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RAPID ENRICHMENT OF PRESYNAPTIC PROTEIN IN BOUTONS UNDERGOING CLASSICAL CONDITIONING IS MEDIATED BY BRAIN-DERIVED NEUROTROPHIC FACTOR

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Abstract

Presynaptic structural modifications are thought to accompany activity-dependent synaptic plasticity and learning. This may involve the conversion of nonfunctional synapses into active ones or the generation of entirely new synapses. Here, using an *in vitro* neural analog of classical conditioning, we investigated presynaptic structural changes restricted to auditory nerve synapses that convey the conditioned stimulus (CS) by tract tracing using fluorescent tracers combined with immunostaining for the synaptic vesicle-associated protein synaptophysin. The results show that the size of presynaptic auditory boutons increased and the area and fluorescence intensity of punctate staining for synaptophysin were enhanced after conditioning. This occurred only for auditory nerve boutons apposed to the dendrites but not the somata of abducens motor neurons. Conditioning increased the percentage of boutons that were immunopositive for synaptophysin and enhanced the number of synaptophysin puncta they contained. Pretreatment with antibodies against brain-derived neurotrophic factor (BDNF) inhibited these conditioning-induced structural changes. There was also a net increase in the number of boutons apposed to abducens motor neurons after conditioning or BDNF treatment. These data indicate that the rapid enrichment of presynaptic boutons with proteins required for neurotransmitter recycling and release occurs during classical conditioning and that these processes are mediated by BDNF.

Keywords

classical conditioning; synaptophysin; presynaptic; structural plasticity; BDNF

Presynaptic modification is one of the mechanisms used to process and store activity-dependent synaptic plasticity (Krueger and Fitzsimonds, 2006). This might occur by the formation of new synapses or by conversion of existing but silent presynaptic terminals to a functional state by inclusion of proteins required for vesicular recycling and release. In recent years, molecular and imaging approaches have been used to examine changes in these two factors during short or long-term synaptic plasticity. Using FM dyes or vesicle proteins conjugated with pHluorins to directly visualize active presynaptic vesicles, evidence has

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suggested that there is rapid activation of silent synapses and slower generation of new synapses in *Aplysia* sensory-motor cocultures during synaptic potentiation (Kim et al., 2003). The formation of new release sites has also been observed in cultured hippocampal neurons by examining the distribution of presynaptic vesicle proteins tagged either with green fluorescent protein (GFP) for live imaging or immunostained for static imaging (Antonova et al., 2001, 2009; Ninan et al., 2006). Although these techniques have revealed presynaptic modifications, molecular mechanisms that initiate these changes have not been clearly identified (de Jong and Verhage, 2009).

Brain-derived neurotrophic factor (BDNF) is implicated in presynaptic modifications that accompany synaptic plasticity. BDNF can be generated and released in an activity-dependent manner from its precursor protein proBDNF (Keifer et al., 2009; Matsuda et al., 2009; Nagappan et al., 2009). BDNF facilitates the generation of long-term potentiation (LTP) when paired with weak stimulation (Kovalchuk et al., 2002) while under high-rate perfusion conditions BDNF treatment alone can result in sustained synaptic potentiation (Kang and Schuman, 1995; Ji et al., 2010). The enhanced synaptic strength induced by BDNF is at least in part attributable to presynaptic changes. BDNF alters the frequency of miniature excitatory postsynaptic currents (mEPSCs) as well as synaptic fatigue and paired-pulse facilitation, an indication of its presynaptic function (Carmignoto et al., 1997; Gottschalk et al., 1998; Li et al., 1998). Using FM1-43 live imaging, Walz et al. (2006) suggested that BDNF is critical for activity-induced facilitation of presynaptic vesicle cycling. Further evidence was obtained by Staras et al. (2010) who found that motility of synaptic vesicles was increased with focal application of BDNF onto single synapses.

