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A Genetic Analysis of Synaptic Development: Pre- and Postsynaptic dCBP Control Transmitter Release at the *Drosophila* NMJ

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Summary

Postsynaptic dCBP (*Drosophila* homolog of the CREB binding protein) is required for presynaptic functional development. Viable, hypomorphic dCBP mutations have a ~50% reduction in presynaptic transmitter release without altering the Ca^{2+} cooperativity of release or synaptic ultrastructure (total bouton number is increased by 25%–30%). Exogenous expression of dCBP in muscle rescues impaired presynaptic release in the dCBP mutant background, while presynaptic dCBP expression does not. In addition, overexpression experiments indicate that elevated dCBP can also inhibit presynaptic functional development in a manner distinct from the effects of dCBP loss of function. Pre- or postsynaptic overexpression of dCBP (in wild type) reduces presynaptic release. However, we do not observe an increase in bouton number, and presynaptic overexpression impairs short-term facilitation. These data suggest that dCBP participates in a postsynaptic regulatory system that controls functional synaptic development.

Introduction

The regulation of synaptic growth and plasticity must include mechanisms that constrain synaptic change within reasonable physiological limits. During development, there is a dramatic proliferation and remodeling of new synaptic connections (Buchs and Muller, 1996; Katz and Shatz, 1996; Engert and Bonhoeffer, 1999). In each system, it is essential that increased synaptic function is moderate and in register with the normal physiological range of the target neuron (Davis and Goodman, 1998a, 1998b). A fundamental question concerns how a neuron is able to generate a modest, yet physiologically relevant, change in synaptic function in response to a developmental or activity-dependent cue.

One hypothesis for regulated control of synaptic strengthening is the activity-dependent release of a highly localized and limited amount of trophic factor or signaling molecule (Arancio et al., 1996; Shieh and Ghosh, 1997). A second possibility is that regulation of synaptic function is achieved locally at the synapse by a biochemical tag that specifies those select synapses that will sustain long-term changes in synaptic function (Frey and Morris, 1997; Martin et al., 1997; Casadio et al., 1999). A third form of regulation is negative feedback within the system that generates increased synaptic function. Such negative feedback could allow for homeostatic control of synaptic strength, thereby preventing excessive synaptic strengthening.

Transcription via the cAMP response element binding protein (CREB) has been implicated in many forms of long-term synaptic plasticity (Bourtchuladze et al., 1994; Bito et al., 1996; Davis et al., 1996; Deisseroth et al., 1996; Silva et al., 1998). Indeed, CREB has been shown to participate in signaling via neurotrophins and may be an essential component of the biochemical tag hypothesis (Casadio et al., 1999). Here, we demonstrate a role for the *Drosophila* homolog of the CREB binding protein (dCBP), a transcriptional coactivator of CREB, in the homeostatic regulation of synaptic functional development.

CBP was identified as a coactivator of CREB-mediated transcription (Chrivia et al., 1993). However, a role for CBP during synaptic development and plasticity has not been thoroughly investigated. CBP function is complex and is not restricted to an interaction with CREB. CBP can act as a transcriptional coactivator with other transcriptional partners (Akimaru et al., 1997; Giordano and Avantaggiati, 1999; Johnston et al., 1999; Waltzer and Bienz, 1999). In addition, CBP has been implicated as a histone acetylase potentially involved in basal transcriptional regulation (Waltzer and Bienz, 1998, 1999; Giordano and Avantaggiati, 1999). The acetylase activity of dCBP has also been implicated in the regulation of Wingless signaling through acetylation of *Drosophila* T cell factor (Waltzer and Bienz, 1998). Thus, CBP has numerous functions, acting as a transcriptional regulator and as an intracellular signaling molecule in the cell nucleus.

Very little is known about the role of CBP at the synapse despite increasing evidence that CREB is an essential element of long-term synaptic plasticity (Davis et al., 1996; Casadio et al., 1999). Experiments in vitro using a GAL4-GFP reporter system indicate that CREB/CBP-mediated transcription is responsive to differential Ca^{2+} entry through voltage-gated Ca^{2+} channels and ligand-gated ion channels (Hardingham et al., 1999; Hu et al., 1999). Rubenstein-Taybi syndrome, a disorder characterized by severe mental retardation and limb malformation, is associated with a truncation of the CBP gene (Tenaka et al., 1997; Taine et al., 1998). Mice heterozygous for mutations in the CBP locus show dominant deficits in learning and memory (Tanaka et al., 1997; Oike et al., 1999). These data implicate CBP as being essential for normal neural development and activity-dependent synaptic plasticity.

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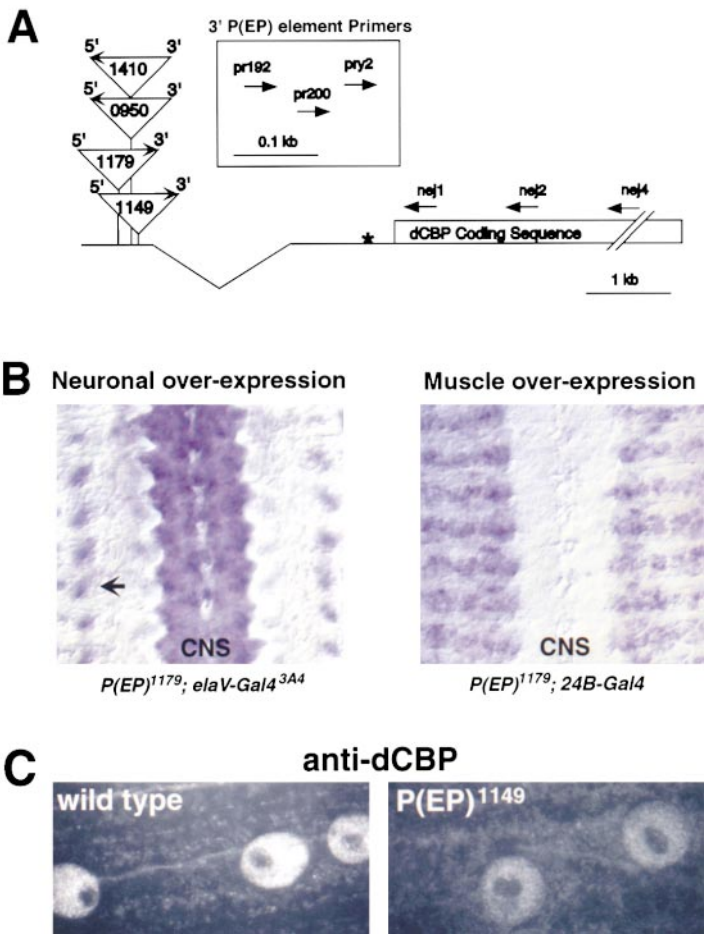

Figure 1. Molecular Characterization of the dCBP P(EP) Insertions

(A) Schematic diagram of the dCBP genomic region showing the P(EP) insertion sites upstream of the published dCBP sequence. The published coding sequence (open box) and position of the 5′ end of the published dCBP sequence (asterisk) are shown. $P(EP)^{1179}$ and $P(EP)^{1149}$ are oriented to overexpress dCBP, while $P(EP)^{1410}$ and $P(EP)^{0950}$ are oppositely oriented. The precise insertion sites for P(EP) elements 1179, 1410, 0950, and 1149, relative to the published dCBP sequence, are 2981 base pairs, 2781 base pairs, 2780 base pairs, and 2747 base pairs upstream of the dCBP start site, respectively. The approximate position and orientation of PCR primers used for PCR amplification are indicated.

