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**CONVERSION OF SILENT SYNAPSES INTO THE ACTIVE POOL BY SELECTIVE GLUR1-3 AND GLUR4 AMPAR TRAFFICKING DURING IN VITRO CLASSICAL CONDITIONING**

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Running title: Selective AMPAR Trafficking in Conditioning

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ABSTRACT

The conversion of silent synapses into active sites is hypothesized to be a primary mechanism underlying learning and memory processes. Here, we used an in vitro model of classical conditioning from turtles that demonstrates a neural correlate of eyeblink conditioning to examine whether the conversion of silent synapses has a role in this form of associative learning. This was accomplished by direct visualization of AMPAR and NMDAR subunits colocalized with synaptophysin (Syn) using immunofluorescence and confocal microscopy. In naive preparations, there was a relatively high level of synapses immunopositive for NR1-Syn alone interpreted to be silent synapses. After early stages of conditioning during acquisition of conditioned responses (CRs), there was a significant increase in the colocalization of GluR1-3 AMPAR subunits at NR1-immunopositive synaptic sites. Later in conditioning, levels of GluR1-3 declined and enhanced colocalization of GluR4-containing AMPAR subunits at synapses was observed. The trafficking of these subunits during conditioning was NMDAR-mediated and was accompanied by protein synthesis of GluR4 subunits. Examination of the PSD fraction confirmed the early and late synaptic insertion of GluR1-3 and GluR4, respectively, during conditioning. These findings suggest that there is differential trafficking of synaptic AMPARs during classical conditioning. Existing GluR1-3 AMPAR subunits are initially delivered to silent synapses early in conditioning to unsilence them followed by synthesis and insertion of GluR4 AMPAR subunits that are required for acquisition and expression of CRs.

INTRODUCTION

Accumulating evidence suggests that long-lasting changes in function of glutamatergic synapses underlie the cellular basis of learning and memory. Recent work has focused attention
on the possibility that alterations in synaptic strength can be due to changes in the number of functional synapses, including a rapid conversion of silent synapses into functional ones. The neurotransmitter glutamate acts on two primary postsynaptic ionotropic glutamate receptors, NMDA receptors (NMDARs) and AMPA receptors (AMPARs). Some of the first studies to show a population of synapses that lacked postsynaptic AMPARs but contained NMDARs, so-called silent synapses, were performed in hippocampal slices (Isaac et al. 1995; Liao et al. 1995) where induction of long-term potentiation (LTP) caused a rapid appearance of AMPAR-mediated responses interpreted to be induced by insertion of synaptic AMPARs. Important support for these observations was obtained from experiments in which AMPARs were observed directly in cultured hippocampal neurons using fluorescence microscopy (Liao et al. 1999; Lissin et al. 1999; Shi et al. 1999; Antonova et al. 2001). In these experiments, activity-dependent redistribution and synaptic insertion of AMPARs was demonstrated. Delivery of AMPARs was rapid and required activation of NMDARs. Studies of the interaction of AMPARs with intracellular proteins that comprise the postsynaptic density (PSD) have furthered an understanding of mechanisms that mediate AMPAR trafficking and surface expression during changes in efficacy of synaptic transmission such as in LTP (for reviews see Malinow and Malenka 2002; Collingridge et al. 2002).