Using an *in vitro* model of the classically conditioned eyeblink response we have shown an increase in levels of the synaptic vesicle-associated proteins synaptophysin and synapsin I during conditioning or BDNF treatment (Mokin and Keifer, 2004; Mokin et al., 2007; Li and Keifer, 2008). Synaptophysin is implicated in regulating endocytosis that controls synaptic vesicle availability during distinct states of neuronal activity (Kwon and Chapman, 2011). In this model of classical conditioning, paired stimulation of the auditory (the “tone” conditioned stimulus, CS) and trigeminal (the “airpuff” unconditioned stimulus, US) nerves was used to evoke a neural analog of conditioned responses (CRs) characteristic of eyeblinks recorded from the abducens nerve (Keifer and Zheng, 2010). In contrast to studies that examined whole populations of synapses, in the present study we used tract tracing to identify presynaptic auditory nerve terminals that convey the CS in apposition to postsynaptic abducens motor neurons and assessed the colocalization of synaptophysin protein specifically to those boutons after conditioning by immunostaining. We found that there is a dramatic increase in the overall level of synaptophysin early in conditioning. Analysis shows that conditioning results in enlargement of boutons apposed to the dendrites but not the somata of abducens motor neurons and induces an increase in the aggregation of synaptophysin protein confined to those boutons. Furthermore, a greater number of boutons apposed to dendrites contain synaptophysin protein while there are fewer boutons that contain no synaptophysin. We also show that these changes are inhibited by bath application of antibodies against BDNF. The results suggest that there is a rapid enhancement in the functional capacity of presynaptic auditory nerve terminals during *in vitro* conditioning by BDNF-induced incorporation of proteins required for synaptic vesicle recycling and release.

EXPERIMENTAL PROCEDURES

Conditioning procedures

Freshwater pond turtles, *Trachemys scripta elegans*, obtained from commercial suppliers were anesthetized by hypothermia until torpid and decapitated. Protocols involving the use of animals complied with the guidelines of the National Institutes of Health and the

Institutional Animal Care and Use Committee. The brain stem was transected at the levels of the trochlear and glossopharyngeal nerves and the cerebellum was removed as described previously (Anderson and Keifer, 1999). The brain stem was continuously bathed in physiological saline (2–4 ml/min) containing (in mM): 100 NaCl, 6 KCl, 40 NaHCO₃, 2.6 CaCl₂, 1.6 MgCl₂ and 20 glucose, which was oxygenated with 95% O₂/5% CO₂ and maintained at room temperature (22–24°C) at pH 7.6. Suction electrodes were used for stimulation and recording of cranial nerves. The US was an approximately twofold threshold single shock stimulus applied to the trigeminal nerve; the CS was a subthreshold 100 Hz, 1 sec train stimulus applied to the ipsilateral auditory nerve that was adjusted to below threshold amplitude required to produce activity in the abducens nerve. Neural activity was recorded from the ipsilateral abducens nerve that projects to the extraocular muscles controlling movements of the eye, nictitating membrane and eyelid. The CS-US interval was 20 ms which is defined as the time between the offset of the CS and the onset of the US. The intertrial interval between the paired stimuli was 30 sec. A pairing session consisted of 50 CS-US presentations (lasting 25 minutes in duration) followed by a 30 minute rest period in which there was no stimulation. Conditioned responses were defined as abducens nerve activity that occurred during the CS and exceeded an amplitude of double the baseline recording level. Conditioned preparations received paired CS-US stimulation whereas pseudoconditioned controls received the same number of CS and US exposures that were explicitly unpaired using a CS-US interval randomly selected between 300 ms and 25 sec.

Tracer injections, imaging, and data analysis

Following the physiological experiments, preparations were injected with anterograde tracer into the auditory nerve and retrograde tracer into the abducens nerve. Anterograde injections were accomplished using a micropipette (tip diameter ~15 µm) filled with 10% fluororuby (FR, red; Invitrogen, Carlsbad, CA, USA) dissolved in physiological saline. A total volume of ~0.2–0.4 µl FR was injected slowly into the cut end of the auditory nerve over a period of 1 h. In the same preparation, retrograde labeling of the abducens motor neurons was achieved by injections of ~0.4 µl of 0.5% Alexa Fluor 405 (AF405, blue; Invitrogen) into the ipsilateral abducens nerve for the same time period. Preparations remained in oxygenated physiological saline for an additional 4 h to allow for transport of the tracers for a total elapsed time of 5 h between the end of training and fixation. Preparations were then immersion fixed in cold 0.5% paraformaldehyde, cryoprotected, and sectioned at 60 µm on a microtome. Sections were probed with a primary antibody to synaptophysin (1:1000; 5768, Sigma, St. Louis, MO, USA) followed by incubation with a Cy2-conjugated secondary antibody (1:50; green). Images were acquired using an Olympus Fluoview 1000 laser scanning confocal microscope from which z-stack optical sections were obtained. Tissue samples were scanned using a 60x 1.4 NA oil immersion objective with triple excitation using 405 nm diode, 488 nm argon, and 543 HeNe lasers. All sections were viewed for the presence of abducens motor neurons with apposing boutons for analysis. Boutons were identified as *en passant* with varicosities along thinner axon shafts or of the *terminaux* type with swellings at the termination of axonal branches. Only boutons observed to be directly overlapping (i.e., having shared pixels) with a portion of a retrogradely labeled motor neuron and in the same focal plane were determined to be in apposition to one another and were included in the analysis (Keifer and Mokin, 2004). Nonapposed boutons were those that were not in contact with an abducens motor neuron but were attached to the same axon as those in apposition and within a range of approximately 100 µm. Synaptophysin puncta chosen for analysis were localized within the boundaries of an anterogradely labeled presynaptic auditory bouton by visualizing them in the x, y and z optical planes. Z-stack images were collapsed into a single image for measurements of boutons and synaptophysin puncta, and images were thresholded to produce equal background intensity for quantification. Synaptophysin puncta that were at least two-fold greater intensity above