(B) $P(EP)^{1179}$ drives specific overexpression of the endogenous dCBP transcript in the embryonic nervous system using the *elaV-GAL4* driver ("Neuronal over-expression") or in embryonic muscle using the *24B-GAL4* driver ("Muscle over-expression").

(C) Expression of dCBP in postsynaptic muscle, as detected by anti-dCBP. Expression in muscle is reduced by ~75%–80% in the hypomorphic loss-of-function mutant background [$P(EP)^{149}$] based on reduced fluorescence intensity.

Here, we present a complete genetic and electrophysiological analysis of dCBP function during synaptic development at the *Drosophila* neuromuscular junction (NMJ). We present evidence that postsynaptic dCBP is necessary for normal presynaptic functional development. In addition, we demonstrate that overexpression of dCBP can act to inhibit presynaptic functional development. We propose that dCBP participates in a postsynaptic homeostatic regulatory system that controls presynaptic function through both positive and negative regulation. This model provides a mechanism that can allow for the precise control of new synapse formation during development.

Results

Molecular and Genetic Identification of dCBP P(EP) Element Mutations

dCBP is expressed both pre- and postsynaptically at the developing larval NMJ. dCBP is ubiquitously expressed based on in situ experiments and protein expression (data not shown). More specifically, an antibody raised against dCBP shows that the protein is present in larval muscle nuclei (Figure 1) and throughout the CNS (data not shown).

We have identified four P(EP) elements that are located in a region ~3 kb upstream of the dCBP open reading frame. Two of these elements—$P(EP)^{1179}$ and $P(EP)^{1149}$—are oriented to overexpress dCBP, and two are in the opposite orientation. P(EP) elements are mobile genetic elements (P elements) containing upstream transcription activation sequences (UAS sequence) that exploit the tendency for P elements to insert in 5′ regulatory regions of a gene. Insertion of such an element in the proper 5′-3′ orientation places random genes under the control of the UAS element, allowing them to be expressed in specific tissues under the control of an appropriate GAL4 driver.

$P(EP)^{1179}$ and $P(EP)^{1149}$ can initiate overexpression of dCBP. Crossing $P(EP)^{1179}$ or $P(EP)^{1149}$ to GAL4 drivers that promote expression in nerve or muscle causes tissue-specific overexpression of dCBP, as detected by in situ hybridization using probes specific to the dCBP gene (Figure 1). Overexpression of dCBP was also demonstrated by RT-PCR using primers from the P(EP) elements and primers within the dCBP open reading frame. Each PCR product isolated by RT-PCR was sequenced to ensure that the correct open reading frame was driven by the P(EP) element.

To ensure that we are not driving overexpression of an additional message located between our P(EP) elements and the start of the dCBP open reading frame, we subcloned and sequenced the entire 3 kb genomic region between $P(EP)^{1179}$ (the furthest insertion upstream of dCBP) and the start of dCBP. This region did not contain an additional transcript. Furthermore, our overexpression phenotype is phenocopied by overexpression of the *dCBP* cDNA under UAS control (see below). Based

on this sequence data and data obtained from RT-PCR from the P(EP)^{1179} and P(EP)^{1149} elements, we present a more complete characterization of the 5′ untranslated region of dCBP that extends to, and most likely beyond, these P(EP) elements. These results also predict that these P(EP) elements will generate a hypomorphic loss of dCBP function.

Genetic, histological, and electrophysiological evidence demonstrate that the P(EP) elements P(EP)^{1149} and P(EP)^{1179} are hypomorphic mutations in dCBP. dCBP expression in muscle nuclei, as detected by an anti-dCBP antibody, is significantly reduced in the P(EP)^{1149} hypomorphic mutant (expression being decreased by ~75%–80%, based on reduced fluorescence intensity; Figure 1). Genetic experiments demonstrate that null or strong hypomorphic mutations in dCBP die as late embryos or first instar larvae (Florence and McGinnis, 1998; Waltzer and Bienz, 1998). Patterning defects are associated with these null mutations (Akimaru et al., 1997; Florence and McGinnis, 1998; Waltzer and Bienz, 1998). Here, we demonstrate that P(EP)^{1179} and P(EP)^{1149} fail to complement previously characterized hypomorphic alleles of dCBP, including *dCBP^{TA57}* and *dCBP^{S342}*, for synaptic transmission defects. P(EP)^{1179} or P(EP)^{1149} trans-heterozygous with *dCBP^{TA57}* or *dCBP^{S342}* are semiviable as third instar larvae. These trans-heterozygous larvae are developmentally delayed by ~1 day and emerge as sluggish third instar larvae. We were therefore able to proceed with anatomical and electrophysiological characterization of these dCBP loss-of-function mutations at the third instar larval synapse.

dCBP Is Necessary for Functional Synaptic Development

Hypomorphic dCBP loss-of-function alleles (reduced function) cause a specific ~50% decrease in presynaptic transmitter release at the NMJ. The homozygous viable P(EP)^{1149} insertion shows a significant decrease in presynaptic transmitter release (quantal content; see Experimental Procedures) without any change in the average quantal size compared with wild-type and heterozygous P(EP)^{1149} element controls (Figure 2). P(EP)^{1179} homozygous viable larvae do not show any change in synaptic structure or function. However, when P(EP)^{1179} is placed in *trans* to known hypomorphic alleles of dCBP, including *dCBP^{TA57}* and *dCBP^{S342}*, we observe a decrease in presynaptic quantal content that is identical to that observed in P(EP)^{1149} homozygous larvae; there is a ~50% decrease in quantal content without any change in quantal size compared with wild-type and heterozygous controls, including P(EP)^{1179}/+, *dCBP^{S342}*/+, and *dCBP^{TA57}*/+ (Figure 2). We never observed a change in the average muscle resting potential or in the average muscle input resistance in any genotype demonstrating the specificity of these phenotypes to changes in presynaptic release.

Previously identified homozygous dCBP mutations are all lethal during early development. We therefore used a previously identified dominant-negative allele of dCBP to further demonstrate the specificity of this dCBP physiological phenotype. The *dCBP^{Q7}* allele has been characterized as an antimorphic allele with a presumed dominant-negative function (Florence and McGinnis, 1988). Consistent with these previously published data,

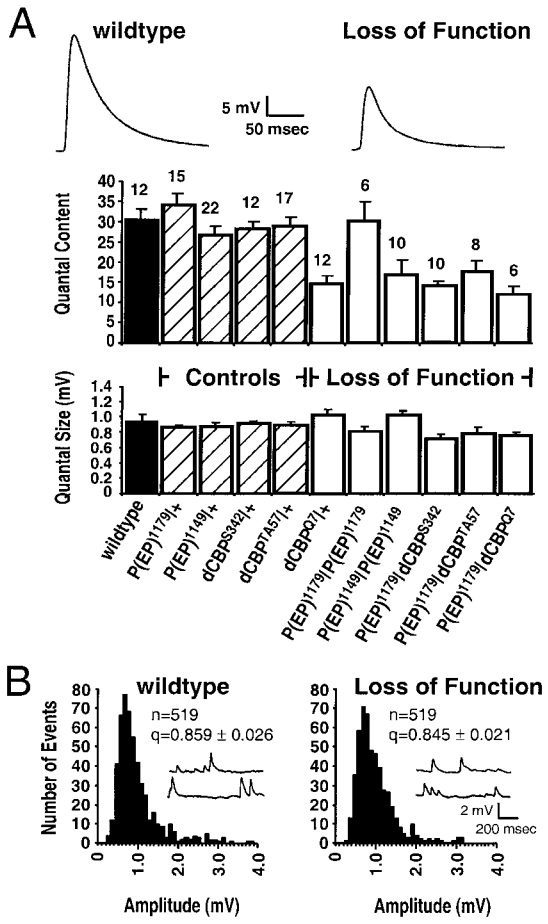

Figure 2. dCBP Loss of Function Decreases Quantal Content without Affecting Quantal Size