Synaptic insertion of AMPARs to activate previously silent synapses during LTP is widely accepted, however, only a few studies have investigated synaptic AMPAR delivery during associative learning. Recent studies of Pavlovian fear conditioning in the amygdala show that this learning shares similarities with LTP in requiring AMPAR trafficking. Rumpel et al. (2005) found that fear conditioning induced the incorporation of GluR1-containing AMPARs into thalamo-amygdala synapses while blockade of GluR1 synaptic incorporation using a
truncated form of the subunit resulted in disruption of the learning process. Furthermore, a fear conditioning-induced increase in surface GluR1 was observed that was dependent on the activation of NMDARs and required new protein synthesis (Yeh et al. 2006). The classically conditioned eyeblink response is also an extensively studied model for understanding the neural mechanisms of associative learning and memory. Our laboratory has developed an in vitro brain stem preparation from turtles that exhibits a neural correlate of classical conditioning during pairing of auditory (the "tone" conditioned stimulus, CS) and trigeminal (the "airpuff" unconditioned stimulus, US) nerve electrical stimulation while recording from the abducens nerve which mediates eyeblinks in turtles (Keifer 2003 for a review). Using this model, we have shown that conditioning is NMDAR-dependent and is associated with the synaptic insertion of GluR4-containing AMPARs in abducens motor neurons (Keifer 2001; Keifer and Clark 2003; Mokin and Keifer 2004; Mokin et al. 2006). Our previous work indicates that the immediate-early gene (IEG) protein Arc is induced shortly after in vitro conditioning and is associated with GluR4 subunits during delivery to synapses in the early stages of conditioning (Mokin et al. 2006). The present study is aimed at further investigation of the cellular mechanisms underlying in vitro conditioning in this preparation. Direct visualization of presynaptic (synaptophysin) and postsynaptic proteins (AMPAR and NMDAR subunits) by immunofluorescence and confocal microscopy allowed us to examine the hypothesis that silent synapses are recruited into the pool of active synapses during classical conditioning. The results show that existing GluR1-3 AMPAR subunits are initially trafficked to silent synapses during the earliest stages of conditioning in order to unsilence them. This is followed by replacement of GluR1-3 with GluR4 AMPAR subunits corresponding with acquisition of conditioned responses (CRs). Therefore, classical conditioning is associated with selective AMPAR subunit trafficking that
serves to initially activate silent synapses thereby allowing for the synthesis and insertion of new subunits that induce and maintain the CRs.

METHODS

Conditioning Procedures

Freshwater pond turtles, *Pseudemys scripta elegans*, obtained from commercial suppliers were anesthetized by hypothermia 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). Therefore, this preparation consisted only of the pons with the cerebellar circuitry and the red nucleus removed. The brain stem was 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; Anderson and Keifer 1999). Suction electrodes were used for stimulation and recording of cranial nerves. The US was an approximately two-times threshold single shock stimulus applied to the trigeminal nerve; the CS was a 100 Hz, 1 s train stimulus applied to the ipsilateral posterior root of the eighth nerve that was below threshold amplitude required to produce activity in the abducens nerve (Keifer et al. 1995; Anderson and Keifer 1999; Keifer 2001). The latter nerve will be referred to as the auditory nerve as it carries predominantly auditory fibers. Neural activity was recorded from the ipsilateral abducens nerve which projects to the extraocular muscles controlling movements of the eye, nictitating membrane, and eyelid. The CS-US interval used in this study was 20 ms which is defined as the time between the offset
of the CS and the onset of the US. This brief trace delay interval was found to be optimal for conditioning, however, conditioning is not supported using longer trace intervals (Keifer 2001). Intertrial interval between the paired stimuli was 30 sec. A pairing session consisted of 50 CS-US presentations followed by a 30 min rest period in which there was no stimulation. Conditioned responses were defined as abducens nerve activity that occurred during the CS and exceeded the amplitude of double the baseline recording level. Conditioned preparations were those that received paired CS-US stimulation whereas pseudoconditioned control preparations received the same number of CS and US exposures that were explicitly unpaired using a CS-US interval randomly selected between 100 ms and 25 sec. At the end of the conditioning experiments, preparations for immunocytochemistry were immersion fixed in cold 0.5% paraformaldehyde whereas those for protein analysis were frozen in liquid nitrogen and stored at -70°C.

**Pharmacology**

The NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (AP-5; 100 µM; Tocris Cookson, St. Louis, MO) was dissolved in physiological saline and perfused through the bath. Preparations underwent the conditioning procedure in normal physiological saline during which time CRs were exhibited. AP-5 was perfused through the bath prior to the next pairing session and remained in the bath for additional pairing sessions until CRs were completely blocked (see Results). Following this treatment preparations were placed in fixative.
**Immunocytochemistry**