background were outlined on the images and measured for area or fluorescence intensity using ImageJ (NIH, Bethesda, MD, USA). Intensity values for synaptophysin puncta were normalized with respect to pseudoconditioning which was set at 100%. Data were analyzed using StatView software using a one-way ANOVA followed by *post-hoc* analysis using Fisher's and Bonferroni's tests. Values are represented as means $\pm$ SEM. Cumulative distribution plots were analyzed using the Kolmogorov-Smirnov test.

Pharmacology

Some preparations were pretreated with BDNF antibodies (5 μ g/ml; 20981, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 5 h prior to application of the conditioning procedure. Other preparations were pretreated by bath application of BDNF (100 ng/ml; Santa Cruz), K252a (200 nM; Calbiochem, La Jolla, CA, USA), a cell-permeable protein kinase inhibitor with actions on receptor tyrosine kinases, Rp-cAMPs (50 μ M), a selective cAMP analog and competitive inhibitor of PKA activation, or PD98059 (50 μ M; Calbiochem), a MEK-ERK inhibitor.

Western blotting

Following the conditioning experiments, preparations were frozen in liquid nitrogen and stored at -70°C . Tissue (40 ng samples) was homogenized in NP-40 buffer containing protease and phosphatase inhibitors, rotated at 4°C for 2 h, and centrifuged at 10,000 g for 20 min. The supernatants were obtained and protein concentrations determined by the Bradford protein assay. Equal amounts of protein sample were denatured in loading buffer containing 125 mM Tris-HCl (pH 6.8), 20% glycerol, 6% SDS, and 5% β -mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE. Proteins were blotted on PVDF membranes and blocked with 5% nonfat dry milk in TBST (20 mM Tris at pH 7.6, 150 mM NaCl, and 0.1% Tween-20) for 1 h. Membranes were probed with an antibody to synaptophysin (1:500; Sigma) overnight at 4°C , rinsed with TBST, and incubated with an HRP-conjugated secondary antibody for 2 h at room temperature. Membranes were reprobed for loading controls with a β -actin antibody (1:1000, Millipore, Temecula, CA, USA) which in our preparation shows a 3-fold change in densitometry readings between 30–50 ng protein. Proteins were detected using chemiluminescence and immunoreactive signals were captured on Kodak X-omatic AR film and quantified by computer-assisted densitometry. Optical densities of the bands for synaptophysin and β -actin were determined relative to background levels. Ratios of synaptophysin and β -actin were obtained for each experiment, averaged, and displayed as a percentage with respect to pseudoconditioning.

RESULTS

Timing of conditioning-induced synaptophysin protein expression

In this *in vitro* analog of classical conditioning, stimulation of the cranial nerves is used in place of natural stimulation using a tone or airpuff. Conditioning consists of paired stimulation of the auditory nerve for one second in duration that precedes a single shock to the trigeminal nerve that evokes a neural correlate of an abducens nerve unconditioned blink response (UR). After about one hour or during the second pairing session, neural discharge is recorded in the abducens nerve during auditory nerve stimulation that represents a neural correlate of a CR. Pseudoconditioning trials consist of the same number of CS and US exposures that are explicitly unpaired and results in no CR acquisition (see Exp. Procedures). To characterize the temporal profile of synaptophysin protein expression during conditioning, Western blot analysis was carried out at several different time points. Levels of synaptophysin were significantly increased after one pairing session or 25 minutes after the onset of the conditioning protocol (C1) but not earlier after 15 minutes (C₁₅; Fig. 1A; $n = 5$ preparations/group, $P = 0.03$, C1 vs. Ps1). Elevated levels of synaptophysin were