(A) Quantification of quantal content, as estimated by dividing the average EPSP by the average mEPSP. Average quantal content is significantly reduced in strong hypomorphic mutations (open bars), including *dCBP^{Q7}*/+ (dominant-negative allele), *P(EP)^{1149}/P(EP)^{1149}*, *P(EP)^{1179}/dCBP^{S342}*, *P(EP)^{1179}/dCBP^{TA57}*, and *P(EP)^{1179}/dCBP^{Q7}* ($p < 0.01$ for all genotypes compared with wild type [closed bar]). Quantal content was not significantly altered in the heterozygous controls (hatched bars), including *P(EP)^{1179}*/+, *P(EP)^{1149}*/+, *dCBP^{S342}*/+, and *dCBP^{TA57}*/+. Quantal size is not significantly altered in any genotype compared with wild type. The number of recordings for each genotype is shown above the bar. Representative EPSPs from wild type and a strong hypomorphic mutation are shown above the graphs. Coding of genotypes in this and subsequent figures are is follows: wild type, closed bars; heterozygous controls, hatched bars; and experimental genotypes, open bars.

(B) Distribution of mEPSPs for recordings of wild type and *P(EP)^{1179}/dCBP^{TA57}* (abbreviations: n, number of events; q, mean quantal size). Inset, sample traces for each genotype. The distribution of mEPSPs is unaltered in strong dCBP hypomorphs.

dCBP^{Q7} shows a dominant reduction in presynaptic transmitter release without any change in quantal size (Figure 2). The anatomical and electrophysiological phenotypes of the *dCBP^{Q7}* allele are identical to the phenotypes of the *P(EP)^{1149}* homozygous larvae and to P(EP)^{1179} in *trans* with *dCBP^{TA57}* and *dCBP^{S342}*. Consistent with *dCBP^{Q7}* having a dominant-negative (antimorphic) activity, the electophysiological phenotype is not significantly enhanced in *trans* to the P(EP)^{1179} loss-of-function insertion (Figure 2). Thus, our recently identified hypomorphic

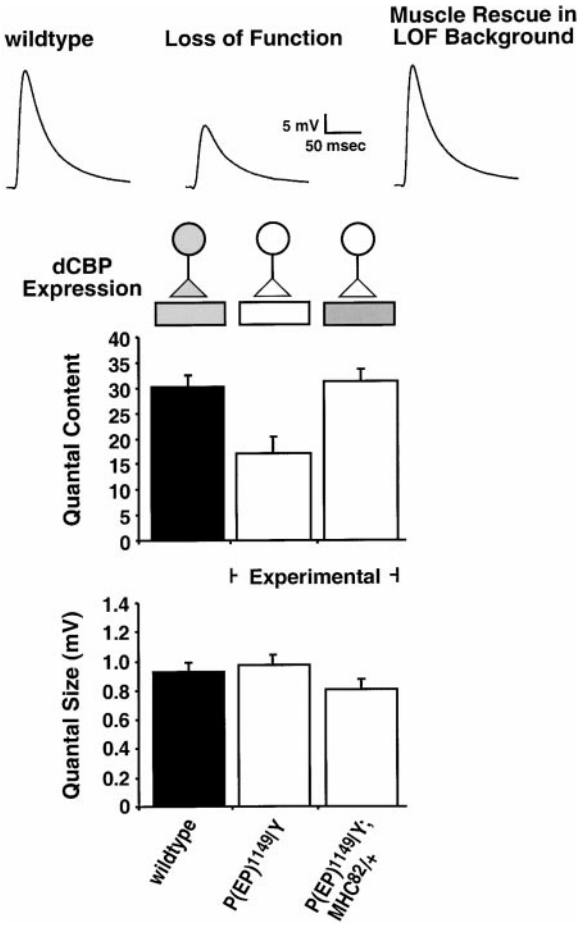

Figure 3. Postsynaptic dCBP Expression Rescues Presynaptic Transmitter Release in the dCBP Mutant Background

Quantal content and quantal size are quantified for dCBP loss of function ("Loss of Function") and when dCBP is expressed in postsynaptic muscle in this loss-of-function genetic background ("Muscle Rescue in LOF Background"). Sample traces are shown at the top for each genetic background (traces are signal averages of 10–20 individual EPSPs). dCBP expression is shown schematically above each bar of the graph.

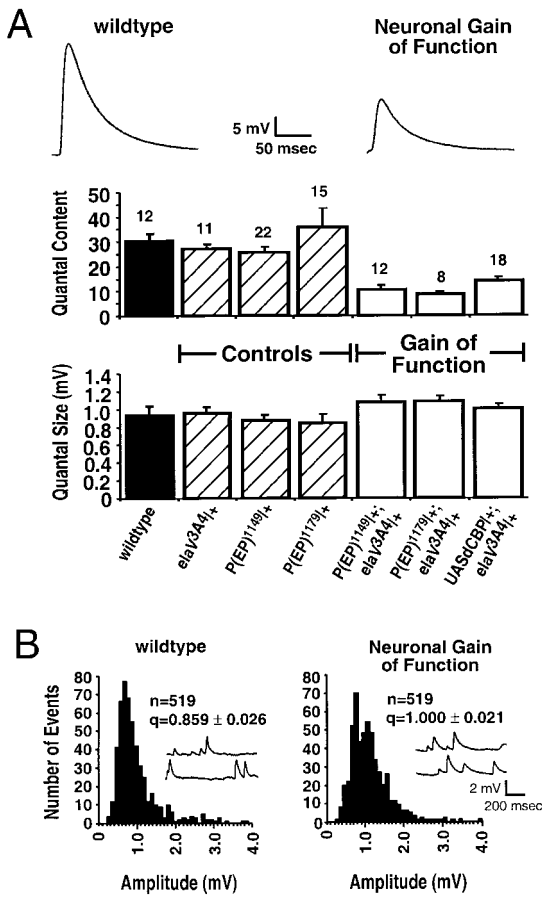

Figure 4. Presynaptic dCBP Overexpression Decreases Quantal Content without Affecting Quantal Size

(A) Quantification of quantal content, as estimated by dividing the average EPSP by the average mEPSP. Average quantal content is significantly reduced in genotypes that overexpress dCBP presynaptically, including *P(EP)^{1149}/+;elaV3A4/+*, *P(EP)^{1179}/+;elaV^{3A4}/+*, and *UAS-dCBP/+;elaV^{3A4}/+*. Quantal size is not significantly altered in any genotype compared with wild type. The number of recordings for each genotype is shown above the bar. Representative EPSPs from wild type and presynaptic overexpressing genotypes are shown above the graphs.