For triple label colocalization studies, nine groups of preparations were examined: naive preparations that were not presented with electrical stimulation \((n = 3)\), those that were presented with paired stimuli and were conditioned for one \((n = 3)\), two \((n = 5)\) or five \((n = 5)\) pairing sessions, those that were presented with unpaired pseudoconditioning stimuli for one \((n = 3)\), two \((n = 3)\), or five \((n = 3)\) sessions, and preparations that were conditioned and treated with AP-5 in the second pairing session during CR acquisition \((n = 3)\) or in sessions three through five during CR expression \((n = 3)\). Lightly fixed tissue sections (0.5% paraformaldehyde) were cut at 30 µm and preincubated in 10% normal goat serum for 1 h followed by incubation in primary antibody overnight at 4°C with gentle shaking. For triple labeling studies of GluR1-3-NR1-Syn (synaptophysin) the primary antibodies used were a polyclonal raised in rabbit that recognizes GluR1 (Chemicon, Temecula, CA; 1:100) and GluR2/3 subunits of the AMPAR (Chemicon; 1:100), a polyclonal raised in goat that recognizes NR1 subunits of the NMDAR (Santa Cruz, Santa Cruz, CA; 1:1000), and a monoclonal antibody raised in mouse that recognizes synaptophysin (Sigma, St. Louis, MO; 1:1000). For studies of GluR4-NR1-Syn, the primary antibodies used were a polyclonal raised in rabbit that recognizes NR1 subunits of the NMDAR (Chemicon; 1:1000), a polyclonal raised in goat that recognizes GluR4 subunits of the AMPAR (Santa Cruz; 1:00), and a monoclonal antibody raised in mouse that recognizes synaptophysin. Following incubation in the primary antibodies overnight, sections were incubated with the appropriate Cy2-, Cy3- or Cy5-conjugated secondary antibodies for 2 h (Jackson ImmunoResearch, West Grove, PA; 1:100).
Imaging and data analysis

Images of triple label staining of abducens motor neurons were obtained using an Olympus Fluoview 500 laser scanning confocal microscope. Tissue samples were scanned using a 60x 1.4 NA oil immersion objective with triple excitation using a 488 nm argon laser, a 543 nm HeNe laser, and a 633 nm HeNe laser. Quantification of punctate staining of at least twofold greater intensity above background was performed using stereological procedures with MetaMorph software (Universal Imaging Corp., Downingtown, PA) which was described in detail in an earlier study (Mokin and Keifer 2006). Briefly, images of two consecutive optical sections were taken using confocal microscopy. Protein puncta were counted in one optical section (sample section) if they were not present in the optical section immediately below the sample section (look-up section) and if they were within the inclusion boundaries of the unbiased counting frame. Colocalized staining was determined when puncta were immediately adjacent to one another or if they were overlapping. Data were analyzed using StatView software (SAS, Cary, NC) by ANOVA and are presented as means ± SEM except where indicated.

Western Blot Analysis

Brain stem preparations underwent conditioning for two \((n = 4)\) or five \((n = 4)\) sessions, or pseudoconditioning for two \((n = 4)\) sessions, and were frozen in liquid nitrogen. Preparations conditioned for two pairing sessions demonstrated a mean of 57 ± 34% (SD) CRs in the second session. Those conditioned for five sessions averaged 87 ± 14% CRs between sessions two through five. Tissue was homogenized and centrifuged at 1000 g for 10 min at 4°C and supernatants recentrifuged at 20,000 g for 20 min. Pellets were resuspended in ice-cold high-
performance liquid chromatography (HPLC) grade water, spun at 7,600 g, resuspended again in HPLC grade water and clarified by centrifugation at 48,000 g for 20 min at 4°C. Final pellets were resuspended in 50 mM HEPES buffer (pH 7.4), aliquoted and stored at -70°C. Protein sample concentrates were solubilized in 2x SDS/β-mercaptoethanol and boiled for 3 min prior to separation by 8% SDS-PAGE. Following electrophoresis, membranes were blocked with 5% nonfat dry milk in PBS/0.1% Tween-20 for 4 h at 4°C. The membranes were incubated with primary antibodies to GluR1, GluR2/3, GluR4 and NR1 (same as used for immunocytochemistry) overnight in PBS/0.1% Tween-20/0.1%BSA at 4°C, washed, and incubated with in HRP-conjugated secondary antibodies for 2 hr at room temperature. Loading controls were performed using primary monoclonal antibodies to actin (Chemicon; 1:500). Proteins were detected using the ECL-Plus chemiluminescence system (Amerisham Pharamcia, Piscataway, NJ). Immunoreactive signals were captured on Kodak X-omatic AR film and quantified by computer assisted densitometry.

