainings using an antibody against *Drosophila* ChAT (Takagawa and Salvaterra, 1996). Figure 1*A* shows the phase-contrast image of a typical giant neuron culture 3 d after plating, and Figure 1*B* presents images of stained cultures viewed by Nomarski optics. The majority of neurons with a variety of morphologies stained positively for ChAT [wild type (WT): mean ± SEM = 75.0 ± 1.0%, 598 cells were examined in six cultures; dnc^1: 78.1 ± 2.4%, 469 neurons in five cultures, data not shown).

Previous studies of dissociated cultures of vertebrate species have shown that growth cones of developing neurons are capable of secreting neurotransmitters (Hume et al., 1983; Young and Poo, 1983). In the present study, we investigated whether cultured *Drosophila* neurons can release ACh from their growth cones. Spherical myocytes (myoballs) derived from *Xenopus laevis* embryos, which had been cultured separately for 1 d, were placed onto contact with the growth cone of *Drosophila* neurons (see Materials and Methods). *Xenopus* myoballs express ACh receptors at their membrane surface and have been used extensively as a detector for ACh release from both neurons and non-neuronal cells in dissociated *Xenopus* embryonic cultures (Girod et al., 1995; Morimoto et al., 1995). The myoballs were voltage-clamped at −70 mV, so that inward currents caused by binding of ACh to the receptors on the muscle surface could be monitored. The configuration in a typical experiment is shown in Figure 1*C*, with the patch-clamped myoball situated on top of a giant neuron growth cone. In this arrangement, pulsatile inward currents were readily detected (Fig. 1*D*). Spontaneous events were obtained from 44 ± 7% (mean ± SEM, representing 26 neurons in 12 cultures) of all tested neurons, a frequency lower than the observed frequency of ChAT-immunoreactive cells in the culture (compare Fig. 1*B*). This result implies that not all ChAT-immunopositive neurons in cultures are capable of spontaneous releasing of ACh.

To confirm that the spontaneous currents detected by *Xenopus* myoballs were caused by ACh released from *Drosophila* giant

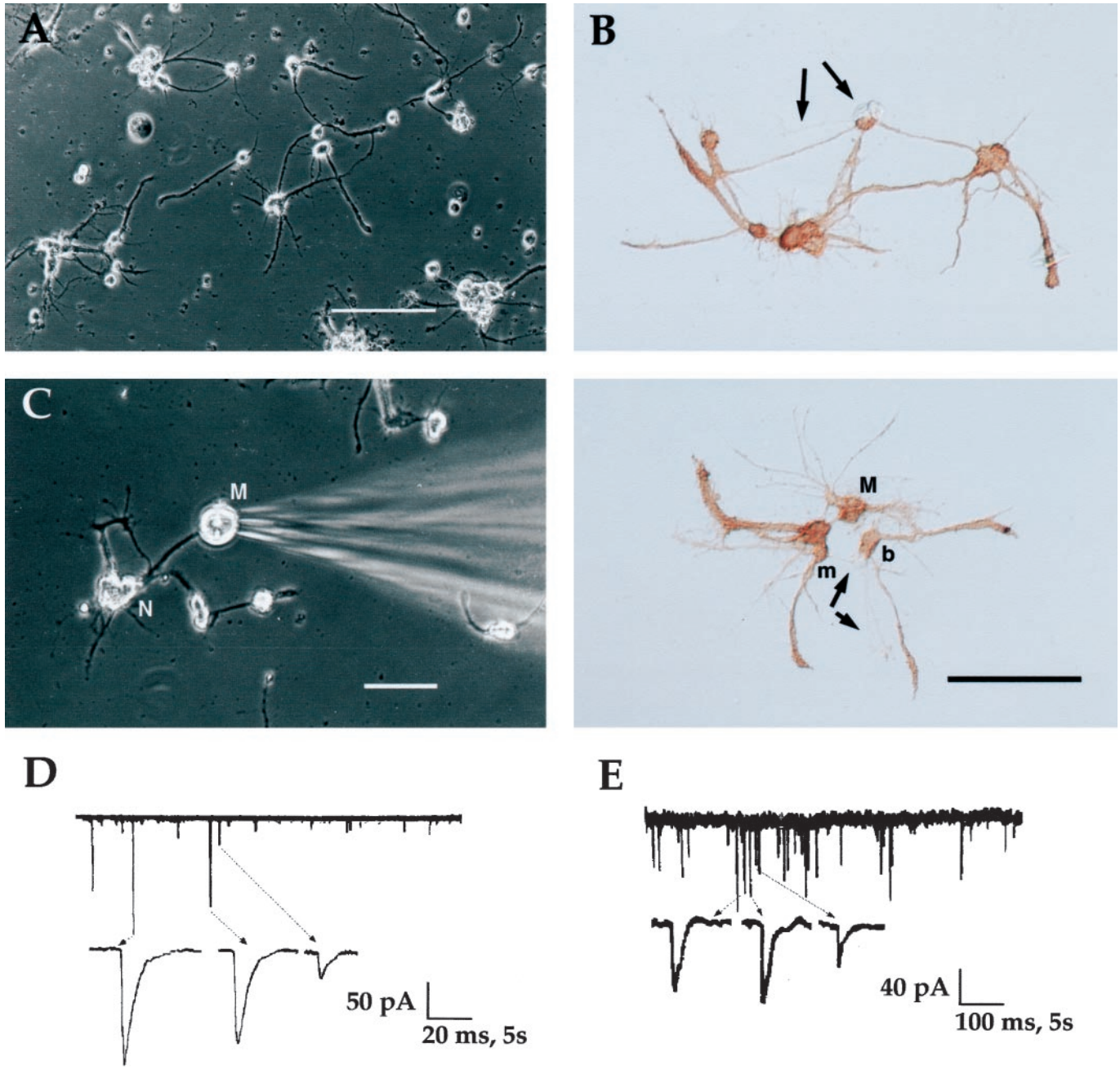

Figure 1. ACh release from *Drosophila* giant neurons. *A*, Phase-contrast image of a 3-d-old culture of *Drosophila* giant neurons. *B*, Nomarski image of a 3-d-old giant culture stained with a monoclonal antibody against *Drosophila* ChAT. The majority of the neurons stain positively for this marker. Examples of immunoreactive monopolar (*m*), bipolar (*b*), and multipolar (*M*) neurons are indicated. The *arrows* point to the soma and neurites of the small fraction of nonexpressing cells. *C*, Phase-contrast image of a patch-clamped *Xenopus* myoball (*M*) situated on the growth cone of a 4-d-old giant neuron (*N*). *D*, Example trace of spontaneous currents recorded from a voltage-clamped myoball ($V_h = -70$ mV) in contact with a Canton S (CS) neuron growth cone. *Bottom trace,* Individual events from the top trace in higher time resolution. *E*, Miniature spontaneous currents recorded from a giant *Drosophila* neuron that had an autaptic connection. *Bottom trace*, Individual events from the top trace in higher time resolution. Scale bars: *A, B*, 100 μm; *C*, 50 μm.

neurons, D-tubocurarine (Fig. 2*A*) and α-bungarotoxin (Fig. 2*B*) were added to the bath. Both antagonists completely abolished the currents. Consistent with previous results (Xie and Poo, 1986), the effects of curare, but not of bungarotoxin, were reversible after washing away the toxin. The Na^+-channel blocker tetrodotoxin (TTX; 0.2 μM), reduced the frequency of the spontaneous events in some cells, but did not abolish the large events (defined as those at least two times larger than the amplitude median; mean ± SEM = 22 ± 8% for pre-TTX and 15 ± 0.6% for post-TTX, $n = 3$ experiments; compare Fig. 2*C*). The Ca^{2+} channel blocker Cd^{2+} had little effect on either the amplitude or the frequency of the spontaneous currents (data not shown). We conclude that these inward currents were caused by the spontaneous release of quantal ACh packets from growth cones, with only a small portion of events triggered by nerve impulses.