(B) Distribution of mEPSPs for recordings of wild type and *P(EP)^{1179}/elaV^{3A4}dCBP^{TA57}* (abbreviations: n, number of events; q, mean quantal size). Inset, sample traces for each genotype. Presynaptic overexpression of dCBP does not alter the distribution of mEPSPs compared with wild type.

P(EP) element insertions in the 5′ UTR of dCBP show an electrophysiological phenotype that is identical to a dCBP dominant-negative allele and fail to complement previously identified hypomorphic alleles of dCBP, indicating that these P(EP) elements disrupt dCBP activity. These data demonstrate that dCBP is necessary for functional synaptic development at the NMJ.

Postsynaptic dCBP Rescues Presynaptic Release in the Loss-of-Function Mutant

To address whether dCBP is necessary in the pre- or postsynaptic cell for normal synaptic development, we have rescued dCBP expression on either side of the synapse in the hypomorphic mutant background. P(EP)^{1149} males show a ~50% reduction in presynaptic transmitter release that is identical to homozygous females and other loss-of-function allelic combinations (Figure 3). We therefore used P(EP)^{1149} males as a hypomorphic mutant background and expressed dCBP with either *MHC-GAL4* (all muscle) or *elaV-GAL4* (all neurons). Expression of dCBP in muscle in the hypomorphic mutant background rescues presynaptic quantal content to wild-type levels (Figure 3). However, expression of dCBP in nerve in the same hypomorphic mutant background resulted in early larval lethality (0.5% survival). Thus, postsynaptic dCBP can rescue presynaptic functional development in the hypomorphic mutant background.

dCBP Overexpression Inhibits Functional Presynaptic Development

Overexpression of dCBP in either the pre- or postsynaptic cell causes a reduction in presynaptic transmitter release (Figures 4 and 5). dCBP was overexpressed in

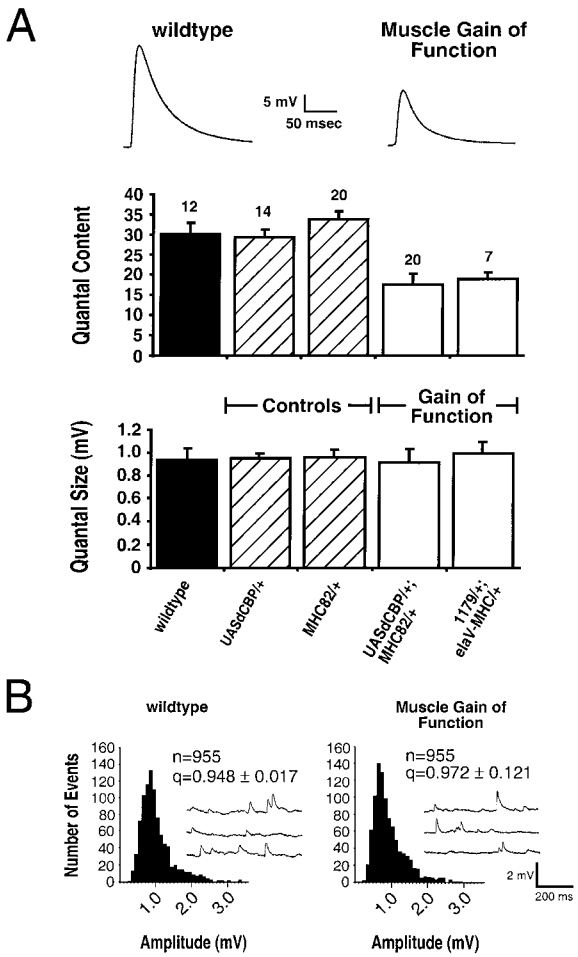

Figure 5. Postsynaptic dCBP Overexpression Decreases Quantal Content without Affecting Quantal Size

(A) Quantal content and quantal size are quantified for two genetic controls and when dCBP is overexpressed in muscle using *MHC-GAL4* and *UAS-dCBP*. A significant difference is observed for quantal content at the *UAS-dCBP/MHC-GAL4*^{82} synapse (expression postsynaptically) and at the *P(EP)*^{1179}/+;*elaV-GAL4*/+;*MHC-GAL4*^{82}/+ synapse (expression both pre- and postsynaptically) compared with all other genotypes ($p < 0.001$, student's t test). Sample traces are shown at top (averages of 10–20 individual EPSPs).

(B) There is no change in the distribution of spontaneous miniature release events when dCBP is overexpressed in muscle. The number of events analyzed (n), the average quantal size (q), and the standard deviation (±) are indicated for each histogram. Inset, sample traces showing spontaneous miniature release events from the distribution quantified in each histogram.

the wild-type background using the *dCBP* cDNA under UAS control (*UAS-dCBP*). Presynaptic overexpression was driven by *elaV-GAL4*. We observe a decrease in presynaptic transmitter release (~70%) without a change in the average size or distribution of the spontaneous miniature release events and without any change in the average resting potential or average input resistance of the postsynaptic muscle (Figure 4). We also overexpressed dCBP from either P(EP)^{1149}/+ or P(EP)^{1179}/+. These heterozygous P(EP) elements are electrophysiologically wild type in the absence of GAL4 (Figure 4). When dCBP is overexpressed from the P(EP) elements using *elaV-GAL4*, we observe a reduction in presynaptic release identical to the overexpression of *UAS-dCBP*. Overexpression of dCBP in muscle, or in both muscle and nerve, also inhibits presynaptic transmitter release (Figure 5). Again, we do not observe any change in quantal size or muscle physiological parameters. We conclude that overexpression of dCBP either pre- or postsynaptically inhibits presynaptic functional development.

Ultrastructural Analysis

We examined the synaptic ultrastructure to determine whether the observed impairment of presynaptic release is due to gross ultrastructural abnormalities. Synaptic ultrastructure appears normal at neuromuscular boutons in both gain- and loss-of-function genetic backgrounds despite reduced presynaptic transmitter release at these synapses. There does not appear to be any alteration in the extent of subsynaptic reticulum or in the integrity of individual active zones (Figure 6). Indeed, there is an increase in the number of presynaptic T bars per active zone in the dCBP loss-of-function synapse ($n = 183$ active zones in P(EP)^{1149}, and $n = 219$ active zones in wild type, $p < 0.001$). It has been hypothesized that T bars may facilitate transmitter release; however, their precise function in synaptic transmission remains unclear. We therefore conclude that gross ultrastructural abnormalities are not the cause of decreased presynaptic release.

Synapse Morphology at dCBP Gain- and Loss-of-Function Mutant Synapses

A morphological analysis demonstrates that reduced transmitter release in the dCBP gain- and loss-of-function backgrounds is not due to a disruption of morphological synaptic development. Synaptic structure and function were both calculated for each individual preparation by fixing and staining each electrophysiological preparation with anti-Synaptotagmin to reveal the nerve terminal boutons. Thus, we can precisely correlate changes in synaptic function with a change in the synapse morphology. Strong loss-of-function allelic combinations and the dominant-negative *dCBP*^{Q7} allele all cause an increase in bouton number (255; 35% increase) at the NMJ of muscles 6 and 7 (Figure 7) and muscle 4 (data not shown). There is only a modest change in the number of synaptic boutons, and, therefore, we suspect that this is a compensatory response due to the observed decrease in synaptic function in these dCBP mutants. These data indicate that the health of the pre- and postsynaptic cells has not been compromised.

In contrast, overexpression of dCBP either pre- or postsynaptically does not alter bouton number despite a reduction in transmitter release that is comparable to that observed at dCBP loss-of-function synapses (Figure 7). Thus, dCBP overexpression inhibits presynaptic release without any morphological compensation.

