cAMP-Dependent Plasticity at Excitatory Cholinergic Synapses in *Drosophila* Neurons: Alterations in the Memory Mutant *Dunce*

Daewoo Lee and Diane K. O'Dowd

Departments of Developmental and Cell Biology, Anatomy and Neurobiology, University of California at Irvine, Irvine, California 92697-1280

It is well known that cAMP signaling plays a role in regulating functional plasticity at central glutamatergic synapses. However, in the *Drosophila* CNS, where acetylcholine is thought to be a primary excitatory neurotransmitter, cellular changes in neuronal communication mediated by cAMP remain unexplored. In this study we examined the effects of elevated cAMP levels on fast excitatory cholinergic synaptic transmission in cultured embryonic *Drosophila* neurons. We report that chronic elevation in neuronal cAMP (in *dunce* neurons or wild-type neurons grown in db-cAMP) results in an increase in the frequency of cholinergic miniature EPSCs (mEPSCs). The absence of alterations in mEPSC amplitude or kinetics suggests that the locus of action is presynaptic. Furthermore, a brief exposure to db-cAMP induces two distinct changes in transmission at established cholinergic synapses in wild-type neu-

rons: a short-term increase in the frequency of spontaneous action potential-dependent synaptic currents and a long-lasting, protein synthesis-dependent increase in the mEPSC frequency. A more persistent increase in cholinergic mEPSC frequency induced by repetitive, spaced db-cAMP exposure in wild-type neurons is absent in neurons from the memory mutant *dunce*. These data demonstrate that interneuronal excitatory cholinergic synapses in *Drosophila*, like central excitatory glutamatergic synapses in other species, are sites of cAMP-dependent plasticity. In addition, the alterations in *dunce* neurons suggest that cAMP-dependent plasticity at cholinergic synapses could mediate changes in neuronal communication that contribute to memory formation.

Key words: *Drosophila*; nAChRs; cAMP-dependent plasticity; mEPSC; *dunce*; cholinergic synaptic transmission

Modulation of transmission at central synapses is important for processes ranging from development to learning and memory in both vertebrates and invertebrates. A search for the molecular mechanisms underlying synaptic modulation has revealed that the cAMP second messenger signaling cascade plays a crucial role in regulating functional plasticity at central synapses. This was initially demonstrated in studies of short-term facilitation at sensorimotor synapses in *Aplysia* (Brunelli et al., 1976; Castellucci et al., 1980). Long-term facilitation at these synapses is also regulated by cAMP signaling (Schacher et al., 1988; Dash et al., 1990; Kaang et al., 1993; Martin et al., 1997; Bartsch et al., 1998). In the rodent hippocampus, cAMP is involved in long-term potentiation, a form of functional plasticity at central excitatory glutamatergic synapses (Frey et al., 1993; Huang et al., 1994; Weisskopf et al., 1994; Nicoll and Malenka, 1995; Silva et al., 1998). In *Drosophila melanogaster*, the cAMP signal transduction cascade has also been implicated in regulating synaptic plasticity based on data obtained at the neuromuscular junction (NMJ), a peripheral glutamatergic synapse, where alterations in facilitation and post-tetanic potentiation were observed in mutants (*dunce* and *rutabaga*) with defects in cAMP signaling (Zhong and Wu, 1991). A number of the *Drosophila* mutants defining genes in the cAMP-signaling cascade were originally identified on the basis of asso-

ciative learning deficits (Dudai et al., 1976; Livingstone et al., 1984; Chen et al., 1986; Levin et al., 1992), suggesting that this second messenger system also regulates plasticity at central excitatory synapses. However, this hypothesis has not been directly tested. Therefore, identification of neuronal synapses at which transmission is regulated by cAMP represents an important step toward understanding the cellular and molecular events regulating functional plasticity in the *Drosophila* CNS.

In the insect CNS acetylcholine, as opposed to glutamate in vertebrates, is thought to be a primary excitatory neurotransmitter (Breer and Sattelle, 1987; Restifo and White, 1990). This is supported by electrophysiological recordings demonstrating that acetylcholine mediates functional transmission in the brain of the adult honeybee (Oleskevich, 1999). While nicotinic AChRs (Schuster et al., 1993; Jonas et al., 1994) and choline acetyltransferase (ChAT)-positive neurons (Yasuyama et al., 1995) are found throughout the *Drosophila* CNS, the location and size of the cholinergic synapses have so far precluded analysis of their functional properties in the fly brain. The recent demonstration in our lab that nAChRs mediate the predominant form of fast excitatory synaptic transmission between neurons grown in cell culture (Lee and O'Dowd, 1999), provides the first opportunity to examine the role of cAMP in regulating plasticity at interneuronal synapses in *Drosophila*. Here we report that cAMP signaling regulates transmission and plasticity at excitatory cholinergic synapses in *Drosophila* neurons. We also observed alterations in basal levels of transmission and cAMP-dependent plasticity at cholinergic synapses in neurons from the memory mutant *dunce*. These data suggest that cAMP-dependent plasticity at cholinergic synapses may contribute to changes in neuronal communication important for learning and memory in the fly.

Received Oct. 6, 1999; revised Dec. 27, 1999; accepted Jan. 4, 2000.

This work was supported by National Institutes of Health Grant NS27501 and Research Career Development Award NS01854 to D.K.O. In addition, this work benefited from a generous gift from Merck. We thank Drs. M. A. Smith and A. Agmon for helpful comments on an earlier version of this manuscript.

Correspondence should be addressed to Diane K. O'Dowd, Department of Anatomy and Neurobiology, University of California at Irvine, Irvine, CA 92697-1280. E-mail: dkodowd@uci.edu.