maintained through the second pairing session or after about two hours ($P = 0.01$, C2 vs. Ps2), which we have shown previously (Li and Keifer, 2008). As is typical of conditioning in this preparation, CRs are generally recorded during the second pairing session. In these cases, CRs averaged 3% after 15 minutes of training, 10% after one pairing session, and 86% after two sessions compared to 0% CRs after pseudoconditioning for all time points. The conditioning-related increase in synaptophysin was inhibited by pretreatment with K252a ($P = 0.03$, K252a vs. C2), a protein kinase inhibitor with actions on receptor tyrosine kinases, that also blocked conditioning to 0% CRs. Confocal images of abducens motor neurons selected from the different treatment groups showing punctate staining for synaptophysin are illustrated in Fig. 1B. It can be seen from the images that synaptophysin puncta are more numerous and appear larger or more aggregated after conditioning compared to pseudoconditioning or treatment with K252a. Quantification of the enhanced number of synaptophysin puncta following conditioning has been reported in earlier studies (Li and Keifer, 2008; Zheng and Keifer, 2008, 2009). We were also interested as to whether a protein synthesis inhibitor prevented the changes in synaptophysin. Bath application of anisomycin (30 μM) for 1.5 hr prior to conditioning suppressed levels of synaptophysin protein compared to either pseudoconditioning or normal conditioning (79% compared to 100% for Ps2 or 137% for C2; $P = 0.003$). These data indicate that significantly enhanced levels of synaptophysin protein are expressed early in the acquisition process of conditioning and precede the timepoint when the majority of CRs are recorded.

Growth of presynaptic boutons during conditioning is regulated by BDNF

Previously (Li et al., 2011), we reported that auditory nerve terminals apposed to abducens dendrites, but not somata, undergo growth during the conditioning process. Whether the conditioning-related increase in synaptophysin expression occurs in the same population of auditory nerve boutons that undergo changes in size was determined here. Tract tracing was employed to anterogradely label auditory nerve boutons with FR (red) and retrogradely label abducens motor neurons with AF405 (blue) followed by immunostaining for synaptophysin (green). In pond turtles, the auditory nerve projects directly to the abducens motor neurons (Keifer and Mokin, 2004). Representative FR-labeled boutons apposed to AF-labeled abducens motor neurons are shown in Fig. 2A. Conditioning for two pairing sessions (to a mean of 82% CRs) resulted in a significant increase in the average area of auditory nerve boutons compared to pseudoconditioning (0% CRs; Fig. 2B, $n = 5$ preparations/group, Total boutons, $P < 0.0001$). In contrast, preparations that underwent treatment with BDNF antibodies applied to the bath followed by conditioning failed to show CR acquisition (0% CRs) and there was no change in bouton size compared to pseudoconditioning ($P = 0.64$, BDNF Ab vs. Ps2). Further analysis showed that the observed bouton growth was restricted to those apposed to the dendrites but not the somata of abducens motor neurons (Fig. 2B, Dendrite, $P < 0.0001$, C2 vs. Ps2). These findings are apparent in the images showing considerably larger boutons apposed to dendrites after conditioning compared to pseudoconditioning or BDNF antibody treatment while there were no changes in those apposed to somata (Fig. 2A, arrows). Interestingly, the increase in bouton size failed to be observed for boutons that were not in direct apposition to abducens motor neurons but were attached to the same axon as those in apposition and located within 100 μm (Fig. 2C; $P = 0.31$, C2 vs. Ps2, Nonapposed boutons; $P < 0.0001$, C2 Total Apposed vs. C2 Nonapposed). Additionally, a significant rightward shift toward larger dendritic bouton area was also seen in the cumulative percentage distribution plots after conditioning (Fig. 2D; $P < 0.0001$, C2 vs. Ps2). BDNF antibody pretreatment prevented this shift resulting in values indistinguishable from pseudoconditioning ($P = 0.23$). These data showing significant conditioning-related auditory nerve bouton growth on abducens motor neuron dendrites confirms the earlier findings of Li et al. (2011).