We have also performed experiments to control for any potential dCBP-dependant alteration in axon guidance, target selection, or alteration in neuronal and glial cell fate at dCBP gain- or loss-of-function backgrounds. Synaptic connectivity in the larval neuromuscular system appears normal based on visual inspection of type

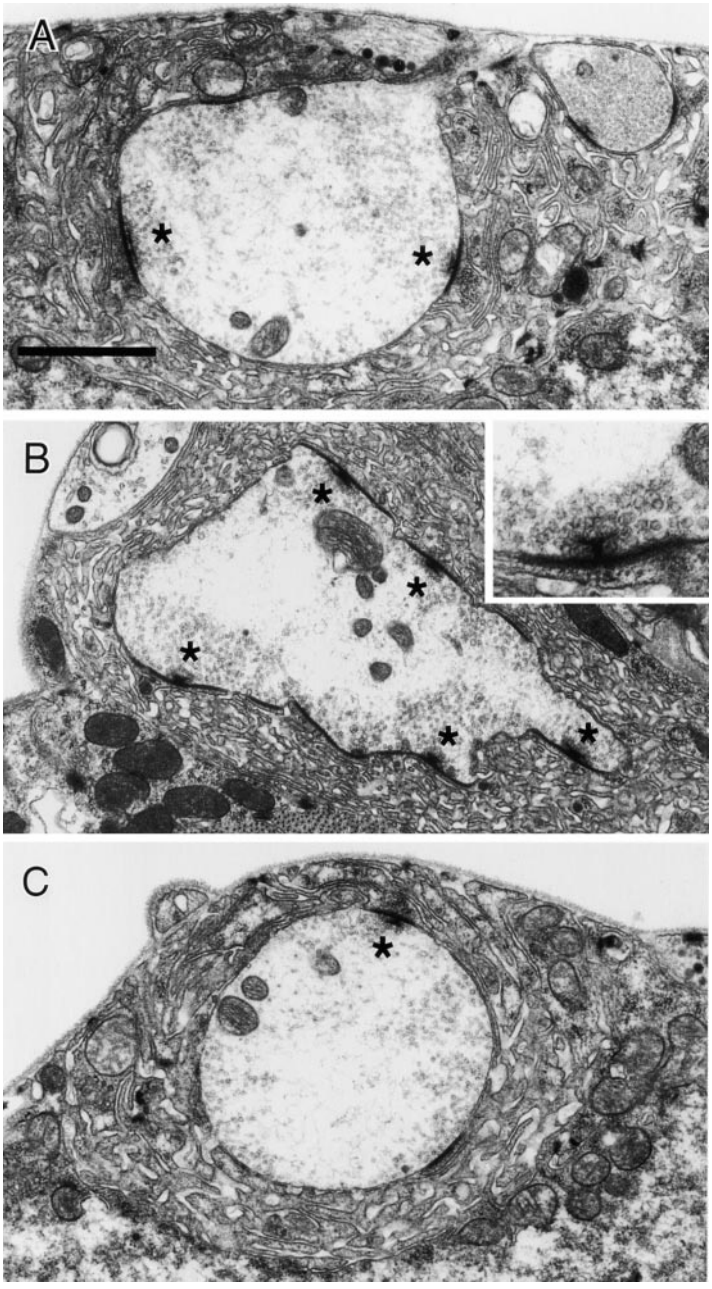

Figure 6. Synaptic Ultrastructure Is Normal in dCBP Mutants and When dCBP Is Overexpressed

Single sections from EM serial reconstruction of third instar synapses on muscle 6 in abdominal segment 3.

(A) Cross-section of a wild-type bouton. Active zones with T bars are indicated (asterisk).

(B) Cross-section of a synaptic bouton from dCBP loss of function, $P(EP)^{1149}/P(EP)^{1149}$. Inset, higher magnification of an active zone with a presynaptic T bar.

(C) Cross-section of a synaptic bouton from a synapse overexpressing dCBP both pre- and postsynaptically [$P(EP)^{1179}$;*elaV-GAL4*; *MHC-GAL4*].

Ia, type Ib, and type II synapses at muscle 6, 7, 13, 12, 4, 3, 2, and 1. These synapses were inspected during the quantitation of bouton number in each genotype (over 400 segments analyzed). We do not observe any alteration in axon guidance in the embryonic CNS, indicating that cell fate and guidance are normal in the CNS (data not shown). In the embryo, we do observe weakly penetrant axon guidance defects in the neuromuscular system (data not shown). In the experiments presented here, we controlled for any defect in target selection by quantitating synapse morphology and bouton number for each electrophysiological preparation. We did not observe any alteration in target selection or innervation at the muscles from which we recorded electrophysiologically. Finally, we have examined the distribution of glial cell bodies in the embryo and larva, visualized with the anti-REPO antibody. We did not observe any change in the number or distribution of glia within the embryonic or larval CNS or PNS (data not shown).

Ca^{2+} Cooperativity and Synaptic Facilitation

We performed additional experiments to determine whether decreased transmitter release in the dCBP gain-of-function and dCBP loss-of-function mutations can be further distinguished. We examined the Ca^{2+} dependence of transmitter release at synapses with either increased or decreased dCBP. At both dCBP gain- and loss-of-function synapses, the Ca^{2+} cooperativity of transmitter release remains in the range of what is considered to be wild type (slope, $\sim$4) despite a shift

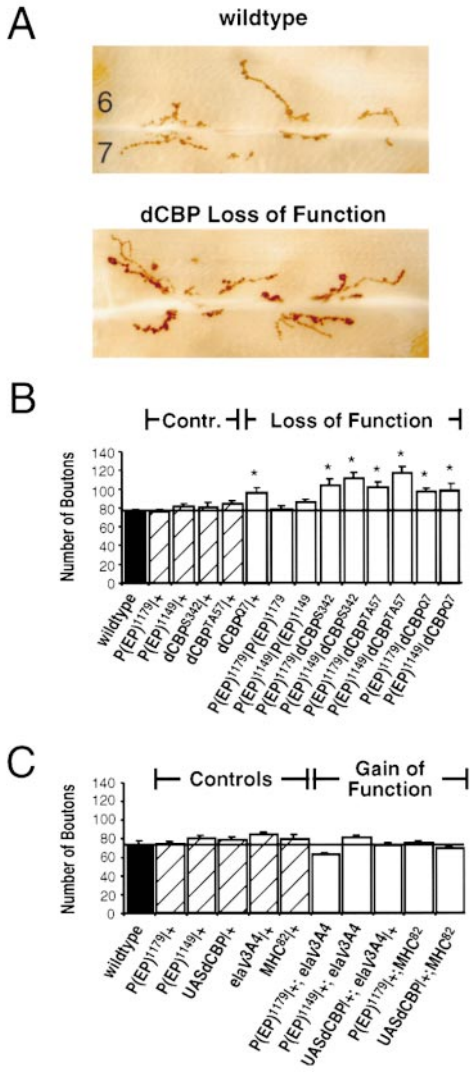

Figure 7. Hypomorphic Mutations in dCBP Cause a Moderate Increase in Synapse Size

(A) Representative light micrographs of muscles 6 and 7 in segment A3 of wild type and dCBP loss-of-function (*P(EP)^{1179}/dCBP^{TA57}*) third instar larvae stained with anti-Fasciclin II and anti-Synaptotagmin. dCBP loss of function causes a moderate (~25%) increase in bouton number.