To examine if synaptic transmission occurs between naturally

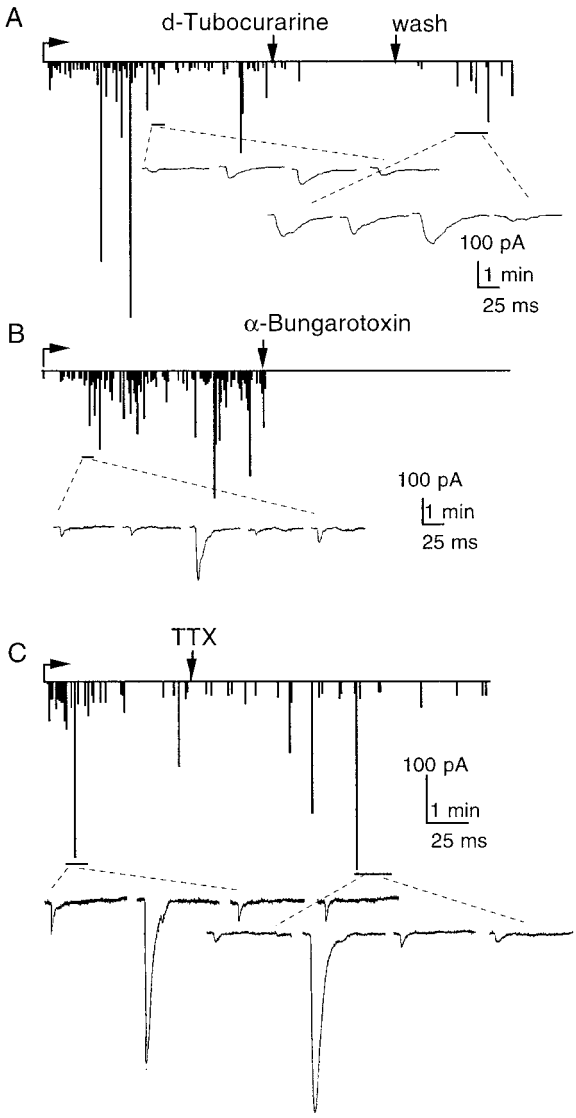

Figure 2. The spontaneous currents are mediated by acetylcholine. *A*, The ACh receptor antagonist D-tubocurarine (1 mM) was added at the time indicated by the *arrow*, causing the disappearance of the spontaneous currents. Currents slowly reappeared after the drug was removed by washing at the time indicated by the second arrow. *B*, The nicotinic ACh receptor blocker α-bungarotoxin (0.25 μM, *arrow*) eliminated the spontaneous currents irreversibly. *C*, The Na^+-channel blocker tetrodotoxin (TTX; 0.2 μM), added at the time indicated by the *arrow*, abolished some of the current pulses as reflected in the decrease in release frequency. In this and the following figures, continuous time courses of spontaneous currents were constructed by plotting the amplitude of each ACh current against the time when it occurred. *Bottom traces* are samples at higher time resolution. Unless otherwise indicated, membrane currents were recorded from a voltage-clamped myoball manipulated into contact with neuronal growth cones. Note also that there were no consistent changes in amplitude and frequency of spontaneous currents after contact.

connected giant neurons, direct patch-clamp recordings were performed on giant neurons with autaptic (Fig. 1*E*) or synaptic input from another neuron (see Fig. 4*C*). Spontaneous currents could be readily detected in voltage-clamped giant neurons (V_h = −80 mV), suggesting that in these cultures giant neurons were also capable of forming functional synapses with one another. In general, these synaptic currents occurred at a higher frequency and showed slower decay kinetics (see Fig. 4*D*), presumably reflecting differences in the properties between the *Drosophila* neuronal ACh channels and vertebrate muscle ACh receptors.

In our culture system, giant neurons can be morphologically categorized according to the number of processes they bear (Wu et al., 1990). Three morphologically distinct cell types, i.e., monopolar, bipolar, and multipolar neurons, can be distinguished but do not coincide with identifiable functional categories, e.g., firing patterns (Saito and Wu, 1991; Yao and Wu, 1995; Zhao and Wu, 1997). As shown in Figure 1*B*, neurons of different morphologies are equally likely to be ChAT-immunoreactive. Furthermore, similar spontaneous events of ACh release were readily detected from neurons with different morphologies (Fig. 3). Analysis of these events revealed similar mean peak amplitudes and release frequencies for the three morphological categories (67.7 pA and 0.17 Hz for monopolar, 69.0 pA and 0.28 Hz for bipolar, and 72.1 pA and 0.12 Hz for multipolar cells, not significant in paired *t* tests). Moreover, in neurons with more than one process, i.e., a bipolar cell (Fig. 3, middle) and two three-process neurons (data not shown), ACh release could be observed from all processes.

We further examined the changes of the spontaneous ACh currents with time after contact of a myoball and a neuronal growth cone for an indication of possible inductive effects of the myoball on the presynaptic cell. In a large number of cells monitored (n = 26), we typically observed the first release event immediately (within seconds) after the manipulation of the myoball into contact with the neuron (compare Figs. 2 and 3; data not shown). Furthermore, there was no consistent increase or decrease in the frequencies and amplitudes of the spontaneous events (in an average of 10 min) after contact in a number of cells analyzed (n = 12; the frequencies and amplitudes of the spontaneous ACh events were measured at the first two and the last two minutes). These results differ from the observations made in the early phases of synaptogenesis between *Xenopus* neurons and myoballs in which both frequency and amplitude of the spontaneous currents increase over time after initial contact (Xie and Poo, 1986; Evers et al., 1989). The absence of such temporal changes in our manipulated giant neuron–myoball pairs argues against the possibility of an inductive influence by the vertebrate muscle surface and suggests that the observed events reflect the intrinsic neuronal secretion mechanisms before synapse formation.