Copyright © 2000 Society for Neuroscience 0270-6474/00/202104-08\$15.00/0

MATERIALS AND METHODS

Fly strains

The wild-type (wt1) population in this study was a Canton-S stock. Two of the *dunce* alleles, *dnc¹* and *dnc²* (*y dnc² ec f*), were generated in a Canton-S background by S. Benzer's group (Dudai et al., 1976; Byers et al., 1981). The third *dunce* allele examined, *dnc^{M11}*, was isolated during an independent mutagenesis (Mohler, 1977) on the basis of female sterility and was subsequently shown to be allelic to *dnc¹* and *dnc²*. *dnc¹* was obtained from T. Tully, *dnc²* from C.-F. Wu, and *dnc^{M11}* from C. Goodman.

Cultures

Cultures were prepared from two midgastrula stage embryos from each genotype indicated, plated on uncoated glass coverslips, and grown in a *Drosophila*-defined medium (DDM1) as described previously (O'Dowd, 1995). db-cAMP (Calbiochem, La Jolla, CA), staurosporine (Sigma, St. Louis, MO), and cycloheximide (Sigma) were added to the DDM1 as indicated in specific experiments. Controls for biochemical manipulations included mock-treated cultures that were exposed to solution changes in parallel with sibling cultures undergoing chemical treatments. There were no statistically significant differences observed in the mock and untreated cultures, and therefore these were pooled for statistical analyses.

Electrophysiology

The whole-cell recording technique was used to record EPSCs using pipettes (3–6 MΩ) filled with internal solution containing (in mM): 120 CsOH, 120 D-glucuronate, 0.1 CaCl₂, 2 MgCl₂, 20 NaCl, 1.1 EGTA, and 10 HEPES, pH 7.2. The external solution contained (in mM): 140 NaCl, 1 CaCl₂, 4 MgCl₂, 3 KCl, and 5 HEPES, pH 7.2. All data were recorded at a holding voltage of −75 mV. EPSCs recorded in the absence of tetrodotoxin (TTX) were defined as spontaneous EPSCs (sEPSCs), and those recorded in the presence of 1 μM TTX (Sigma) were defined as mEPSCs. Bath application of D-tubocurarine (100 nM; Aldrich, Milwaukee, WI) blocked the EPSCs, whereas bath application of CNQX (5 μM), APV (50 μM), bicuculline (2 μM), and picrotoxin (10 μM) did not affect these currents. Data were collected using an Axopatch 1D (Axon Instruments, Foster City, CA) amplifier, a Digidata analog-to-digital converter (Axon Instruments), a Pentium-equipped Gateway 2000 computer, and either pClamp6 (Axon Instruments) or SCAN (SES; University of Strathclyde, Scotland) software.

All whole-cell recordings were performed between 3 and 9 d *in vitro* (DIV). The neurons selected for electrophysiological analysis were physically contacted by neurites from neighboring cells. More than 50% of the recordings were obtained blind with respect to genotype and/or treatment condition. There was no significant difference in the blind versus nonblind data sets, and therefore these were grouped for statistical analyses.

sEPSC incidence. The incidence of detecting cholinergic sEPSCs was determined by monitoring activity in each neuron for at least 2 min, in which a stable whole-cell recording was obtained. Neurons in which sEPSC frequency was <0.1 Hz were scored as negative, whereas cells with sEPSC frequency of ≥0.1 Hz, were scored as positive. The incidence of positive neurons on each day was determined by dividing the number of sEPSC-positive neurons by the total number of neurons examined.

sEPSC, mEPSC frequency. These were calculated for each neuron in which synaptic currents occurring at ≥0.1 Hz were observed. Frequency was determined by recording 20–100, 500 msec current traces, filtered at 2 kHz, acquired using pClamp6 software. Individual events were detected using an automated mini-detection software (Mini Analysis Program, Synaptosoft, NJ) with threshold criteria having an amplitude of 10 pA (fourfold greater than the RMS noise level of ~2.5 pA) and a charge transfer of 7 fC. All records were obtained between 2 and 7 min after breaking into the whole-cell configuration. During this time there was no significant run down in frequency in any of the genotypes or conditions examined. mEPSC biophysical properties were determined from records acquired at 20 kHz using SCAN, a trigger-based detection program with an amplitude threshold of 10 pA. The mean amplitude and rise time were determined by averaging the values obtained from 25 or more single events in each neuron. Decay time constants were determined by fitting a single exponential distribution to the falling phase of the averaged mEPSC from each neuron. mEPSCs with slow rise and decay kinetics caused by electrotonic decay were excluded from this analysis.

Figure 1. Elevated cholinergic sEPSC frequency in *dnc* mutant neurons. *A*, Examples of typical recordings of rapid transient sEPSCs obtained from a single wt1 and a single *dnc¹* neuron at 5 DIV. Each record represents four superimposed current traces. Currents in both genotypes are reversibly blocked by bath perfusion of 100 nM curare. *B*, The average sEPSC frequency is significantly higher in *dnc¹* neurons when compared with wt1 (Student's *t* test, *p* < 0.01). All recordings were obtained between 3 and 9 DIV. Error bars indicate SEM; number of neurons indicated in parentheses.

RESULTS

Altered transmission at cholinergic synapses in *dnc* mutant neurons

To determine if cAMP signaling is important in regulating synaptic transmission at central synapses, we examined cholinergic transmission at synapses formed between cultured neurons from *dunce¹* (*dnc¹*) embryos in which a defective phosphodiesterase leads to chronically elevated levels of cAMP (Davis et al., 1995). sEPSCs, composed of both action potential (AP)-dependent and AP-independent components, were recorded in *dnc¹* neurons between 3 and 9 DIV (Fig. 1*A*). These currents were blocked by bath application of curare, indicating that they were mediated by nicotinic AChRs (Fig. 1*A*). The incidence of detecting cholinergic sEPSCs in *dnc¹* neurons was 48% (23 of 48), not different than the incidence rate 51% (77 of 152) observed in wild-type (wt1) neurons. In contrast, the mean sEPSC frequency in *dnc¹* mutant neurons was significantly higher than in wt1 neurons (Fig. 1*A,B*). These data suggested that a mutation in the *dnc* gene alters AP-dependent and/or AP-independent transmission at cholinergic synapses.