Conditioning-related enrichment of auditory boutons with synaptophysin

Next, the area and fluorescence intensity of synaptophysin puncta localized specifically within auditory nerve presynaptic boutons was examined. To confirm that synaptophysin was localized within a bouton, confocal images were acquired in the *x*, *y* and *z* planes. As illustrated in Fig. 3, a bouton labeled with FR (red) and immunostained to reveal synaptophysin protein clusters or puncta (green) were judged to be colocalized (appearing yellow) when the bouton was examined in *xz* and *yz* planes and the puncta was within the boundaries of the bouton in three dimensions. In contrast, synaptophysin puncta located near a bouton but outside of its boundaries were not included in further analysis. Measurements of synaptophysin puncta localized within auditory nerve boutons that were in apposition to abducens motor neurons showed that conditioning resulted in a significant increase in puncta area (Fig. 4A, $n = 5$ preparations/group; Total puncta, $P < 0.0001$) and fluorescence intensity (Fig. 4D; $P < 0.0001$) compared to pseudoconditioning. When boutons were analyzed according to localization on abducens motor neurons, a significant increase in area and intensity of synaptophysin puncta was observed for boutons apposed to the dendrites but not to somata (Fig. 4A,D; Dendrite, $P < 0.0001$, C2 vs. Ps2 for both area and intensity). Pretreatment with BDNF antibodies suppressed the enhanced area and staining intensity of synaptophysin puncta resulting in values similar to pseudoconditioning, suggesting that BDNF is involved in these structural changes. For comparison, we also assessed whether the observed changes in area and staining intensity of synaptophysin puncta was specific to boutons that were not directly apposed to abducens motor neurons but were located nearby. Compared with pseudoconditioning or BDNF antibody application, conditioning had no effect on the area (Fig. 4B; $F_{2,310} = 1.5$, $P = 0.24$, all Nonapposed groups; $P < 0.0001$, C2 Total Apposed vs. C2 Nonapposed) or fluorescence intensity (Fig. 4E; $F_{2,310} = 0.2$, $P = 0.83$, all Nonapposed groups; $P = 0.02$, C2 Total Apposed vs. C2 Nonapposed) of nonapposed synaptophysin puncta. Further analysis of synaptophysin puncta localized to boutons apposed to dendrites using cumulative percentage distribution plots revealed a significant rightward shift of puncta toward larger size and higher fluorescence intensity after conditioning (Fig. 4C,F; $P < 0.0001$, C2 vs. Ps2 for both area and intensity). This shift returned to control values by BDNF antibody pretreatment. These data suggest that conditioning induces enhanced aggregation of synaptophysin protein within auditory nerve boutons that are specifically in apposition to dendrites of abducens motor neurons and that BDNF mediates this response.

Changes in synaptophysin distribution within boutons

Analysis also revealed that auditory nerve boutons contain a greater proportion of synaptophysin-positive puncta after conditioning. The percentage of boutons in apposition to abducens motor neurons containing no synaptophysin puncta (empty boutons) was significantly reduced after conditioning compared to pseudoconditioning (Fig. 5; $n = 5$ preparations/group, $P < 0.01$). Correspondingly, the percentage of boutons having two synaptophysin puncta after conditioning was significantly increased ($P < 0.01$, C2 vs. Ps2) as were boutons having three puncta ($P < 0.001$) or even four ($P = 0.03$). As with the alterations in bouton and puncta size, the changes in puncta distribution was attributable to boutons apposed to the dendrites but not to somata. These conditioning-induced changes in bouton distribution were inhibited by BDNF antibody pretreatment.

Evidence for conditioning- and BDNF-induced synaptogenesis

Finally, we examined whether there is a net increase in the number of boutons apposed to abducens motor neurons after conditioning or BDNF treatment. To accomplish this, all anterogradely-labeled boutons apposed to abducens motor neurons were counted and this value was divided by the total number of retrogradely labeled motor neurons for each preparation and averaged. The results showed that the mean number of boutons per neuron

increased significantly after conditioning (Fig. 6; $n = 5$ preparations/group, Total boutons, $P < 0.01$, C2 vs. Ps2) or bath application of BDNF for 80 minutes, the equivalent time period of two pairing sessions ($P = 0.02$, BDNF2 vs. Ps2). The enhanced number of boutons per neuron induced by conditioning was significantly attenuated by treatment with the PKA inhibitor Rp-cAMPs ($P = 0.006$, Rp vs. C2) or the MEK-ERK inhibitor PD98059 ($P = 0.01$, PD vs. C2). Similar findings were obtained for the BDNF-induced increase in boutons per neuron ($P = 0.03$, Rp vs. BDNF2; $P = 0.02$, PD vs. BDNF2). The increase in the total number of boutons was attributable to those apposed to the dendrites rather than to somata (Fig. 6; Dendritic boutons, $P = 0.04$, C2 vs. Ps2, $P = 0.03$, BDNF2 vs. Ps2). These data suggest that conditioning or BDNF treatment induces an overall increase in the number of boutons specifically apposed to the dendrites of abducens motor neurons.