Characterization of myoball currents induced by ACh release from *Drosophila* growth cones

We have analyzed the amplitude, kinetics, and frequency of spontaneous myoball currents after contact with *Drosophila* neurons. The results were compared with the parameters observed in cell pairs of *Xenopus* neurons and myoballs, as well as with *Drosophila* neuronal synapses. Figure 4*A* presents histograms for the spontaneous events collected from a typical wild-type neuron, showing the rise and decay times and peak amplitudes. In addition to the amplitude and kinetics, the event frequency and the total charge movement associated with individual events are also summarized in Table 1. It is evident that the ACh events showed a considerable degree of heterogeneity even within the same cell. As indicated in Figure 4*A*, the peak amplitude displayed a skewed distribution toward smaller values. Application of TTX (0.2 μM) did not significantly change the skewed distribution and did not abolish the large events (data not shown). Thus, the spontaneous currents, regardless of their size, were likely to be quantal secretions from the giant neurons. It has been shown that a skewed

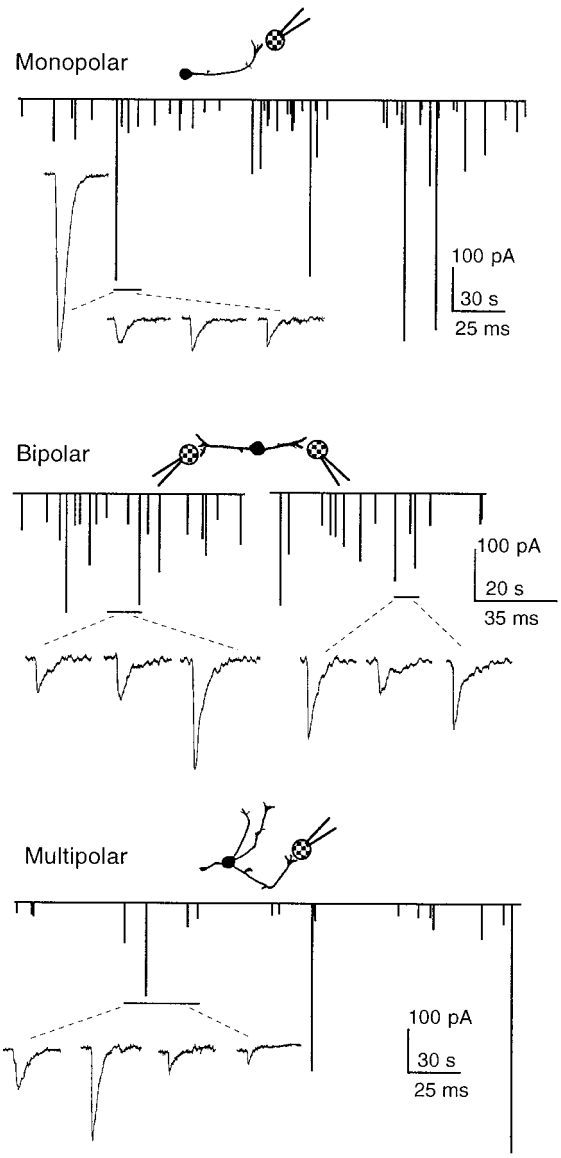

Figure 3. Spontaneous ACh release from neurons with different morphologies. Representative traces obtained from myoballs in contact with neurons of monopolar (*top*), bipolar (*middle*), and multipolar (*bottom*) morphologies. The schematic drawings of neurons represent typical morphologies and do not necessarily correspond to the actual neurons from which the recordings were made. *Top traces*, Time courses of spontaneous currents; *bottom traces*, selected individual events in higher time resolution. For the bipolar cell shown, growth cones of both processes were continuously releasing ACh, as tested by sequential recordings from a myoball manipulated into contact with both neuritic endings of the bipolar neuron.

amplitude distribution of spontaneous miniature synaptic currents is a typical feature for developing neuromuscular junctions *in situ* (Ohmori and Sasaki, 1977) or in culture (Kidokoro et al., 1980; Evers et al., 1989; Song et al., 1997). A skewed amplitude distribution of quantal events is also a feature of synapses in the CNS of vertebrates (Bekkers et al., 1990). The data obtained for contacts between *Xenopus* neurons and myoballs under our culture and recording conditions are consistent with previous experiments where a skewed amplitude distribution of quantal events was observed (Fig. 4*B*). Interestingly, synaptic currents recorded from naturally formed *Drosophila* neuronal synapses also revealed a skewed amplitude distribution of spontaneous synaptic ACh events (confirmed by curare inhibition; data not shown), but to a lesser degree.

Presumably, the variable amount of ACh in each packet, uneven distribution of ACh receptors on the apposing muscle membrane, and distance between the neuron–muscle membrane surfaces for ACh diffusion may give rise to variations in the amplitude and time course of ACh currents. We found no strict correlation between rise times and amplitudes among all the currents from any neuron–muscle pairs (data not shown), suggesting that diffusion could not be the major factor contributing to the variations observed. The ACh current heterogeneity caused by intrinsic neuronal properties (e.g., ACh package size) could be explored by employing the same myoball to detect qualitative differences in transmission from different releasing sources.

ACh secretion from different cellular compartments of neurons

By placing the same myoball onto different regions of a *Drosophila* neuron, we were able to reveal spontaneous secretion in different compartments of developing neurons. Figure 5*A* demonstrates that significant differences in the releasing properties could be observed when a single myoball was manipulated sequentially into contact with the growth cone, neurite, and soma of a monopolar neuron. The results of such experiments are summarized in Table 2. We found that the peak amplitude and integrated charge of ACh currents were significantly greater at the soma than at the growth cone and the neurite ($p < 0.001$), and ACh events from the soma showed a more skewed amplitude distribution than those from the growth cone and the neurite (Fig. 5*B*). The rise and decay kinetics of the spontaneous currents were significantly slower at the soma ($p < 0.001$). In contrast, the differences for both the peak amplitude and current kinetics were smaller when the events of growth cones and neurites were compared (Table 2). The frequency of the spontaneous currents was lower at the soma (Fig. 5, Table 2).

These results suggest that the machinery for spontaneous quantal ACh secretion is present throughout the entire neuron, whereas the ACh packets at the soma are considerably greater than those at growth cones. In addition, the distinct rise and decay times of the quantal currents suggest potential differences in the molecular machinery for ACh release between the soma and the growth cone.