Figure 2. Elevated cholinergic mEPSC frequency in *dnc* mutant neurons. *A*, mEPSC recordings obtained from indicated genotype in the presence of 1 μ M TTX. Averaged mEPSC on a faster time scale from a single neuron is shown for each genotype (traces were normalized to the same peak current amplitude). *B*, The mEPSC frequencies in *dnc*¹ and *dnc*² neurons are significantly higher (fourfold) than wt1 (ANOVA, * p < 0.05; ** p < 0.005 Fisher's protected least significant difference). All recordings obtained between 3 and 9 DIV. *C*, Overlapping cumulative probability amplitude histograms at a holding potential of -75 mV constructed from wt1 (1895 individual mEPSCs recorded from 13 wt1 neurons) and *dnc*¹ (763 mEPSCs from 8 *dnc*¹ neurons).

Previous studies have documented changes in the properties of voltage-gated potassium currents (Delgado et al., 1998) and excitability of *dnc* mutant neurons (Zhao and Wu, 1997) that may contribute to the difference observed in sEPSC frequency between wt1 and *dnc*¹ neurons. To determine if there were changes in synaptic transmission, independent of possible alterations in presynaptic excitability, miniature excitatory postsynaptic currents (mEPSCs) resulting from AP-independent transmitter release were examined. The average mEPSC frequency recorded in *dnc*¹ mutant neurons was significantly higher (fourfold) than wt1 neurons (Fig. 2*A,B*). The mean mEPSC frequency observed in neurons from embryos homozygous for a second mutant allele at the *dunce* locus (*dnc*²) was similar to that recorded from *dnc*¹ (Fig. 2*A,B*), supporting the hypothesis that this phenotype is directly related to mutations in the *dunce* gene. However, because these two *dnc* alleles were isolated in the same screen, it is possible that a second mutation in the background stock is responsible for the phenotype we report. Therefore, to confirm that the changes in EPSCs are caused by mutations in the *dunce* gene, we examined *dnc*^{M11}, an independently isolated *dunce* mutant (Mohler, 1977). The *dnc*^{M11} flies are sterile as homozygotes and therefore are maintained over a balancer chromosome (FM7c). Because we were unable to find a marked balancer stock for identification of

homozygous mutant embryos at midgastrula stage, we prepared single cultures from four embryos harvested from the *dnc*^{M11}/FM7c parental stock. These cultures contained a genetically heterogeneous population of neurons in which, on average, 25% were homozygous for the *dnc*^{M11} mutation. Analysis of mEPSC frequency revealed a significant increase (p < 0.01, Student's *t* test) in neurons in the *dnc*^{M11} cultures (2.5 ± 0.82 Hz) when compared to wt1 neurons (1.1 ± 0.17 Hz). These data, in conjunction with the similar changes we observed in neurons within the genetically homogeneous *dnc*¹ and *dnc*² cultures, strongly support the hypothesis that the increase in EPSC frequency is caused by a mutation in the *dunce* locus, as opposed to an unidentified background mutation.

In contrast to the increase in frequency, no significant differences were observed in the biophysical properties of the mEPSCs recorded from mutant and wild-type neurons indicated by similar mean amplitudes and kinetic properties (Fig. 2*A*, Table 1). In addition, cumulative probability amplitude distributions indicate a similar distribution of event sizes in *dnc*¹ and wild-type neurons (Fig. 2*C*). These data demonstrate that mutations in the *dnc* gene alter AP-independent transmission at interneuronal cholinergic synapses. The increased mEPSC frequency in the absence of alterations in amplitude or kinetic properties further suggests that the locus of the change is presynaptic.

Chronic elevation of cAMP levels in wild-type neurons results in a *dnc* phenocopy

If the changes in cholinergic transmission observed in *dnc* mutant neurons are directly related to a constitutive increase in neuronal cAMP levels, then it should be possible to mimic the mutant phenotype by increasing basal cAMP levels in wild-type neurons. Neurons, dissociated from wt1 embryos at midgastrula stage, were plated in a defined medium (DDM1) containing 0.5–500 μ M dibutyryl-adenosine-3',5'-cyclic monophosphate (db-cAMP). Cholinergic transmission was examined in cultured neurons between 3 and 9 DIV, in normal physiological saline, for the 4 hr after removal from high-cAMP growth media. These analyses revealed a concentration-dependent increase in sEPSC frequency that saturated at ~ 100 μ M db-cAMP (Fig. 3*A,B*).

To assess the effects of differentiation in high cAMP on AP-independent transmission, mEPSCs were monitored in wt1 neurons chronically exposed to 100 μ M db-cAMP. These studies revealed a high mEPSC frequency (~ 7 Hz) in db-cAMP-treated neurons examined between 3 and 8 DIV (Fig. 3*D*). As previously reported (Lee and O'Dowd, 1999), a stable mEPSC frequency of ~ 1 Hz was observed in the untreated wt1 neurons over this same time period (Fig. 3*D*). Data pooled for the whole time period demonstrate that the mean mEPSC frequency in db-cAMP-treated neurons (6.63 ± 0.81 Hz; $n = 40$) was significantly higher than untreated neurons (1.12 ± 0.17 Hz; $n = 56$) (p < 0.001, Student's *t* test). There were no changes in the sEPSC incidence rate of 52% (50 of 96) or in the mEPSC biophysical properties in wt1 neurons differentiating in presence or absence of 100 μ M db-cAMP (Table 1). The similarity between the mean sEPSC and mEPSC frequencies in wt1 neurons grown in db-cAMP (Fig. 3*B,D*) indicate that the predominant change involves an upregulation in the AP-independent component of the synaptic currents. These data suggest that the chronically elevated level of neuronal cAMP is likely to be the primary defect resulting in the increase in AP-independent transmission at cholinergic synapses in *dnc* mutant neurons.