Subcellular fractionation

Crude postsynaptic density (PSD) fractions were obtained by subcellular fractionation according to previously published procedures (Dunkley et al. 1988). All procedures were performed at 4°C in the presence of protease inhibitors (complete Mini/EDTA-free; Roche, Welwyn Garden City, UK). Brain stems that underwent pseudoconditioning or conditioning for one, two or five sessions were homogenized in HEPES buffer (4 mM HEPES/NaOH, 0.32 M sucrose, pH 7.4). Three similarly responding preparations per group were combined to obtain an adequate amount of tissue to produce the PSD fraction. Two complete sets of these groups were obtained and analyzed. The homogenate was centrifuged at 1,000 g for 10 min and the
The supernatant was centrifuged in discontinuous Percoll gradient (5%-10%-23%) at 33,000 g for 8 min and the interface between 10% and 23% Percoll was collected to yield the crude synaptosomal fraction. This fraction was mixed with PSD buffer (PSDB; 40 mM HEPES/NaOH, pH 8.1) and centrifuged at 20,000 g for 20 min. The pellet was resuspended in PSDB to obtain the final synaptosomal fraction. This was mixed with PSDB diluted 1:1 with 1% Triton X-100 and centrifuged at 40,000 g for 1 hr to yield the PSD fraction. Protein sample concentrations were determined using Bradford Reagent assay and the fractionation confirmed by Western blotting using antibodies against the presynaptic marker synaptophysin and the postsynaptic protein PSD-95 (Santa Cruz).

RESULTS

Synaptic localization of AMPARs and NMDARs during early and late stages of conditioning

Acquisition curves from preparations examined for synaptic glutamate receptor localization by immunocytochemistry are summarized in Fig. 1. Representative abducens nerve recordings taken from a preparation before and after the acquisition of CRs is shown. A recording taken at the beginning of the conditioning procedure shows the UR alone (Fig. 1A, upper record) while later in conditioning a burst discharge characteristic of abducens CRs in response to the CS is recorded (Fig. 1A, lower record, arrow) followed by the UR. Acquisition curves for preparations conditioned or pseudoconditioned for one, two, or five pairing sessions are illustrated in Fig. 1B. Preparations presented with paired stimuli for one session showed relatively few CRs (Fig. 1B, Cond 1). Those conditioned for two sessions, however, showed a significant increase in the percentage of CRs in the second pairing session as is typically observed (Fig. 1B, Cond 2). The acquisition curve shown after five pairing sessions (Fig. 1B,
Cond 5) illustrates the two phases of in vitro conditioning we have identified: CR acquisition, which occurs in sessions 1-2, and CR expression, which occurs in later stages of conditioning when the percentage of CRs reaches asymptotic levels. Pseudoconditioned control preparations failed to generate CR acquisition in all cases.

Using immunostaining and confocal imaging, we tracked the synaptic localization of AMPARs containing either GluR1-3 or GluR4 subunits and NMDARs on abducens motor neurons during early and late stages of conditioning. Images of abducens motor neurons triple labeled for GluR1-3-NR1-Syn are shown (Fig. 2) as are quantitative data for punctate staining of individual proteins (Fig. 4) and their colocalization (Fig. 5). Overall levels of punctate staining for GluR1-3 or NR1 did not change with conditioning. This is apparent when comparing images in Fig. 2 for GluR1-3 (red) and NR1 (blue) across the pseudoconditioned and conditioned cases. These findings are also demonstrated by the analysis shown in Fig. 4A-B in which there were no significant differences in GluR1-3 or NR1 across conditioning $\left( F(6,195) = 1.8, P = 0.09; F(6, 403) = 1.6, P = 0.13, \text{for GluR1-3 and NR1, respectively} \right)$. In contrast, there was a significant increase in synaptophysin punctate staining during conditioning after two and five sessions (Fig. 4C; $F(6,403) = 57.1, P < 0.0001$). We have consistently observed enhanced levels of synaptophysin during in vitro conditioning in previous studies (Mokin and Keifer 2004; Mokin et al. 2006; Keifer et al. 2007). Analysis of colocalization revealed that pseudoconditioned or naive preparations immunostained for GluR1-3-NR1-Syn showed that the majority of synaptophysin puncta colocalizing with NR1 did not also colocalize with GluR1-3 puncta (Fig. 2A, arrows). After one or two sessions of conditioning, colocalization of all three proteins was significantly enhanced above naive and pseudoconditioned levels (Fig. 5A; $F(4,151) = 12.5, P < 0.0001$). As shown in Fig. 2B, triple label colocalization appeared as white pixels (arrowheads)
indicating synapses with both NR1-containing NMDARs and GluR1-3-containing AMPARs. However, later in conditioning after five sessions the majority of NR1-immunopositive synapses no longer contained GluR1-3 (Fig. 2C, arrows; Fig. 5A; $F(1,65) = 8.3$, $P = 0.005$, differences between Cond 2 vs Cond 5). These findings indicate that in the early stages of conditioning GluR1-3-containing AMPARs become transiently localized to NMDAR-containing synapses but withdraw from such synapses later in conditioning.