It should be noted that all of the above observations were obtained from isolated neurons to eliminate potential postsynaptic influences. We also examined neurons that had made connections with their neighbors and found that ACh release from the soma became extremely rare (Table 2). In addition, the peak current and release frequency detected at free neuritic endings of the same connected neurons were significantly suppressed (Table 2). Regardless of the culture age (from 2 to 10 d), the soma of isolated neurons released ACh much more frequently than those in neuronal networks. Thus, interactions between presynaptic and postsynaptic elements apparently exert influence to confine ACh release to restricted synaptic sites.

cAMP regulation and ACh secretion

Mutations in the cAMP signaling pathway have been shown to impair learning behavior (Dudai et al., 1976; Byers et al., 1981; Tully and Quinn, 1985; DeZazzo and Tully, 1995; Davis, 1996). The *dunce* (*dnc*) gene, which encodes a cAMP-specific phospho-

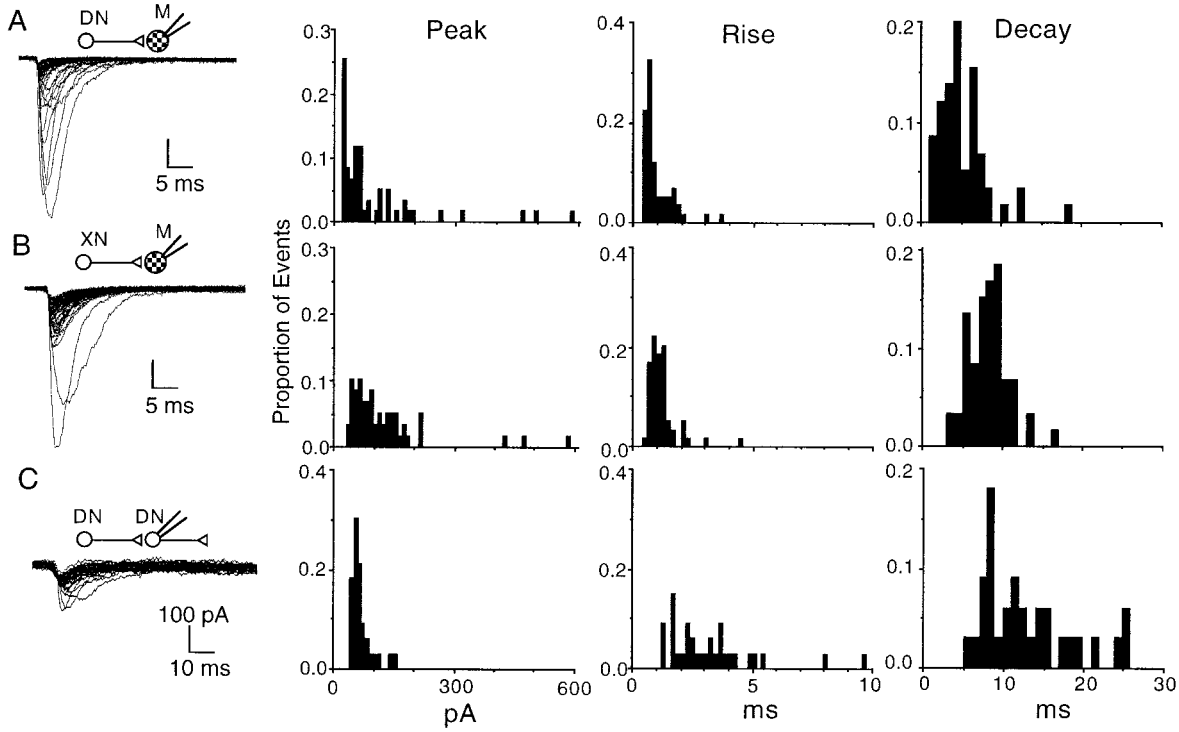

Figure 4. Amplitude, rise time, and decay time distributions of spontaneous ACh currents. Superimposed spontaneous currents collected from one *Drosophila* neuron–myoball contact (*A*), one *Xenopus* spinal neuron–myoball contact (*B*), and one *Drosophila* synapse naturally developed in a 3 d culture (*C*), are shown in the leftmost panels. Parameters of their spontaneous currents are summarized in the histograms of peak amplitudes (Peak), rise times (Rise), and 90% decay times (Decay). The schematic drawings above the current traces show the experimental configurations. *DN*, *Drosophila* neuron; *XN*, *Xenopus* spinal neuron; *M*, myoball. Note that spontaneous currents recorded from the *Drosophila* synapse showed a less skewed amplitude distribution.

Table 1. ACh secretion properties in different lines of *Drosophila* giant neurons and *Xenopus* neurons

	Peak (pA)	Rise (msec)	Decay (msec)	Charge (pC)	Frequency (Hz)
Drosophila neurons					
Wild type (16)	67.5 ± 6.0	1.02 ± 0.06	6.22 ± 0.58	0.36 ± 0.03	0.18 ± 0.03
dnc^1 (5)	25.6 ± 4.7**	2.18 ± 0.19**	9.14 ± 1.45*	0.25 ± 0.06	0.16 ± 0.08
dnc^2 (17)	61.2 ± 8.1	1.34 ± 0.15*	8.07 ± 1.06	0.43 ± 0.09	0.12 ± 0.03
PKA-RI (7)	36.8 ± 10.7**	1.51 ± 0.20*	10.88 ± 2.49*	0.37 ± 0.20	0.26 ± 0.16
Xenopus neurons (4)	96.9 ± 21.5	0.99 ± 0.13	5.34 ± 1.97	0.43 ± 0.16	0.78 ± 0.71

Data presented are mean ± SEM for the number of cells indicated in parentheses. ACh currents were collected from wild-type, *dnc*^{1}, *dnc*^{2}, and *PKA-RI* giant neurons, and from *Xenopus* spinal neurons. All events were obtained from growth cones (at least 20 events per cell) and analyzed for their peak amplitudes (Peak), total charge (Charge, amplitude integrated over the time course), rise time to the peak (Rise), decay time to 10% (Decay), and event frequency (Frequency). Comparisons of electrophysiological parameters are made against wild-type values. *$p < 0.05$; **$p < 0.01$ (Student's *t* test).

diesterase II responsible for the degradation of the second messenger, cAMP, has been shown to regulate activity-dependent plasticity at larval neuromuscular junctions (Zhong and Wu, 1991), habituation of synaptic connections in an escape circuit (Engel and Wu, 1996), spike frequency coding in cultured giant neurons (Zhao and Wu, 1997), and growth cone motility and nerve terminal arborization (Zhong et al., 1991; Kim and Wu, 1996). A downstream effector in this signaling pathway, protein kinase A (PKA), is thought to confer the cAMP regulation of the memory process (Tully et al., 1996). A mutation of the regulatory subunit of PKA (*PKA-RI*) affects classical conditioning (Goodwin et al., 1997). Using the myoball detection system we were able to study how alterations in the cAMP cascade by mutations of *dnc* and a downstream effector, *PKA-RI*, affect the properties of neurotransmitter secretion in central neurons before synapse formation.