Table 1. Biophysical properties of cholinergic mEPSCs in wild-type and *dunce* neurons

Genotype	Amplitude (pA)	Rise time (msec)	Tau (msec)	<i>n</i>
wt1	23.7 ± 2.0	0.61 ± 0.05	2.2 ± 0.2	24
wt1+db-cAMP	20.9 ± 1.7	0.71 ± 0.08	2.3 ± 0.4	6
<i>dnc</i> ¹	21.4 ± 1.0	0.66 ± 0.05	2.3 ± 0.4	8
<i>dnc</i> ²	20.2 ± 0.9	0.56 ± 0.06	2.0 ± 0.3	5

wt+db-cAMP, Cells were plated directly into DDM1 containing 100 μ M db-cAMP and were grown in this medium for 3–9 d.

Figure 3. Differentiation of wild-type neurons in db-cAMP results in a *dnc* phenocopy. *A*, Ten superimposed sEPSC recordings obtained from two wt1 neurons (4 DIV) that were grown in either 1 μ M (top) or 100 μ M (bottom) db-cAMP. *B*, Dose-dependent increase in sEPSC frequency. Neurons were grown in the indicated concentration of db-cAMP and examined between 3 and 9 DIV. A sigmoidal fit to the data indicated an EC_{50} of $17.7 \pm 8.1 \mu$ M. *C*, mEPSCs recorded from four wt1 neurons grown in the absence of db-cAMP (first trace), presence of 100 μ M db-cAMP (second trace), and 1 (third trace) or 2 (fourth trace) days after removal of db-cAMP at 3 DIV. *D*, The average mEPSC frequency in wt1 neurons grown in the presence of 100 μ M db-cAMP (squares) was approximately sevenfold higher than that observed in the untreated wt1 neurons (circles) at all ages examined. In contrast, wt1 neurons that were grown in the presence of 100 μ M db-cAMP for 3 DIV and then switched to db-cAMP-free media (triangles), showed an elevated mEPSC frequency at 1 d after removal of db-cAMP that returned to baseline levels by 2 d. All recordings were obtained in normal external saline at room temperature. Error bars indicate SEM. All data points represent values obtained from between 8 and 38 neurons examined in three or more separate platings.

In both the *dnc* neurons and wt1 neurons chronically treated with db-cAMP, the alterations in mEPSC frequency could be caused by permanent changes induced during neuronal differentiation in the presence of high cAMP levels or could alternatively be linked to the time or duration of exposure. To distinguish between these possibilities, wild-type neurons were grown for 3 d in high cAMP and then returned to control growth medium for varying length of time. A time-dependent decrease in mEPSC

frequency after removal of the db-cAMP was observed, and by 48 hr the mEPSC frequency was indistinguishable from control neurons (Fig. 3*D*). These data demonstrate that high cAMP levels during differentiation do not irreversibly alter transmission at cholinergic synapses.

Modulation of cholinergic transmission by transient elevation in cAMP in differentiated wild-type neurons

To further explore the temporal relationship between elevation of cAMP levels and cholinergic transmission, we asked if transmission at established synapses could be altered by brief elevations in cAMP levels. To address this question, wt1 neurons were plated in DDM1, allowed to develop in the absence of db-cAMP for 3 d, then transferred to DDM1 containing 100 μ M db-cAMP for exposure periods ranging from 30 min to 8 hr (Fig. 4). After a rapid wash in fresh DDM1, sEPSCs were monitored in normal physiological recording saline for the next 4 hr. Although the mean sEPSC frequency was slightly elevated after a 30 min and 1 hr db-cAMP exposure, a 2 hr treatment was necessary to induce a significant increase over control cultures (Fig. 4). Both a 4 and 8 hr exposure produced a threefold increase in sEPSC frequency. This increase was blocked in the presence of staurosporine, a general protein kinase inhibitor (Fig. 4). These findings demonstrate that acute elevation of cAMP levels mediates a rapid onset, protein-kinase dependent modulation of AP-dependent transmission at cholinergic synapses, that persists for up to 4 hr after termination of the stimulus, in differentiated wild-type neurons.

In contrast to the increase in sEPSC frequency, there was no significant change in the mEPSC frequency when monitored 0–4 hr after termination of a 4 hr db-cAMP exposure (Fig. 4). However, analysis of mEPSCs for 36 hr after a transient increase in cAMP levels revealed a progressive, time-dependent increase in frequency (Fig. 5). The peak increase (fourfold) occurred at 24 hr after termination of the stimulus. The increase in mEPSC frequency was blocked when cycloheximide, a protein synthesis inhibitor, was present during the 4 hr db-cAMP exposure and subsequent 20 hr before recording. This blockade was not simply attributable to a nonspecific toxic effect of the inhibitor because there were no changes in the mEPSC frequencies in sister cultures exposed to cycloheximide alone (Fig. 5). These results clearly demonstrate that a brief elevation of cAMP levels in differentiated wild-type neurons can induce a delayed-onset, protein synthesis-dependent increase in AP-independent transmission at cholinergic synapses.