(B and C) Quantification of synapse size in dCBP loss-of-function (B) and overexpression (C) genotypes. Synapse size was quantified by staining with anti-Fasciclin II and anti-Synaptotagmin and counting boutons at muscles 6 and 7 of segment A3 in third instar larvae. Strong dCBP loss-of-function mutations show a significant increase in synapse size, including *P(EP)^{1179}/dCBP^{S342}*, *P(EP)^{1149}/dCBP^{S342}*, *P(EP)^{1179}/dCBP^{TA57}*, *P(EP)^{1149}/dCBP^{TA57}*, *dCBP^{Q7}/+* (dominant-negative), *P(EP)^{1179}/dCBP^{Q7}*, and *P(EP)^{1149}/dCBP^{Q7}* ($p < 0.01$). One presynaptic overexpression genotype (*P(EP)^{1179}/+;elaV^{3A4}/+*) showed a significant decrease in synapse size ($p = 0.013$).

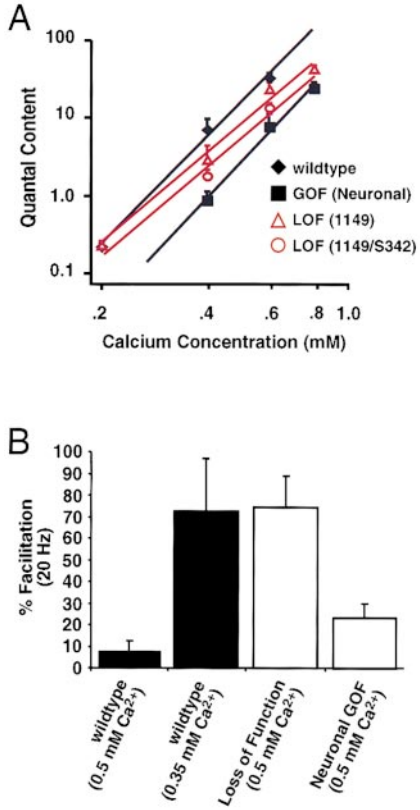

Figure 8. Ca^{2+} Cooperativity and Facilitation in dCBP Gain and Loss of Function

(A) Ca^{2+} cooperativity data are shown for both dCBP loss of function [*P(EP)^{1149}/P(EP)^{1149}* ($R^2 = 0.9894$) and *P(EP)^{1149}/dCBP^{S342}* ($R^2 = 0.9626$)] and presynaptic overexpression [*UAS-dCBP/+;elaV^{3A4}/+* ($R^2 = 0.9925$)] compared with wild type ($R^2 = 0.9951$). Cooperativity is maintained within a normal range (slope, ~4) despite a consistent reduction in cooperativity in the two loss-of-function genetic backgrounds.

(B) Short-term facilitation is examined at wild-type synapses (closed bars) at two different Ca^{2+} concentrations (0.5 mM and 0.35 mM). Facilitation is also examined at 0.5 mM Ca^{2+} in both dCBP loss of function [P(EP)^{1149}/P(EP)^{1149}] and when dCBP is overexpressed presynaptically in the wild-type genetic background [*UAS-dCBP/+; elaV-GAL4/+*].

toward smaller quantal contents at all but the lowest Ca^{2+} concentrations used (Figure 8). There is a consistent reduction in the cooperativity of release in the loss-of-function background compared with wild-type and dCBP overexpression. However, this change in cooperativity is relatively minor. These data demonstrate that the decrease in presynaptic release in the dCBP gain- and loss-of-function backgrounds is not due to a substantial alteration in the Ca^{2+}-dependent transmitter release mechanism (Figure 8).

We also analyzed short-term facilitation in these dCBP gain- and loss-of-function backgrounds. Short-term facilitation was induced with five stimuli delivered with an interpulse interval of 50 ms (20 Hz). Facilitation was calculated by the percent change in excitatory postsynaptic potential (EPSP) amplitude from the first to the fifth EPSP (Davis et al., 1996). In the dCBP mutant background, there is 74% facilitation compared with 9% facilitation in wild type at 0.5 mM extracellular Ca^{2+}. However, the initial EPSP in the dCBP mutant background is 50% of that of wild type. When we reduced the first EPSP in wild-type larvae to a similar level (10.0 ± 1.2 mV) by recording in 0.35 mM extracellular Ca^{2+}, we observed 73% facilitation. These data indicate that altered facilitation in the dCBP mutant background is due to a reduction in the initial EPSP amplitude. These data further

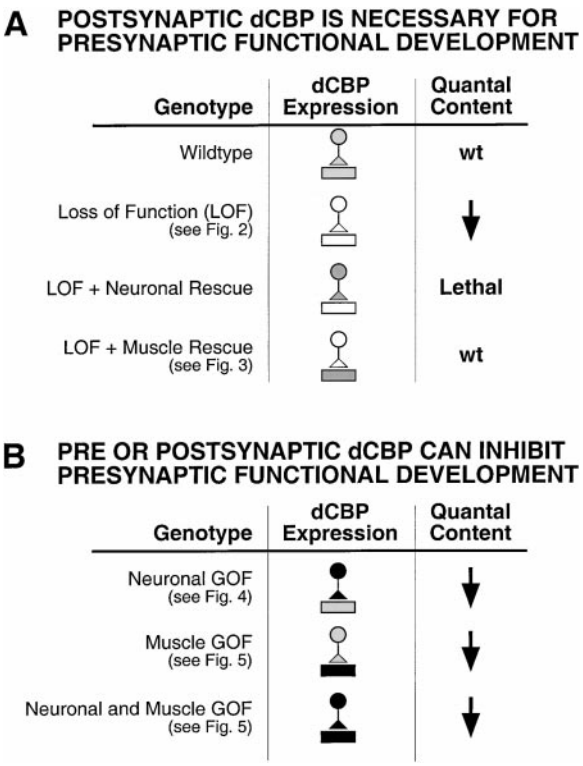

Figure 9. dCBP Both Positively and Negatively Regulates Presynaptic Functional Development

(A) Summary of results from loss-of-function and rescue experiments.

(B) Summary of experiments overexpressing dCBP either pre- or postsynaptically in the wild-type genetic background. A reference to primary data is indicated below each experiment. Levels of dCBP expression are indicated schematically, with darker shading indicating higher levels of expression.

suggest that reduced transmitter release in the dCBP loss-of-function background may be due to a reduction in the probability of presynaptic release.

Facilitation at synapses with presynaptic dCBP overexpression is not increased despite a ~50% reduction in the initial EPSP amplitude. At 0.5 mM extracellular Ca^{2+}, we observe 23% facilitation. This is significantly less facilitation than observed at dCBP loss-of-function synapses and at wild-type synapses in 0.35 mM Ca^{2+}, which have a comparable initial EPSP amplitude. Thus, the reduced initial EPSP amplitude in dCBP neuronal gain of function does not correlate with increased facilitation, suggesting that decreased transmitter release may be due to a reduction in the number of functional active zones. However, we do not observe a change in the frequency of spontaneous vesicle fusion events (mEPSPs) in dCBP neuronal gain of function compared with wild type (data not shown). In conclusion, reduced transmitter release in dCBP gain- and loss-of-function is likely due to different mechanisms.