In our assay, both *dnc*^{1} and *dnc*^{2} alleles were examined (Fig. 6, Table 1). We found in *dnc* neurons that the ACh currents were reduced in peak amplitude. Furthermore, there were events with markedly slower kinetics of rise and decay in *dnc* neurons. These phenotypes appeared to be more extreme in *dnc*^{1} than *dnc*^{2} neurons (Fig. 6*A*, Table 1). The reduction in peak amplitude based on statistical analysis was confirmed in experiments using the same myoball to detect release from both wild-type and mutant growth cones grown on two coverslips that were placed side by side in the same recording dish (Fig. 6*B*). In all cases studied ($n = 4$; WT-*dnc*^{2} pairs), the mean ACh current size from each mutant growth cone was smaller than that from the corresponding wild-type growth cone. Collectively, the ACh events from these *dnc*^{2} and wild-type cells were significantly different in size (55.3 ± 8.2 pA for *dnc*^{2} vs 93.6 ± 5.6 pA for wild type; $p < 0.001$, *t* test). In control experiments to test the reproducibility of

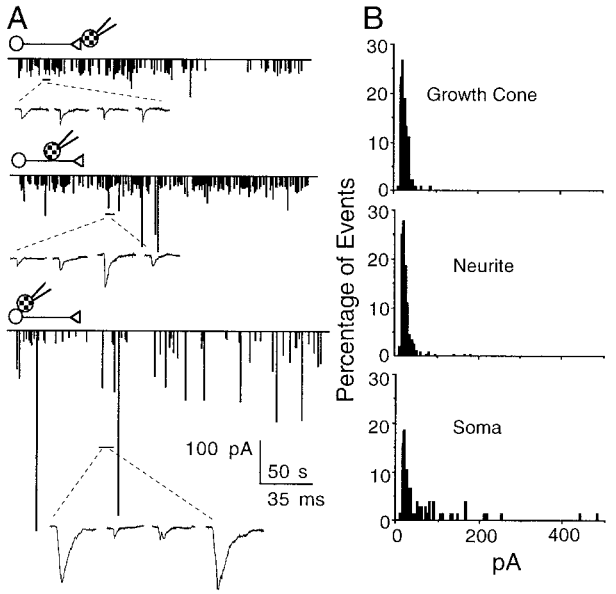

Figure 5. Quantal ACh secretion from different compartments of giant neurons. *A*, Continuous tracing of the membrane currents recorded from a myoball that was sequentially manipulated into contact with the growth cone (*top trace*), the neurite (*middle trace*), and the soma (*bottom trace*) of a WT neuron. *Bottom traces* show sample spontaneous currents at higher time resolution. Spontaneous currents recorded from the soma occurred less frequently and had larger amplitudes and slower kinetics than the ones recorded from the growth cone or the neurite, which did not differ significantly from each other. *B*, Amplitude distribution histograms of the spontaneous currents collected from the growth cone, the neurite and the soma shown in *A*.

myoball detection, a myoball was manipulated into contact with a wild-type neuron, lifted up to traverse in the bath, then returned to the same contact site of the neuron (Fig. 6*B*). The results demonstrated that the myoball produced stable and consistent ACh currents among repeated manipulations. Thus, the observed differences between mutant and wild-type cells are not likely to be caused by variations among different myoballs. Interestingly, statistical analyses shown in Table 1 indicate that the mean total charge movements associated with individual events were not different between wild-type and mutant neurons. Thus, on average, the total amount of ACh delivered by each packet was similar despite the differences in peak current amplitude, presumably because of altered release kinetics as reflected by the slower rise and decay time of ACh currents in mutant neurons (Table 1). In contrast, the frequency of spontaneous ACh release in *dnc* mutant neurons was similar to that of wild-type neurons (Table 1). This was true even in older mutant cultures in which the elevated cAMP effects should have been chronically maximized.

The phenotypes of *PKA-RI* neurons, in which PKA activity is constitutively enhanced (Goodwin et al., 1997), paralleled those of *dnc* neurons, with reduced peak and slower rise and decay time of ACh currents (Fig. 6*A*, Table 1). Furthermore, acute bath application of db-cAMP, a membrane-permeable cAMP analog (0.5 mM), prolonged the time course and reduced the amplitude of ACh currents in wild-type neurons ($n = 4$) within minutes (Fig. 6*C*). These results further support the idea that the effect of altered cAMP metabolism on ACh release is preferentially mediated by certain downstream targets of PKA.

One hallmark for the ACh release by *dnc* (and *PKA-RI)* neuronal growth cones was the occurrence of markedly prolonged events, characterized by slow rise and decay. These events intermittently appeared amid the fast events in the same experiment (Fig. 6*A,B*). Figure 7*A* compares the distribution histograms of rise and decay times for data collected from 19 dnc^2 and 12 wild-type cells. Notably, the distributions of rise and decay times in *dnc* neurons showed excessive prolonged events, which were scarce in wild-type neurons. Apparently, in dnc^2 neurons there was an additional population of abnormally slow events in addition to the regular wild-type population (Fig. 7*A*). The percentage of such abnormally prolonged rise (>3 msec) or decay (>20 msec) time courses were significantly higher in dnc^2 neurons (Fig. 7*B*).

A closer examination of this population of slow ACh events revealed two types of distinct waveforms: smooth and notched (Fig. 7*C*). The smooth slow events could arise from slow presynaptic release of single quanta or increased diffusion distance of released ACh. In contrast, the notched slow events were apparently caused by coupled releases of multiple quanta. We found that both types were very rare in wild-type neurons and that neither type dominated in dnc^2 neurons (Fig. 7*C*).

Aberrant spontaneous action potentials have been reported in the soma of cultured *dnc* neurons (Zhao and Wu, 1997). To examine the possibility that the prolonged ACh currents are caused by abnormal nerve action potentials, we applied TTX (0.2 μM) to block action potentials in giant neurons (Saito and Wu, 1991, 1993). There were no apparent changes in the amplitude distribution after TTX application, and prolonged ACh events remained in *dnc* neurons (data not shown). In addition, application of the Ca^{2+}-channel blocker, Cd^{2+}, did not decrease the

Table 2. ACh secretion from different compartments of developing *Drosophila* neurons

	Peak (pA)	Rise (msec)	Decay (msec)	Charge (pC)	Frequency (Hz)
Isolated neurons					
Growth cone (4, 233)	49.4 ± 5.3	0.99 ± 0.05	5.59 ± 0.17	0.27 ± 0.06	0.31 ± 0.45
Neurite (3, 278)	33.1 ± 1.78*	0.78 ± 0.03**	5.29 ± 0.17	0.13 ± 0.01**	0.22 ± 0.17
Soma (4, 206)	127.3 ± 12.6**	1.93 ± 0.09**	9.67 ± 0.29**	1.51 ± 0.25**	0.13 ± 0.1
Connected neurons					
Growth cone (5, 67)	20.0 ± 2.52	1.03 ± 0.07	4.32 ± 0.43	0.15 ± 0.02	0.034 ± 0.007
Soma (4, 2)	ND	ND	ND	ND	0.003 ± 0.003*

Values in parentheses are the number of cells examined and the total number of events collected, respectively. Data are presented as mean ± SEM for all events collected for each case. Events are grouped according to subcellular regions and analyzed for their peak amplitudes, total charge, rise time, 90% decay time, and frequencies. Note that ACh release from the soma of neurons that had made connections with their neighbors became extremely rare (frequency = 0.003). *$p < 0.01$; **$p < 0.001$, student's *t* tests. Comparisons are made against the growth cone data in the corresponding category. ND, Not determined because of very rare events.