Repetitive, spaced application enhances persistence of cAMP-induced increase in mEPSC frequency in differentiated wild-type neurons

The increase in mEPSC frequency seen after a single 4 hr treatment with db-cAMP peaked at 24 hr and by 48 hr declined to levels not different than seen in control cultures (Fig. 6*B*). Previous studies in *Aplysia* have demonstrated that the persis-

Figure 4. Acute elevation in cAMP levels mediates a rapid onset, protein kinase-dependent increase in AP-dependent transmission at cholinergic synapses in wild-type neurons. *A*, Schematic of treatment protocol. sEPSCs were recorded in standard external saline 0–4 hr after termination of the db-cAMP pulse. *B*, Increasing the duration of cAMP exposure, between 0.5 and 4 hr, induced an increasing elevation in sEPSC frequency when compared with controls (* $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$, ANOVA, Scheffé's *post hoc* analysis). There was no further increase in sEPSC frequency when the db-cAMP exposure time was increased to 8 hr. When 50 nM staurosporine was present during the 4 hr db-cAMP exposure, the increase in sEPSC frequency was completely blocked, indicating a protein kinase-dependent mechanism. The mEPSC frequency was not significantly different in neurons immediately after 4 hr db-cAMP exposure compared with controls (value at db-cAMP concentration of 0). Error bars indicate SEM. All means represent data obtained from between 10 and 23 neurons examined in three or more separate platings.

tence of plasticity mediated by cAMP signaling at sensorimotor synapses is related to the temporal pattern of the stimulus: repetitive, spaced application of 5-HT, a stimulus that activates cAMP signaling, induced a longer lasting increase in synaptic strength than a single pulse (Montarolo et al., 1986; Glanzman et al., 1990). To determine if there was a similar relationship between the temporal pattern of db-cAMP exposure and the persistence of cellular changes at the *Drosophila* cholinergic synapses, differentiated wt1 neurons were repetitively exposed to db-cAMP. The total exposure time remained at 4 hr but was administered as four, 1 hr applications, separated by 30 min intervals in normal media (Fig. 6*A*, *Spaced*). The mean mEPSC frequency at 24 hr was slightly higher than that induced by a single 4 hr treatment with db-cAMP (Fig. 6*B*). However, more dramatic was the persistent increase in mEPSC frequency induced by the spaced treatment paradigm evident at 2 and 3 d after exposure (Fig. 6*B*). These findings suggest that the rest intervals between short db-cAMP exposures are important in mediating persistent alterations in mEPSC frequency.

Figure 5. Acute elevation in cAMP levels mediates a delayed onset, protein synthesis-dependent increase in AP-independent transmission at cholinergic synapses in wild-type neurons. *A*, Schematic of treatment protocol. mEPSCs were recorded in standard external saline at the indicated times (± 2 hr) after termination of the db-cAMP pulse. *B*, There was a gradual increase in mEPSC frequency as a function of increasing time after termination of the db-cAMP treatment. The peak mEPSC frequency (a fourfold increase) was observed at 24 hr and subsequently declined over the next 8 hr. When 100 μ M cycloheximide was present during the 4 hr db-cAMP exposure as well as the subsequent 20 hr, the increase in mEPSC frequency was completely blocked. Cycloheximide (CXM) alone for 24 hr resulted in mEPSC frequencies that were not different than the controls. Error bars indicate SEM. All data points represent values obtained from between 8 and 32 neurons examined in three or more separate platings.

Reduced cAMP-dependent plasticity in *dnc* mutant neurons

We next asked if, in addition to changes in basal level of cholinergic transmission, the ability of *dnc* neurons to respond to transient changes in cAMP levels was compromised. In *dnc*¹ neurons, both a single pulse and repetitive, spaced 4 hr exposure to db-cAMP resulted in a small, although not statistically significant, increase in mEPSC frequency over control levels 1 d after treatment (Fig. 6*C*). However, in marked contrast to the results obtained in wild-type neurons, no persistent increase in mEPSC frequency in *dnc* mutant neurons was observed after spaced exposure to db-cAMP (Fig. 6*C*). It seems unlikely that the lack of ability to induce a persistent change in *dnc* neurons with spaced db-cAMP application is attributable to a ceiling effect because the basal mEPSC frequency seen in both *dnc* mutant alleles was 3–4 Hz, well below the maximal frequency that could be induced in wt1 neurons after either chronic or spaced exposure to 100 μ M db-cAMP. These data demonstrate altered cAMP-dependent plasticity at cholinergic synapses in *dnc* mutant neurons.

DISCUSSION

In this study we demonstrate that neuronal cAMP levels can regulate functional plasticity, independent of differentiation, at

Figure 6. A persistent increase in mEPSC frequency induced by repetitive, spaced db-cAMP in differentiated wild-type neurons is absent in neurons from the memory mutant *dunce*. **A**, db-cAMP treatment and recording paradigm. **B**, A single 4 hr exposure to db-cAMP results in a delayed onset increase in mEPSC frequency that peaks at 24 hr and returns to control levels 2 d after treatment in wt1 neurons. In contrast, repetitive space application of db-cAMP resulted in a slightly higher mEPSC frequency at 24 hr with a persistent elevation in frequency maintained for up to 3 d after treatment. (* $p < 0.05$, ** $p < 0.01$, Student's t test, treated vs control). These data suggest that rest intervals are important in mediating persistent changes initiated by elevation in cAMP levels. **C**, *dunce* neurons have a higher basal mEPSC frequency than wt1. However, the mEPSC frequencies in *dnc¹* neurons treated for 4 hr with db-cAMP (single and repetitive spaced exposure) were only slightly elevated at 1 d after treatment when compared with the control *dnc¹* neurons. The mean mEPSC frequencies in *dnc¹* neurons at 2–3 d after either db-cAMP treatment paradigm were not different than the control neurons, indicating a reduction in cAMP-induced plasticity in *dnc* mutants. Error bars indicate SEM. All data points represent values obtained from between 9 and 32 neurons examined in three or more separate platings.