DISCUSSION

Presynaptic morphological correlates of synaptic plasticity and learning

Previously, we documented that the presynaptic proteins synaptophysin and synapsin I are significantly increased after two pairing sessions of conditioning (Mokin and Keifer, 2004; Mokin et al., 2007; Li and Keifer, 2008). Here we show that synaptophysin is increased during *in vitro* conditioning as early as one pairing session, or after 25 minutes, but is not enhanced after only 15 minutes of pairing. Immunostaining for synaptophysin localized specifically to auditory nerve boutons reveals an increase in the size and fluorescence intensity of synaptophysin puncta, and an enhanced number of boutons containing one or more puncta, after conditioning. The observed changes in synaptophysin are also accompanied by significant enlargement of auditory nerve presynaptic terminals. Importantly, these presynaptic modifications are selectively confined to synaptic contacts that are in direct apposition to the dendrites of abducens motor neurons but not to the somata. Boutons located nearby but not in direct apposition to motor neurons fail to show these conditioning-related alterations. Our findings indicate that structural modifications in presynaptic terminals that convey the CS occur rapidly and accompany the acquisition of CRs during classical conditioning. Synaptophysin has been implicated in controlling distinct phases of synaptic vesicle availability through regulation of endocytosis during neuronal activity (Kwon and Chapman, 2011) thereby having a key role in presynaptic function. Enhanced levels of synaptophysin and alterations in its distribution may therefore be a critical component that accompanies synapse modification during learning.

While structural manifestations of activity-dependent synaptic plasticity have been well documented, studies have largely focused on postsynaptic remodeling with less attention to presynaptic modifications (Holtmaat and Svoboda, 2009). Morphological alterations include changes to existing structures that might occur rapidly and formation of new synapses occurring over longer time periods. The increased size and fluorescence intensity of synaptophysin protein aggregates and the enhanced number of boutons containing synaptophysin after conditioning likely results from *de novo* protein synthesis as evidenced by our Western blot data. However, it is also possible that existing synaptophysin protein is redistributed during conditioning. Rapid clustering of synaptophysin was observed in cultured hippocampal neurons within minutes of the onset of long-lasting potentiation suggesting that reorganization of presynaptic proteins may accompany synaptic plasticity (Antonova et al., 2001). Redistribution of presynaptic proteins has also been observed in cultured hippocampal neurons following tetanus in which diffuse staining for vesicle-associated membrane protein 2 (VAMP2) becomes aggregated and there is budding of preexisting release sites containing VAMP2 puncta to form new sites (Ninan et al., 2006). These morphological changes correspond with enhanced neurotransmitter release. Synaptic vesicles have also been observed in time-lapse studies to be transported between adjacent or even distant boutons (Darcy et al., 2006; Staras et al., 2010). In *Aplysia* sensory-motor

neuron cocultures, repeated application of serotonin to the sensory neurons induces two morphologically and temporally distinct changes: an early rapid filling of preexisting varicosities with synaptic vesicles and a slower formation of new synapses (Kim et al., 2003). These changes accompany enhanced EPSPs and contribute to intermediate and long-term forms of synaptic facilitation. A shift in the relative percentage of boutons that contain no synaptophysin puncta (empty boutons) toward those that contain one or more puncta after conditioning observed here suggests that there is rapid filling of presynaptic nerve terminals with proteins required for vesicular recycling and release of neurotransmitter. Moreover, the observation of a greater number of boutons apposed to the dendrites of abducens motor neurons after conditioning or BDNF treatment suggests the formation of entirely new synapses. Expansion of existing synapses involving the generation of multiple-synapse boutons (MSBs), that is boutons containing multiple presynaptic active zones that form separate synapses with two or more postsynaptic sites, might occur during *in vitro* conditioning as it does after LTP or associative learning (Toni et al., 1999; Geinisman et al., 2001). Filling of nonfunctional boutons with synaptic vesicle recycling and release machinery could take place rapidly, would not necessarily require protein synthesis, and might precede slower changes such as bouton growth or synaptogenesis. While increases in both synaptophysin protein and bouton size occur early in conditioning it remains to be determined if these two events are related or have distinct time courses.