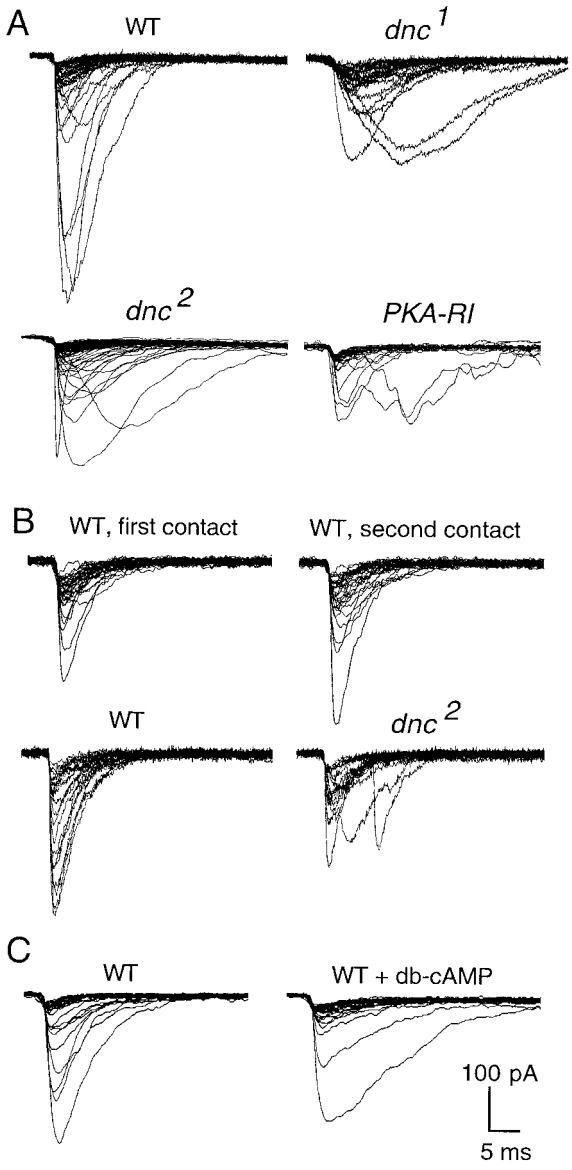

Figure 6. Effects of the cAMP pathway mutations on quantal ACh release from growth cones. *A*, Example traces of spontaneous currents collected from WT, *dnc*[1], *dnc*[2], and *PKA-RI* growth cones. Subsets of ACh currents detected from myoballs in contact with mutant growth cones had prolonged or irregular time courses. The ACh current amplitudes were smaller in mutants. *B*, Direct comparison of the spontaneous ACh currents from a WT and a *dnc*[2] growth cone detected by the same myoball. In control experiments, a myoball was manipulated into contact with a WT neuron (WT, first contact), lifted up, then returned to the same contact site (*WT, second contact*). The ACh currents remained the same between the first and the second contact (mean ± SEM = 59.5 ± 53.1 pA for the first contact and 63.1 ± 46.2 pA for the second contact). In a separate experiment, same myoballs were manipulated into contact with a WT and *dnc*[2] neurons, which were situated side by side in the same recording dish. Currents detected with the same myoball from WT, and *dnc* neurons showed clear differences in amplitudes and kinetics (see traces and Results) C, Acute application of membrane-permeable db-cAMP (0.5 mM) to WT neurons prolonged the time course and reduced the amplitude of the ACh currents.

frequency of the prolonged event (data not shown) in *dnc*. Thus the slow ACh events, either smooth or notched, are independent of nerve action potentials resulting from regenerative Na^+ or Ca^{2+} currents.

Effect of nerve stimulation on quantal secretion

An interesting phenotype of *dnc* mutations is the alteration in activity-dependent neuronal plasticity. Neuronal firing and synaptic efficacy after previous activity are markedly altered in *dnc* (Zhong and Wu, 1991; Engel and Wu, 1996; Zhao and Wu, 1997). We examined the effects of nerve stimulation (10 V, 1 msec duration, 0.5 Hz, delivered to the soma) on the secretion process in wild-type and *dnc*[2] neurons. We found that the amplitude and kinetics of ACh currents were not markedly modified by nerve stimulation in both genotypes (Fig. 8*A*). However, a striking difference was seen in the frequency of ACh release events in *dnc*[2] growth cones after soma stimulation.

In these cultures, reliable release evoked by extracellularly applied stimuli was not observed in most of the cells examined. However, at 0.5 Hz stimulation there was an increase in the frequency of spontaneous release that could be maintained for minutes (Fig. 8). Interestingly, in *dnc*[2], the low-frequency (0.5 Hz) extracellular stimulation achieved a much greater enhancement on ACh release after a few electric stimuli than in wild type. This enhancement was sustained throughout the duration of stimulation (2 min) and then dropped to a lower level of enhancement that persisted for minutes (Fig. 8*C*). Subsequent repeated cycles of stimulation could reproduce the same phenomenon (data not shown). The enhancement seemed to require extracellular Ca^{2+} as the Ca^{2+} channel blocker, Cd^{2+}, abolished the enhanced frequency of release (data not shown). Thus, Ca^{2+} influx may play a role in the altered activity-dependent regulation of spontaneous release in *dnc*[2] neurons.

DISCUSSION

In this paper, we describe a novel bioassay system to detect the release of the neurotransmitter ACh from cultured *Drosophila* central neurons by using a voltage-clamped vertebrate myocyte. We detected spontaneous ACh secretion from the growth cone, neurite, and soma of developing giant neurons. We demonstrated that this method is sufficiently sensitive to reveal distinct release characteristics at different regions of the neuron as well as the altered release properties of neurons from learning mutants, *dnc* and *PKA-RI*. Our results indicate that the cAMP pathway, modified by these mutations, exerts striking effects on the release process in nerve terminals even before contact with their native postsynaptic targets. Given the vast number of available mutations affecting neuronal functions, this system should be especially useful for elucidating presynaptic mechanisms in the absence of postsynaptic influence.