cholinergic synapses between cultured *Drosophila* neurons. Although numerous studies have demonstrated that the cAMP signaling cascade plays a role in modulating plasticity at central glutamatergic (hippocampus) and presumed glutamatergic (*Aplysia*) (Trudeau and Castellucci, 1993; Murphy and Glanzman, 1997) synapses, this is the first direct evidence that cAMP regulates plasticity at central cholinergic synapses mediating fast ex-

citatory transmission. As acetylcholine appears to be a primary excitatory neurotransmitter in the fly brain, these data are consistent with the hypothesis that cAMP-dependent plasticity at cholinergic synapses mediates changes in neuronal communication in the *Drosophila* CNS. Although the majority of fast excitatory synaptic transmission in the mammalian brain is mediated by glutamate, recent reports indicate the presence of fast synaptic signaling via nAChRs in both the rodent hippocampus (Frazier et al., 1998) and visual cortex (Roerig et al., 1997). In light of our findings in *Drosophila*, it seems likely that cAMP may also be important in modulating fast cholinergic synaptic transmission in the mammalian CNS.

Our data demonstrating a significant increase in mEPSC frequency in three different *dunce* alleles, isolated in two independent screens, strongly support the hypothesis that mutations in *dunce*, a gene encoding a cAMP phosphodiesterase, result in the alterations in cholinergic synaptic transmission we report. Previous studies have demonstrated that the phosphodiesterase activity is higher in the *dnc¹* versus *dnc²* mutant (Tully and Gold, 1993). However, the cAMP levels in these two *dunce* alleles are similar and significantly higher than wild-type (Byers et al., 1981). The similarity in the mEPSC frequency in *dnc¹* and *dnc²* and the observation that chronic exposure to db-cAMP induces a concentration-dependent increase in mEPSC frequency in wild-type neurons, are consistent with the suggestion that elevated levels of cAMP in the mutant regulates mEPSC frequency. The smaller increase in mEPSC frequency in neurons within the *dnc^{M11}*, compared to *dnc¹* and *dnc²*, cultures is not unexpected. The *dnc^{M11}* cultures were genetically heterogenous with only 25% of the neurons homozygous for the mutant allele. In contrast, in the *dnc¹* and *dnc²* cultures, all of the neurons were genetically homozygous for the mutations in the *dnc* locus. Assessment of mEPSC frequency after experimental manipulations resulting in a reduction in cAMP levels in *dnc* mutant neurons will be important in further examining the role of cAMP in regulation of synaptic transmission in the mutant neurons.

In our study, brief exposures of cultured wild-type neurons to db-cAMP demonstrated that the cAMP-signaling cascade is involved in both short-term and long-lasting modulation of activity at cholinergic synapses in *Drosophila*. The short-term change was characterized by a rapid onset increase in cholinergic sEPSC frequency in differentiated wild-type neurons. Because there were no immediate changes in AP-independent synaptic transmission, and the increase in sEPSC frequency was blocked in the presence of a protein kinase inhibitor (staurosporine), it suggests that the alterations involve posttranslational modifications of existing proteins that do not affect the synaptic machinery involved in mediating constitutive release. The increase in AP-dependent release was transient in that the majority of the elevation in synaptic current frequency observed 24 hr after cAMP exposure could be accounted for by AP-independent synaptic currents. In *Aplysia*, it has been shown that cAMP, through protein kinase A (PKA), mediates a phosphorylation-induced reduction in conductance of existing potassium channels resulting in a rapid, transient increase in neuronal excitability contributing to short-term facilitation (Byrne and Kandel, 1995). Previous studies have demonstrated that cAMP-dependent phosphorylation induces a rapid and reversible decrease in outward potassium currents in *Drosophila* neurons (Wright and Zhong, 1995; Delgado et al., 1998). This suggests that an increase in AP duration and/or excitability, similar to that seen in *Aplysia* sensory neurons, may contribute to

the rapid cAMP-induced increase in sEPSC frequency at cholinergic synapses in *Drosophila* neurons.

The long-lasting change induced by brief db-cAMP exposure in cultured *Drosophila* neurons was characterized by a delayed onset increase in mEPSC frequency that peaked 24 hr after exposure and was blocked by cycloheximide. The time course, requirement for *de novo* protein synthesis, and the indication that the changes are presynaptic (absence of changes in the biophysical properties of each mEPSC), are consistent with cAMP-inducing synaptic growth resulting in an increase in number of functionally identical cholinergic release sites. A similar mechanism has been proposed to underlie cAMP-induced long-term facilitation in *Aplysia* where studies in cell culture have revealed that enhancement of synaptic strength between identified sensory and motor neurons, observed at 24 hr after the stimulus, requires protein and RNA synthesis and the growth of new synaptic connections between the neurons (Montarolo et al., 1986; Schacher et al., 1988; Castellucci et al., 1989; Dash et al., 1990; Glanzman et al., 1990; Bailey and Kandel, 1993). However, further analysis will be necessary to determine if cAMP regulates evoked transmitter release at cholinergic synapses in *Drosophila* and if so whether the mechanism involves regulation of the number of synaptic sites or affects other processes such as efficacy of transmission at each bouton.