BDNF mediates presynaptic structural changes in conditioning

BDNF has been shown to promote axonal branching, dendritic outgrowth, and formation of synapses particularly during development (Lu et al., 2005). Earlier we showed that BDNF-TrkB is required for enhanced synaptophysin protein expression thereby implicating them in presynaptic structural modifications (Li and Keifer, 2008, 2009). Antibodies against the BDNF receptor TrkB, but not related receptors TrkA or TrkC, inhibited conditioning-induced synaptophysin synthesis and BDNF application alone promoted its expression. These results were confirmed by immunocytochemical findings showing that BDNF increased synaptophysin punctate staining that was counteracted by coapplication of BDNF with the inhibitor K252a. In the present study, we show that BDNF antibodies suppress conditioning and the associated increase in the area and staining intensity of synaptophysin puncta as well as the number of synaptophysin-containing auditory nerve boutons apposed to the dendrites of abducens motor neurons. How does BDNF mediate conditioning-induced changes in synaptophysin that occur only in boutons apposed to dendrites? There are several possibilities. Because BDNF functions predominantly through binding to the TrkB receptor (Lu et al., 2005), regulation of the cellular localization of TrkB receptors in conditioning could be an important factor. In our model system, it is unknown whether there are low densities of TrkB on abducens motor neuron somata and high densities on dendrites. Additionally, differential intracellular transcription and release of BDNF itself might also be a candidate for regulation of localized BDNF function. Using cultured neurons, An et al. (2008) found selective targeting of BDNF transcripts containing long 3' untranslated regions (UTRs) to dendrites while those with short 3' UTRs are restricted to somata. Neurons deficient in the long 3' UTR BDNF RNAs show impaired BDNF secretion and hippocampal LTP. Those authors suggested that somatic BDNF encoded from the short 3' UTR mRNAs is responsible for neuronal survival whereas dendritic BDNF derived from long UTRs is involved in synaptic plasticity. Cellular responses to BDNF signaling also depend on the kinetics of BDNF release, that is, acute or sustained release may have different structural and functional consequences (Ji et al., 2010).

The Eph/ephrin trans-synaptic signaling system has been implicated in promoting structural synaptic modifications during *in vitro* conditioning (Li et al., 2011) and elsewhere (Kayser et al., 2006; Lim et al., 2008; McClelland et al., 2009). Signaling may proceed in forward or

reverse directions, or bidirectionally (Klein, 2009), which makes this system ideally suited to coordinate pre- and postsynaptic structural alterations that accompany synaptic plasticity. Interaction between BDNF-TrkB and Eph/ephrin signaling has been shown to regulate axonal branching and synaptogenesis (Marler et al., 2008). We recently obtained evidence for ephrin-B-induced EphB forward signaling in auditory nerve presynaptic bouton enlargement associated with *in vitro* classical conditioning (Li et al., 2011). In conditioning, the Eph/ephrin system interacts with BDNF-TrkB to induce bouton growth possibly through Src family kinases. This is coordinated with postsynaptic delivery of AMPARs containing GluA1 and GluA4 subunits that mediate the acquisition of CRs (Keifer and Zheng, 2010). Whether Eph/ephrin signaling is also involved in synaptic enrichment of presynaptic boutons with recycling and release proteins to recruit and enhance their functional capacity during learning, or whether other mechanisms are required, is currently unknown.

In the present study and an earlier report (Li et al., 2011) we have shown that the enhanced growth and number of presynaptic boutons observed after conditioning or BDNF application is inhibited by the PKA antagonist Rp-cAMPs and the MEK-ERK blocker PD98059. From earlier work on postsynaptic changes after conditioning, we provided evidence that both PKA and ERK are required for AMPAR synaptic incorporation and conditioning, and that the activation of ERK is downstream from PKA (Zheng and Keifer, 2009). The effect of suppression of PKA activation on presynaptic structural changes is not surprising since PKA initiates a cascade of signal transduction events including activation of ERK that results in coordinated pre- and postsynaptic modifications (Li and Keifer, 2008; Li et al., 2011). Postsynaptically, ERK is thought to be activated by BDNF-TrkB signaling and is involved in AMPAR trafficking during conditioning (Zheng and Keifer, 2009). However, the current data indicate that ERK, whether directly or indirectly, also has presynaptic effects, but how it participates in structural plasticity is unclear. MEK-ERK may interact postsynaptically with the Eph/ephrin system to affect trans-synaptic signaling to the presynaptic side, or have direct actions presynaptically through activation of TrkB by extracellularly released BDNF induced by conditioning. One may speculate that activation of presynaptic TrkB may be a basis for early changes in conditioning such as filling of boutons with presynaptic release proteins, while later changes including bouton growth and synapse formation utilize Eph/ephrin signaling. Further studies are needed to clarify the signal transduction processes involved in presynaptic structural remodeling in conditioning and the precise timing of these events in relation to postsynaptic modifications.