Neurotransmitter secretion from developing central neurons in *Drosophila*

The ability of the growth cone of developing neurons to secrete neurotransmitter has been reported for both vertebrate (Hume et al., 1983; Young and Poo, 1983) and invertebrate (Haydon and Zoran, 1989) neurons. Unlike mature synapses, neuromuscular synapses in dissociated cultures of *Xenopus* embryos exhibit a skewed amplitude distribution of ACh quantal currents (Kidokoro et al., 1980; Evers et al., 1989). As development progresses and the synapse matures, the amplitude distribution gradually becomes bell-shaped. This developmental progression in the quantal size has also been observed in an *in vivo* preparation of embryonic tunicate neuromuscular junctions (Ohmori and Sasaki, 1977) and in mammalian hippocampal synapses (Bekkers et al., 1990) when preparations from animals of different ages are compared. In our experiments, a skewed amplitude distribution

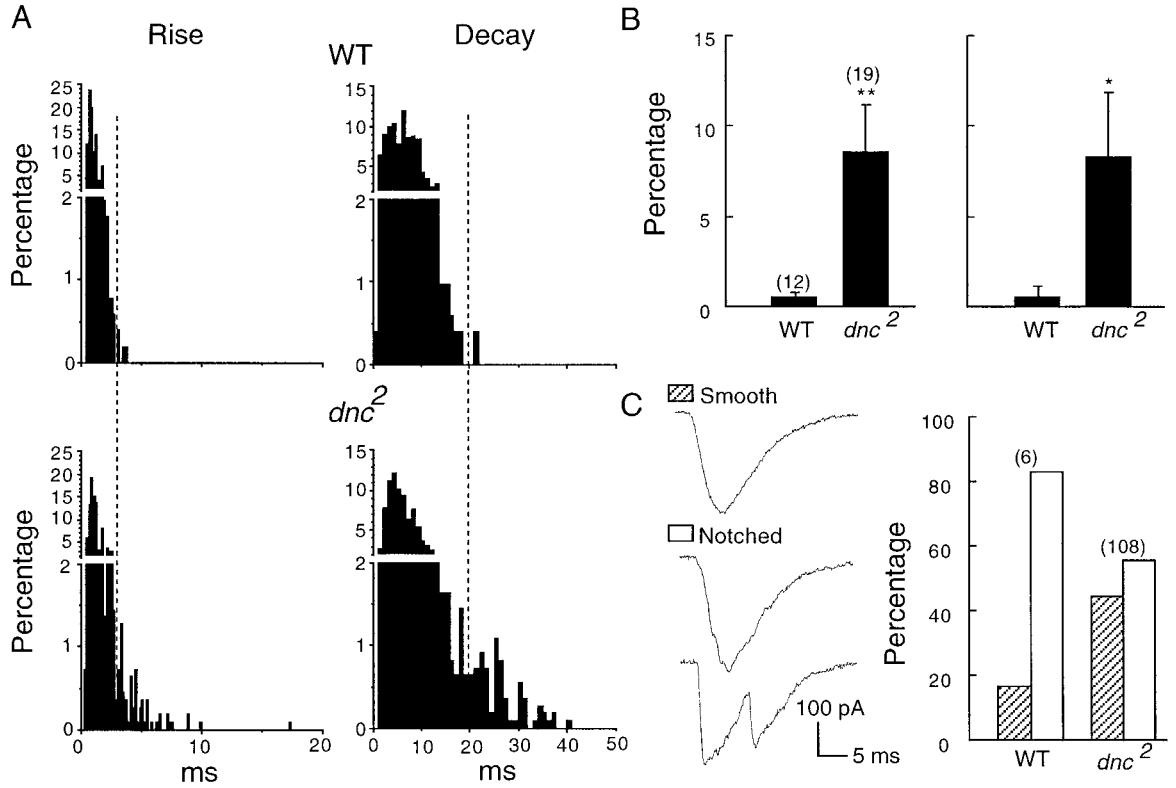

Figure 7. Altered kinetics of quantal ACh secretion in *dnc*^{2} mutant growth cones. *A*, Distribution of rise and decay time of spontaneous ACh currents in WT and *dnc*^{2} neurons. Data include 514 wild-type and 1101 *dnc* events. *Dashed lines* separate the prolonged events with slower rise (>3 msec) and decay (>20 msec) times from the regular events typical of WT neurons. *B*, ACh currents in *dnc*^{2} contained a significantly higher portion of prolonged events. The percentage of the spontaneous events that displayed prolonged rise (>3 msec, *left*) or decay (>20 msec, *right*) time courses are compared between wild type and *dnc*^{2}. $*p < 0.05$, $**p < 0.01$, *t* tests. *C*, Two distinct types of prolonged events in WT and *dnc*^{2} growth cones. Both smooth and notched waveforms were observed in *dnc*^{2} but rarely in WT. The *numbers* in *parentheses* (6 for WT and 108 for *dnc*^{2}) indicate slow events with prolonged rise or decay time. See Results for more detail.

was also observed for spontaneous ACh release in isolated, developing giant neurons of *Drosophila* (Fig. 4*A*). Interestingly, when synapses between neurons or autapses onto the same neuron were recorded postsynaptically, the amplitude distribution of synaptic currents displayed a significantly reduced degree of skewness (Fig. 4*C*). The difference in the amplitude distribution could reflect the differentiation of synaptic vesicles at the nerve terminals after synapse formation.

The spontaneous release of ACh was detected from all regions of the giant neurons tested (Fig. 5), indicating that releasable transmitter vesicles and the corresponding secretion machinery are present throughout the neuron. The release process in the soma is markedly different. Events of large amplitude and prolonged time course were far more prevalent, resulting in a more skewed amplitude distribution (Fig. 5*B*) and a greater charge transfer per event as compared to those in the growth cones and neurites (Table 2). Because synaptic vesicles are biogenically related to recycling vesicles (Kelly, 1993), which vary greatly in size, these observations support the idea that the vesicular components associated with soma release are immature recycling vesicles. Previous studies using manipulated nerve–muscle preparations in *Xenopus* have also demonstrated the presence of transmitter release from the soma of isolated spinal neurons. The release disappears after establishment of synaptic contact (Chow and Poo, 1985), suggesting that target interactions influence the development and differentiation of the secretory machinery at different regions of the neuron. This phenomena was also observed in cultured *Drosophila* neurons (Table 2).

In our study, we found no evidence for specific surface interactions between *Xenopus* myoballs and *Drosophila* neuronal growth cones. Spontaneous release events were detected immediately on contact between the neuron and the myoball, and in most cases, no appreciable changes in either the release frequency or peak amplitude were observed over time (Fig. 2). This is in contrast to studies of manipulated *Xenopus* nerve–muscle synapses, where there was an increase in both of these parameters, paralleled by an increase in the adhesion between the two cell types over the time course of the experiment (Evers et al., 1989). These cellular changes have been interpreted to reflect the process of synaptic maturation, possibly through recognition and interaction of specific surface molecules on the two cell types (Xie and Poo, 1986; Sun and Poo, 1987; Evers et al., 1989; Dai and Peng, 1993; Popov and Poo, 1993). In our experiments, there was no detectable adhesion between the giant neurons and the myoballs because the neuronal processes were never seen to be distorted after lifting off of the myoball. Furthermore, we have not observed consistent evoked release of ACh after soma stimulation even after prolonged periods of contact. In *Xenopus* neuron–myoball pairs, the evoked release increases in reliability and amplitude after the initial contact (Evers et al., 1989). Considering the phylogenetic distance between the two organisms used, a lack of inductive effects of the myoball is not surprising.