It was first demonstrated almost 15 years ago, in *Aplysia* cell culture, that while a single pulse (1×) of serotonin could induce cAMP-dependent short-term facilitation, repetitive spaced (5×) application of serotonin was necessary to induce cAMP-dependent long-term facilitation (Montarolo et al., 1986). Subsequent studies revealed that consolidation of changes induced by spaced stimuli involved cAMP response element-binding protein (CREB)-mediated gene transcription (Dash et al., 1990; Kaang et al., 1993; Bartsch et al., 1995, 1998). A role for cAMP-CREB-mediated transcription has also been demonstrated in long-term potentiation at glutamatergic synapses in the rodent hippocampus (Frey et al., 1993; Abel et al., 1997). Furthermore, studies in *Drosophila* indicate that cAMP-initiated changes in CREB activity play a role in long-term synaptic enhancement at peripheral glutamatergic synapses (Davis et al., 1996). Our observation that repetitive spaced treatments with db-cAMP induced a more persistent change than a single db-cAMP treatment suggests that plasticity at cholinergic synapses induced by spaced exposure to db-cAMP in *Drosophila* involves activation of CREB-mediated gene transcription. It will be possible to test this hypothesis by examining cAMP-dependent modulation of cholinergic transmission in neurons from transgenic flies carrying CREB activators (Yin et al., 1995) and CREB inhibitors (Yin et al., 1994).

Our data clearly demonstrate that cAMP plays an important role in short-term modulation of transmission, as well as initiating events that contribute to long-lasting synaptic changes requiring new protein synthesis, at interneuronal cholinergic synapses in *Drosophila*. Several lines of evidence support the hypothesis that the cAMP-dependent regulation of cholinergic plasticity we report in the cultured neurons is likely to represent a mechanism involved in modulation of functional transmission important for behavior in the adult fly. First, we found that repetitive spaced exposure to db-cAMP induced a more persistent increase in mEPSC frequency than an equivalent length single exposure in wild-type *Drosophila* neurons. These results represent a remarkable parallel to those of behavioral studies in *Drosophila* demonstrating that repetitive spaced trails, where an olfactory stimulus is paired with a foot shock, induced more persistent memory than

an equivalent number of training trials presented in the absence of a rest interval between trials in wild-type flies (Tully et al., 1994). Second, our data from *dnc* mutant neurons reveals that chronic disruption of cAMP signaling in neurons, previously shown to result in associative learning deficits in the fly (Dubnau and Tully, 1998), alters the basal levels of AP-independent transmission at cholinergic synapses. Even more significant was the finding that we were unable to induce a persistent increase in mEPSC frequency by spaced exposure to db-cAMP in the *dnc* mutant neurons. The inability of *dnc* neurons to respond to transient, activity-induced changes in cAMP levels that are likely to occur during olfactory training episodes in the adult fly, could contribute to the reduced performance index when compared with wild-type. Finally, although there are no studies directly demonstrating cholinergic synaptic transmission in the adult *Drosophila* CNS, a recent report documents cholinergic transmission at synapses in the mushroom body of the adult honeybee (Oleskevich, 1999), an anatomical structure critical for learning and memory in *Drosophila* (Davis, 1993) as well as the honeybee (Menzel and Muller, 1996). Taken together these findings support the hypothesis that cholinergic synapses in *Drosophila*, similar to central glutamatergic synapses in other species (Nicoll and Malenka, 1995; Milner et al., 1998), are sites of cAMP-dependent synaptic plasticity important for learning and memory. Insights gained from functional studies in this *Drosophila* culture system, well suited to molecular genetic and biochemical manipulations, will be useful in delineating the molecular mechanisms underlying modulation of central synaptic transmission, a process thought to contribute to learning and memory in all animals.

REFERENCES

- Abel T, Nguyen PV, Barad M, Deuel TAS, Kandel ER, Bourchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* 88:615–626.
- Bailey CH, Kandel ER (1993) Structural changes accompanying memory storage. *Annu Rev Physiol* 55:397–426.
- Bartsch D, Ghirardi M, Skehel PA, Karl KA, Herder SP, Chen M, Bailey CH, Kandel ER (1995) *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. *Cell* 83:979–992.
- Bartsch D, Casandio A, Karl KA, Serodio P, Kandel ER (1998) CREB1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation. *Cell* 95:211–223.
- Breer H, Sattelle DB (1987) Molecular properties and functions of insect acetylcholine receptors. *J Insect Physiol* 33:771–790.
- Brunelli M, Castellucci V, Kandel ER (1976) Synaptic facilitation and behavioral sensitization of the gill-withdrawal reflex in *Aplysia*: possible role of serotonin and cyclic AMP. *Science* 194:1178–1181.
- Byers D, Davis RL, Kiger JA (1981) Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. *Nature* 289:79–81.
- Byrne JH, Kandel ER (1995) Presynaptic facilitation revisited: state and time dependence. *J Neurosci* 16:425–435.
- Castellucci VF, Kandel ER, Schwartz JH, Wilson FD, Nairn AC, Greengard P (1980) Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase stimulates facilitation of transmitter release underlying behavioral sensitization in *Aplysia*. *Proc Natl Acad Sci USA* 77:7492–7496.
- Castellucci VF, Blumenfeld H, Golet P, Kandel ER (1989) Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of *Aplysia*. *J Neurobiol* 20:1–9.
- Chen C-N, Denomen S, Davis RL (1986) Molecular analysis of cDNA clones and the corresponding genomic encoding sequences of the *Drosophila dunce* gene, the structural gene for cAMP phosphodiesterase. *Proc Natl Acad Sci USA* 83:9313–9317.
- Dash PK, Hochner B, Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. *Nature* 345:718–721.