Discussion

Our data demonstrate that postsynaptic dCBP is necessary and may also be sufficient for the development of normal presynaptic transmitter release at the NMJ. This indicates that dCBP participates in a retrograde signaling pathway that controls presynaptic development. We further demonstrate that dCBP can inhibit the development of presynaptic transmitter release when overexpressed either pre- or postsynaptically. This indicates that dCBP-dependent mechanisms can act as both positive and negative regulators of presynaptic development. We hypothesize that postsynaptic dCBP participates in the homeostatic control of presynaptic function.

dCBP Is Necessary for Presynaptic Functional Development

We have analyzed five different loss-of-function alleles of dCBP. In each viable genetic combination in which dCBP activity is reduced, there is a 45%–50% decrease in presynaptic transmitter release. The observed decrease in presynaptic release is severe, being comparable to phenotypes observed in Synaptotagmin mutant larvae (Littleton et al., 1993; DiAntonio and Schwarz, 1994). The loss of dCBP does not alter the health of the synapse, as assayed by light microscopy and ultrastructural analysis, and is not due to altered muscle innervation or altered glial or neuronal cell fate. Thus, dCBP loss of function dramatically and specifically impairs functional synaptic development.

Genetic rescue experiments indicate that the developmental requirement for dCBP is postsynaptic. Exogenous expression of dCBP in muscle in the loss-of-function genetic background rescues presynaptic functional development to wild type. Since transmitter release is wild type despite reduced presynaptic dCBP, we conclude that postsynaptic dCBP expression is necessary for normal functional synaptic development. These postsynaptic rescue data also suggest that postsynaptic dCBP may be sufficient for presynaptic functional development. However, these rescues are conducted in hypomorphic loss-of-function backgrounds, and, as such, there remains a residual level of dCBP presynaptically (Figures 2, 3, and 9).

Expression of dCBP presynaptically in the loss-of-function background causes lethality. We hypothesize that this lethality is due to an additive effect of dCBP loss of function postsynaptically and an inhibitory effect of presynaptic dCBP overexpression. Overexpression presynaptically in the wild-type background (normal muscle dCBP) inhibits transmitter release. Additional data indicate that the inhibitory effect of dCBP overexpression may act via a mechanism different from dCBP loss of function (see next section). Therefore, the effects of presynaptic overexpression and postsynaptic loss of function are likely to be additive. However, these data also raise the possibility that the ratio of dCBP expression in the presynaptic neuron, relative to the postsynaptic target, may also be an important parameter. We are unable to assay expression levels accurately enough to address this issue.

dCBP Overexpression Inhibits Presynaptic Functional Synaptic Development

Overexpression of dCBP in the postsynaptic muscle or the presynaptic nerve inhibits presynaptic transmitter

release (Figures 4, 5, and 9b). Anatomical, ultrastructural, and electrophysiological data indicate that dCBP overexpression does not poison the synapse. One possibility is that overexpression of wild-type dCBP in the wild-type genetic background can somehow act as a dominant-negative and therefore mimics the loss-of-function phenotype. However, postsynaptic dCBP expression can rescue the loss-of-function phenotype, indicating that exogenous dCBP can function correctly during synaptic development. In addition, the gain- and loss-of-function phenotypes can be distinguished by differential changes in synapse morphology and short-term facilitation. Therefore, we interpret our overexpression data as indicating that dCBP can function normally to inhibit presynaptic functional development by a mechanism that is distinct from dCBP loss of function.

We observe increased synapse morphology in the loss-of-function background, while there is no change in bouton number when dCBP is overexpressed either pre- or postsynaptically. Since the increased morphology in the dCBP mutant background is moderate, we hypothesize that this is a compensatory response due to reduced presynaptic release. This implies that dCBP overexpression blocks this form of compensation. However, it is unclear whether this is a direct or indirect effect of dCBP overexpression.

dCBP gain- and loss-of-function phenotypes can also be distinguished by analysis of short-term facilitation. There is a reduction in the initial EPSP amplitude in both dCBP gain and loss of function. At 0.5 mM extracellular Ca^{2+}, the reduced initial EPSP is correlated with increased facilitation at the dCBP loss-of-function synapse, whereas facilitation is normal in the dCBP gain-of-function synapse (despite a reduced initial EPSP). When the initial EPSP amplitude at a wild-type synapse is experimentally reduced by reducing extracellular Ca^{2+} (to 0.35 mM Ca^{2+}), we observe facilitation that is similar to that observed at a dCBP loss-of-function synapse recorded at 0.5 mM Ca^{2+}. These data indicate that the EPSP amplitude is reduced by different mechanisms in the dCBP gain- and loss-of-function genetic backgrounds. We suspect that dCBP overexpression inhibits some aspect of presynaptic release that is independent of the probability of presynaptic release.

dCBP and the Homeostatic Regulation of Synaptic Function

At the larval NMJ, an experimental decrease in postsynaptic excitation causes a compensatory enhancement of presynaptic release (Petersen et al., 1997; Davis et al., 1998). These previous experiments define a homeostatic regulatory system that maintains postsynaptic excitation through a retrograde signal(s) from muscle to nerve. A homeostatic regulatory system will likely include mechanisms that can monitor postsynaptic excitation and transduce this information through a retrograde signal to modulate presynaptic transmitter release. In principle, homeostatic regulation will require both positive and negative regulation of synaptic function.

We hypothesize that dCBP is centrally involved in the mechanisms that monitor postsynaptic activity. We identified the dCBP P(EP) element mutations described here in a screen for mutations in genes that participate in the homeostatic regulation of presynaptic transmitter release (G. W. Davis et al., submitted). We demonstrate here that perturbations in postsynaptic dCBP can affect presynaptic transmitter release. Furthermore, postsynaptic dCBP can act as both a positive and negative regulator of synaptic function. Finally, CBP in *Drosophila* and other systems is well suited to participate in a system that monitors postsynaptic activity. CBP function can be regulated by Ca^{2+} influx through voltage-gated channels and ionotropic receptors (Hardingham et al., 1999; Hu et al., 1999). Furthermore, CBP can act as a transcriptional coactivator with CREB and other transcription factors, as well as function as an intracellular signaling molecule via acetylase activity (Waltzer and Bienz, 1998). In conclusion, we propose that dCBP is an essential component of the postsynaptic homeostatic mechanism that monitors activity and regulates presynaptic transmitter release.

We have previously proposed that the homeostatic retrograde increase in presynaptic release is due to a signal that can enhance presynaptic transmission, similar to that proposed for the presynaptic expression of long-term potentiation (Bliss and Collingridge, 1993). Our current data suggest an additional model, that a homeostatic increase in presynaptic transmission could also be achieved by relieving an inhibitory signal derived from postsynaptic dCBP function.

Homeostatic control of presynaptic function at the *Drosophila* NMJ ensures that presynaptic release is precisely coupled to the growth of the postsynaptic muscle throughout development. To achieve constant muscle depolarization during development, homeostatic signaling must achieve a progressive and gradual increase in synaptic function. A progressive and gradual increase in synaptic function could be achieved through dCBP-dependent mechanisms since it can both promote and inhibit synaptic development. Activation of dCBP could promote synaptic strengthening, while sustained dCBP activation could inhibit further synaptic development, preventing runaway excitation. This is consistent with our demonstration that postsynaptic dCBP is necessary for normal synaptic development but that sustained overexpression of dCBP can inhibit presynaptic functional development.