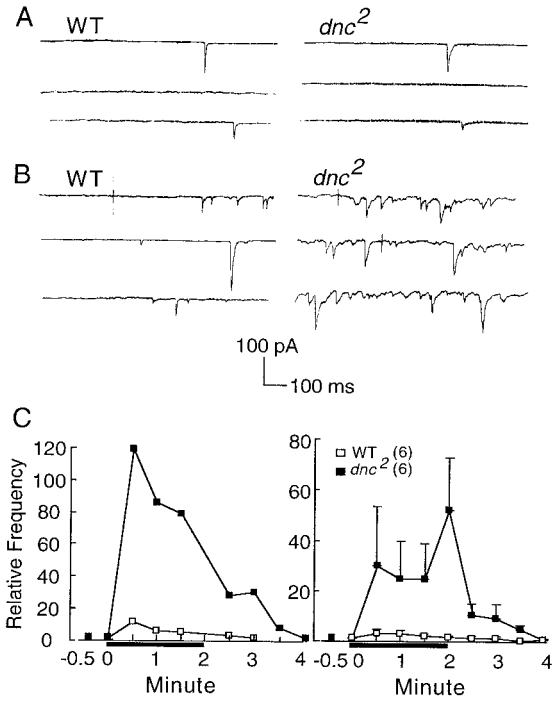

Figure 8. Effects of nerve stimulation on spontaneous ACh secretion in WT and *dnc* growth cones. *A*, *B*, Oscilloscope traces of spontaneous ACh currents from a WT cell and a dnc^2 cell before (*A*) and during (*B*, taken 30 sec after the onset of the stimulus train) low-frequency electric stimulation. The stimulus train (10 V, 0.5–1 msec, 0.5 Hz for 1 min) was delivered at the soma while recordings were made on myoballs manipulated into contact with the growth cones. Note the marked increase in spontaneous release frequency during the stimulus train, especially in the dnc^2 neuron (*B*). Stimulus artifacts are seen on some of the sweeps. *C*, Enhancement of spontaneous release frequency after nerve stimulation (*bar*) in WT and dnc^2 neurons. The time course of enhancement, which is presented as the relative frequency (normalized to the frequency before stimulation) against time, is compared between a WT and dnc^2 neuron (*left panel*). Mean ± SEM of relative release frequencies obtained from six WT and six dnc^2 neurons are shown in the *right panel*. Data point represents events collected in 30 sec bins.

There is a close resemblance in the transmitter release processes between the developing *Drosophila* growth cones and immature vertebrate presynaptic terminals. Both exhibit a skewed amplitude distribution toward smaller values, a lack of effective machinery for evoked release, and the capability of release from nonterminal regions. The rise and decay kinetics and the amount of charge movement per release in *Drosophila* growth cones also closely resemble those in *Xenopus* growth cones (Table 1), suggesting that similar amounts of ACh molecules are packaged into vesicles in neurons of these two species. In contrast, the release process from non-neuronal cells differs markedly from the spontaneous secretion processes in neurons. For example, ACh secretion from *Xenopus* myocytes and fibroblasts artificially loaded with ACh is far more prolonged and irregular as compared to ACh release from neuronal growth cones (Girod et al., 1995). This suggests that the neuronal release process may be highly conserved across phyla and distinctly different from the constitutive secretion in non-neuronal cells.

Function of the cAMP cascade in quantal ACh secretion and activity-dependent defects in mutant growth cones

It is now well established that *Drosophila* neurons share many molecular components of the transmitter release machinery with vertebrate neurons (Südhof, 1995; Wu and Bellen, 1997; Weber et al., 1998). The release of neurotransmitter is a multistep process that involves actions of proteins associated with the synaptic vesicle and the plasma membrane, as well as cytoplasmic proteins (Südhof, 1995, Weber et al., 1998). Some of these proteins, e.g., synapsin (Huttner et al., 1983), αSNAP (Hirling and Scheller, 1996), and Ca^{2+} channels (Leveque et al., 1994) are known to be the downstream targets of PKA. Phosphorylation of these proteins may be important for the regulation of vesicle mobilization, docking, and fusion.

In *Drosophila dnc* mutants, increased cAMP levels caused by the disruption of a phosphodiesterase lead to abnormalities in channel function and nerve excitability (Zhong and Wu, 1993; Zhao and Wu, 1997; Delgado et al., 1998), synaptic transmission and plasticity (Zhong and Wu, 1991; Engel and Wu, 1996), growth cone motility (Kim and Wu, 1996), and nerve arborization (Zhong et al., 1991). Using the present heterologous detection system, we were able to examine the altered transmitter release process in developing growth cones of *dnc* central neurons in isolation from the influence of postsynaptic targets. Examination of *PKA-RI* neurons suggests that the *dnc* defects in ACh secretion might be mediated by PKA. These results established a role for the cAMP cascade in the regulation of the secretion process in developing neurons before synaptogenesis. In light of the profound alterations in synaptic efficacy and activity-dependent modulation observed in mature synapses of *dnc* mutants (Zhong and Wu; 1991), the cAMP pathway may be involved throughout the maturation process of the synapse.

The effects of decreased cAMP levels on synaptic transmission have also been extensively studied in *Drosophila* (Zhong and Wu, 1991; Cheung et al., 1999). Intracellular recordings at the peripheral larval neuromuscular junction have revealed that chronically lowering cAMP causes reduced neurotransmitter release, likely because of reduction of innervation rather than impairment of transmitter release (Cheung et al., 1999). These results do not contradict our results obtained from developing central neurons. It will be important to determine how reduction in cAMP concentration affects neurotransmitter releases in the *Drosophila* central neurons in future studies.

The prolonged ACh currents of *dnc* and *PKA-RI* neurons may be attributable to increased ACh diffusion distance and altered presynaptic release mechanisms, as discussed above. A reduced efficiency in the formation of the exocytotic fusion pore and/or a disrupted fusion machinery may account for the prolonged release events for synaptic vesicles containing similar amounts of ACh. Exocytotic efficiency may be regulated by PKA-dependent phosphorylation of vesicular, cytoplasmic, and plasma membrane proteins involved in exocytosis. Additional mutational analysis will be required to identify the specific proteins that are targeted by PKA in this process.

Although the spontaneous release in neurons of all genotypes examined did not require Ca^{2+} influx, the activity-dependent increase in release frequency in *dnc* neurons after repetitive nerve stimulation appeared to depend on the external Ca^{2+}. It has been proposed that nerve activity regulates cAMP levels, possibly mediated by intracellular accumulation of Ca^{2+} through repetitive nerve spikes, which can trigger the Ca^{2+}/CaM activation of adenylyl cyclase (Zhong et al., 1991). The activity-dependent modification of transmission at mature synapses is known to be altered in *dnc* mutants (Zhong and Wu, 1991; Engel and Wu, 1996). Our results suggest that the cAMP pathway may mediate such activity-dependent regulation in developing neu-

rons before synaptogenesis as well, lending support to the notion that the cAMP pathway is important in a wide variety of neuronal processes throughout development.

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