- Davis GW, Schuster CM, Goodman CS (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* 17:669–679.
- Davis RL (1993) Mushroom bodies and *Drosophila* learning. *Neuron* 11:1–14.
- Davis RL, Cherry J, Dauwalder B, Han PL, Skoulakis E (1995) The cyclic AMP system and *Drosophila* learning. *Mol Cell Biochem* 149/150:271–278.
- Delgado R, Davis R, Bono MR, Latorre R, Labarca P (1998) Outward currents in *Drosophila* larval neurons: *dunce* lacks a maintained outward current component downregulated by cAMP. *J Neurosci* 18:1399–1407.
- Dubnau J, Tully T (1998) Gene discovery in *Drosophila*: new insights for learning and memory. *Annu Rev Neurosci* 21:407–444.
- Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) *dunce*, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci USA* 73:1684–1688.
- Frazier CJ, Buhler AV, Weiner JL, Dunwiddie TV (1998) Synaptic potentials mediated via α -Bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. *J Neurosci* 18:8228–8235.
- Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* 260:1661–1664.
- Glanzman DL, Kandel ER, Schacher S (1990) Target-dependent structural changes accompanying long-term synaptic facilitation in *Aplysia* neurons. *Science* 249:799–802.
- Huang Y-Y, Li X-C, Kandel ER (1994) cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late phase. *Cell* 79:69–79.
- Jonas PE, Phannavong B, Schuster R, Schroder C, Gundelfinger ED (1994) Expression of the ligand-binding nicotinic acetylcholine receptor subunit D α 2 in the *Drosophila* central nervous system. *J Neurobiol* 25:1494–1508.
- Kaang BK, Kandel ER, Grant SG (1993) Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons. *Neuron* 10:427–435.
- Lee D, O'Dowd DK (1999) Fast excitatory synaptic transmission mediated by nicotinic acetylcholine receptors in *Drosophila* neurons. *J Neurosci* 19:5311–5321.
- Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, Reed RR (1992) The *Drosophila* learning and memory gene *rutabaga* encodes a Ca^{2+} /calmodulin-responsive adenylyl cyclase. *Cell* 68:479–489.
- Livingstone MS, Sziber PP, Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylyl cyclase of *rutabaga*, a *Drosophila* learning mutant. *Cell* 37:205–215.
- Martin K, Michael D, Rose JC, Barad M, Casadio A, Zhu H, Kandel ER (1997) MAP kinase translocates to the nucleus of the presynaptic cell and is required for long-term facilitation in *Aplysia*. *Neuron* 18:899–912.
- Menzel R, Muller U (1996) Learning and memory in honeybees: from behavior to neural substrates. *Annu Rev Neurosci* 19:379–404.
- Milner B, Squire LR, Kandel ER (1998) Cognitive neuroscience and the study of memory. *Neuron* 20:445–468.
- Mohler JD (1977) Developmental genetics of the *Drosophila* egg. Identification of 59 sex-linked cistrons with maternal effects of embryonic development. *Genetics* 85:259–272.
- Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* 234:1249–1254.
- Murphy GG, Glanzman DL (1997) Mediation of classical conditioning in *Aplysia californica* by long-term potentiation of sensorimotor synapses. *Science* 278:467–471.
- Nicoll RA, Malenka RC (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* 377:115–118.
- O'Dowd DK (1995) Voltage-gated currents and firing properties of embryonic *Drosophila* neurons grown in a chemically defined medium. *J Neurobiol* 27:113–126.
- Oleskevich S (1999) Cholinergic synaptic transmission in insect mushroom bodies *in vitro*. *J Neurophysiol* 82:1091–1096.
- Restifo LL, White K (1990) Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. *Adv Insect Physiol* 22:115–219.
- Roerig B, Nelson DA, Katz LC (1997) Fast synaptic signaling by nicotinic acetylcholine and serotonin 5-HT₃ receptors in developing visual cortex. *J Neurosci* 17:8353–8362.
- Schacher S, Castellucci VF, Kandel ER (1988) cAMP evokes long-term facilitation in *Aplysia* sensory neurons that requires new protein synthesis. *Science* 240:1667–1669.
- Schuster R, Phannavong B, Schroder C, Gundelfinger ED (1993) Immunohistochemical localization of ligand-binding and a structural subunit of nicotinic acetylcholine receptors in the central nervous system of *Drosophila melanogaster*. *J Comp Neurol* 335:149–162.
- Silva AJ, Kogan JH, Frankland PW, Kida S (1998) CREB and memory. *Annu Rev Neurosci* 21:127–148.
- Trudeau LE, Castellucci VF (1993) Excitatory amino acid neurotransmission at sensory-motor and interneuronal synapses of *Aplysia californica*. *J Neurophysiol* 70:1221–1230.
- Tully T, Gold D (1993) Differential effects of *dunce* mutations on associative learning and memory in *Drosophila*. *J Neurogenet* 9:55–71.
- Tully T, Preat T, Boynton SC, Del Vecchio M (1994) Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79:35–47.
- Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA (1994) Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. *Science* 265:1878–1882.
- Wright ND, Zhong Y (1995) Characterization of K⁺ currents and the cAMP-dependent modulation in cultured *Drosophila* mushroom body neurons identified by lacZ expression. *J Neurosci* 15:1025.
- Yasuyama K, Kitamoto T, Salvaterra PM (1995) Immunocytochemical study of choline acetyltransferase in *Drosophila melanogaster*: Analysis of cis-regulatory regions controlling expression in the brain of cDNA-transformed flies. *J Comp Neurol* 361:25–37.
- Yin JC, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* 79:49–58.
- Yin JC, Del Vecchio M, Zhou H, Tully T (1995) CREB as a memory modulator induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* 81:107–115.
- Zhao ML, Wu CF (1997) Alterations in frequency coding and activity dependence of excitability in cultured neurons of *Drosophila* memory mutants. *J Neurosci* 17:2187–2199.
- Zhong Y, Wu CF (1991) Altered synaptic plasticity in *Drosophila* memory mutants with a defective cyclic AMP cascade. *Science* 251:198–201.