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Highlights

- We studied presynaptic structural changes during *in vitro* classical conditioning
- Synaptophysin protein and punctate staining is rapidly induced after conditioning
- These presynaptic boutons are confined to dendrites and also undergo enlargement
- The presynaptic structural changes require BDNF
- Rapid increased functional capacity of presynaptic boutons accompanies conditioning

Fig. 1.

Expression of synaptophysin protein during conditioning. (A) Levels of synaptophysin protein expression were significantly increased after conditioning for one pairing session (C1, or 25 minutes) but not after 15 minutes of conditioning (C₁₅). Expression remained elevated through the second pairing session (C2) and was inhibited by treatment with K252a. Data were normalized with respect to the loading control β -actin. Ps, pseudoconditioning. (B) Confocal images showing representative punctate staining for synaptophysin of abducens motor neurons from preparations that were pseudoconditioned for two pairing sessions (Ps2), conditioned for two sessions (C2), or underwent conditioning in the presence of K252a. For these images, staining was not confined to presynaptic boutons. Scale bar = 4 μ m.

* Indicates significant differences from Ps for all figures. *P* and *n* values are given in the text.

Fig. 2.

Conditioning results in enlargement of presynaptic auditory nerve boutons apposed to abducens motor neuron dendrites. (A) Confocal images of anterogradely labeled auditory boutons with FR (red) apposed to retrogradely labeled abducens motor neurons with AF405 (blue). Boutons in apposition (arrows) to motor neuron somata and dendrites from preparations that were pseudoconditioned (Ps2), conditioned for two pairing sessions (C2), or pretreated with bath application of BDNF antibodies for the equivalent time period of two pairing sessions (BDNF Ab) are shown. Scale bar = 4 μm . (B) Conditioning resulted in a significant increase in the mean area of the total sample of boutons compared to pseudoconditioning. Further analysis showed that enlarged boutons were those apposed to dendrites but that there were no changes in boutons apposed to the somata. Pretreatment with BDNF antibodies (BDNF Ab) prior to conditioning blocked this response. (C) No significant differences were observed in bouton area for those located nearby but not apposed to motor neurons. (D) Cumulative distribution plot of the area of dendritic boutons shows a significant rightward shift toward larger boutons after conditioning compared to the other groups.

Fig. 3.

Three dimensional image analysis was used to localize synaptophysin puncta to auditory nerve boutons. Separate images are shown of a bouton labeled with FR (red) and an immunostained synaptophysin puncta (green). In the overlapped image, colocalization of the synaptophysin puncta within the bouton is suggested by punctate staining appearing yellow. The z -stack images (3D) and resultant three-dimensional reconstruction (3D-R) unequivocally indicate that the synaptophysin puncta is localized within the boundaries of the bouton.

Fig. 4.

Increased size and intensity of staining of synaptophysin puncta after conditioning is mediated by BDNF. (A,C) Conditioning resulted in a significant increase in the area (A) and intensity of fluorescence (D) of synaptophysin puncta that was inhibited by pretreatment with BDNF antibodies. Separate analysis showed that these increases were derived from puncta localized to boutons apposed to motor neuron dendrites but not somata. (B,E) There were no effects of conditioning on the area (B) or staining intensity (E) of synaptophysin puncta localized to auditory boutons not in apposition to motor neurons. (C,F) Cumulative distribution plots of synaptophysin puncta area (C) and staining intensity (F) revealed a significant rightward shift toward greater values for the conditioned group compared to pseudoconditioning or treatment with BDNF antibodies.

Fig. 5.

The percentage of auditory boutons that contain synaptophysin is increased after conditioning and is inhibited by application of BDNF antibodies. The percentage of empty boutons (those with 0 synaptophysin puncta) was significantly decreased after conditioning, while boutons having two, three, and four synaptophysin puncta was increased. These changes were derived from boutons apposed to dendrites and were inhibited by BDNF antibody pretreatment.

Fig. 6.

An increase in the overall number of boutons apposed to abducens motor neurons accompanies conditioning and bath application of BDNF. The number of boutons apposed to the total number of retrogradely labeled abducens motor neurons was determined for each preparation and averaged. Increased bouton number was attributable to boutons apposed to motor neuron dendrites rather than somata. The observed increase in bouton number was inhibited by bath application of the PKA antagonist Rp-cAMPs or the MEK-ERK blocker PD98059.