CBP/CREB Function during Synaptic Development and Activity-Dependent Plasticity

Our results are consistent with previous data examining CREB activity in *Drosophila*. Neither CREB nor dCBP directly alters synaptic morphology (Davis et al., 1996). Here, we demonstrate a role for dCBP in regulated functional synaptic development. However, we have previously demonstrated that CREB is unlikely to be involved in synaptic development based on heat shock overexpression of dCREB2b (Davis et al., 1996). Thus, dCBP may regulate synaptic development though non CREB-dependent mechanisms.

In vertebrates, CBP has been broadly implicated as being necessary for normal brain development, as well as learning. CBP mutant mice show dominant defects in neuronal development, as well as learning and memory. In humans, CBP mutations are associated with Rubenstein-Taybi syndrome, a disorder that is characterized by developmental limb defects and severe mental

retardation (Tenaka et al., 1997). Our data implicate dCBP as having a specific role at the synapse. A similar role for CBP in vertebrate systems could contribute to our understanding of the observed phenotypes.

CBP is a transcriptional coactivator for CREB. CREB has been investigated in detail for a role in activity-dependent synaptic plasticity (Bartsch et al., 1995, 1998; Deisseroth et al., 1996; Yin and Tully, 1996; Casadio et al., 1999). CREB has also been implicated in the mechanisms of learning in the mouse (Silva et al., 1998). In Aplysia, presynaptic CREB is considered necessary for seratonin- and protein synthesis–dependent long-term synaptic facilitation (Bartch et al., 1995, 1998; Casadio et al., 1999). Experiments on vertebrate neurons in cell culture implicate a role for postsynaptic CREB in activity-dependent plasticity and neurotrophin-dependent neuronal survival (Deisseroth et al., 1996; Finkbeiner et al., 1997). CBP has also been implicated in the neurotrophin-dependent survival mechanisms (Liu et al., 1998). However, the precise relationship between CREB and CBP in the control of these processes remains unclear. For example, CREB and CBP may be differentially phosphorylated in response to different sources of postsynaptic Ca^{2+} influx (Hardingham et al., 1999; Hu et al., 1999). In addition, CBP is known to act as a coactivator with other transcription factors and as a signaling molecule via acetylase activity (Johnston et al., 1999; Waltzer and Bienz 1998, 1999). *Drosophila* now opens the possibility for a forward genetic analysis of CBP function at the synapse.

Experimental Procedures

Electron Microscopy

Wild-type and mutant larvae were prepared for electron microscropy (EM) according to procedures previously described (Schuster et al., 1996). Serial sections were taken at 0.1 μm, as described previously. EM for dCBP loss-of-function analysis was done on homozygous $P(EP)^{1149}$ third instar larvae.

Genetics

$P(EP)^{1149}$ and $P(EP)^{1179}$ were obtained from the Berkeley *Drosophila* Genome Project. $dCBP^{S342}$/*Fm7c*, $dCBP^{TA57}$/*Fm7c*, and $dCBP^{Q7}$/*FM7c* were obtained from the laboratory of William McGinnis. Homozygous lethal dCBP alleles were maintained over an *Fm7c-kruppel*-GFP balancer (provided by Tom Kornberg). dCBP mutant chromosomes were identified in the larvae based on malpigian tubule and mouth hook color (Schuster et al., 1996) and based on the absence of the *FM7c*-GFP balancer. Muscle overexpression of dCBP was achieved using the $MHC\text{-}GAL4^{82}$ line (Winberg and C. S. G., unpublished data). Presynaptic overexpression of dCBP was achieved using the $elaV\text{-}GAL4^{3A4}$ line. *UAS-dCBP* is a complete dCBP open reading frame cloned into the P-UAS transformation vector and inserted on the X chromosome.

Histology

Following dissection and electrophysiological recording, each larval fillet was fixed in 3.7% formaldehyde in HL3 saline (Stewart et al., 1994). The NMJ was visualized with anti-Synaptotagmin and anti-Fasciclin II double staining, as described previously (Davis et al., 1997). Bouton numbers were quantified at muscles 6 and 7 in larval segment A3, as described previously (Davis et al., 1997), allowing direct correlation between the electrophysiology and anatomy for each neuromuscular synapse. All bouton counts were done blind. Glia were stained using the anti-Repo antibody, provided by C. S. Goodman. Axon guidance was assayed in the embryonic nervous system using anti-Fasciclin II (Goodman) and the monoclonal antibody BP102 (Goodman). dCBP expression was assayed using a dCBP antibody (chicken). dCBP imaging was done on a DeltaVision deconvolution confocal microscope (Applied Precision). Relative fluorescence intensity was measured using the line profile tool of DeltaVision software, allowing comparison of pixel intensity between the muscle and nuclear dCBP fluorescence. Averages were take from 30–40 muscle nuclei from 24–30 separate synapses in wild-type and $P(EP)^{1149}$ homozygous mutant larvae.

Molecular Reagents

Genomic sequences flanking the P element insertion were amplified by PCR from $P(EP)^{1179}$ genomic DNA using a primer from the 3′ end of the P element (pry2) and primers from dCBP (nej1, nej2, and nej4). PCR products were cloned into pGEM-T (Promega) and sequenced. For analysis of the P element–driven transcript, $P(EP)^{1179}$ females were crossed to heat shock GAL4 males. Embryos were heat shocked at 37°C for 2 hr, and the RNA isolated (Qiagen). RT-PCR was performed (GIBCO) using the following primer pairs: first round, pr192 and nej2; second round, pr200 and nej1. In situ hybridizations were performed on embryos derived from two GAL4 enhancer lines, *24B-GAL4* and $elaV\text{-}GAL4^{3A4}$, crossed to $P(EP)^{1179}$ females. Embryos were probed with a dCBP probe. The dCBP probe was generated from bases 766 to 1279 of the known dCBP sequence and subcloned into pGEM-T, and the digoxigenin-labeled RNA probe was synthesized accordingly (Roche Molecular Biochemicals). The sequences for the PCR primers designated in Figure 1 are as follows: pry2, CTTGCCGACGGGACCACCTTATGTTATT; pr192, GAGTTAATTCAA ACCCCACGGACATGC; pr200, CTCTAGACAAGCATACGTTAAGTG GATGTC; nej1, CGAGGACACCAGCTCATC; nej2, GGATTCCCGCT TACCAG; and nej4, GTGGTGCTGGAATGTTGC.

Electrophysiology

All recordings were made from muscle fiber 6 in abdominal segment A3 in 0.5 mM Ca^{2+} HL3 saline, as previously described, except where indicated (Davis et al., 1996). For each recording, the resting membrane potential and input resistance were recorded. Only recordings with resting potentials of at least −60 and input resistances of at least 8 MΩ were included in our analysis. Quantal content was calculated by dividing the average maximal EPSP amplitude by the average amplitude of the spontaneous miniature release events (mEPSP). Quantal size was determined by the average amplitude of the spontaneous release events recorded in the absence of stimulation. To compare the distribution of spontaneous release events between different mutant lines, mEPSPs were pooled for at least five recordings from at least five preparations in which the muscle input resistance and resting membrane potentials were closely matched (RMP, between −65 and −72; R_{in}, between 8 and 9 MΩ). Frequency-dependent facilitation experiments were performed and quantified as described previously (Davis et al., 1996). Measurements of maximal EPSP amplitude were done by hand, as described previously. Measurements of spontaneous miniature release events were semiautomated using MiniAnalysis software (Jaejin) and edited by hand.

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