We next examined abducens motor neurons triple labeled for GluR4-containing AMPARs, NR1 subunits, and synaptophysin. After early stages of conditioning or pseudoconditioning, preparations showed synaptic contacts that contained NR1, as indicated by colocalized blue and green puncta, but lacked GluR4 AMPAR subunits (Fig. 3A-B, arrows). Quantitatively, there was minimal colocalization of GluR4-NR1-Syn after one pairing session which was not significantly different from naive (Fig. 5B; $F(1,53) = 0.36$, $P = 0.79$) or pseudoconditioned controls. However, there was a significant increase in GluR4 subunit punctate staining after two (Fig. 4D; $F(1,64) = 21.1$, $P < 0.0001$) and five sessions of conditioning (Fig. 4D; $F(1,56) = 37.4$, $P < 0.0001$, Cond vs Pseudo). This is illustrated by the increased number of GluR4 puncta (red) shown for the preparation conditioned for five sessions compared to those pseudoconditioned or conditioned for one session (Fig. 3A-C). Likewise, there was a significant increase in triple labeling in which synapses contained both NR1 and GluR4 subunits after two (Fig. 5B, $F(1,64) = 7.2$, $P = 0.001$) and five sessions (Fig. 5B, $F(1,56) = 15.9$, $P < 0.0001$, Cond vs Pseudo) of conditioning. These are visualized as white puncta in Fig. 3C (arrowheads). Taken together the results suggest that during the earliest stages of conditioning after one to two pairing sessions there is a significant increase in GluR1-3 AMPARs into NR1-immunopositive synapses which effectively serves to unsilence these
synapses. As conditioning progresses, this is followed by withdrawal of synaptic GluR1-3 and insertion of GluR4 subunits which parallels the expression of CRs.

*Conditioning-induced increase in the number of active synapses is NMDAR-dependent*

In order to determine whether NMDAR activation was required for conversion of silent synapses into active ones, triple labeling for glutamate receptor subunits and synaptophysin was also performed on preparations conditioned in the presence of the selective NMDAR antagonist AP-5. Two groups were examined, preparations treated with AP-5 early in conditioning during CR acquisition and those treated later in conditioning during CR expression. For early conditioning, preparations were first presented with paired stimuli in normal physiological saline for one session after which time they acquired a mean 17 ±13% CRs to show that they could undergo conditioning. Once CRs were acquired, the NMDAR antagonist AP-5 was applied to the bath prior to the second pairing session resulting in complete blockade of CRs to a mean of 0 ± 0% CRs by the end of second session. For preparations treated later in conditioning, CRs were acquired after two sessions to a mean of 64 ± 20% followed by the application of AP-5 during sessions 3 to 5 resulting in complete CR blockade to a mean of 0 ± 0%. Analysis of the colocalization of punctate staining after AP-5 treatment demonstrated that there were no significant changes in any of the proteins examined in any phase of conditioning relative to pseudoconditioned controls (open squares, Figs. 4-5). For example, colocalization of GluR1-3-NR1-Syn was not significantly different from pseudoconditioned controls after two or five sessions of conditioning ($F(3,93) = 2.3, P = 0.08$). Similar results were observed for GluR4-NR1-Syn staining ($F(3,114) = 1.8, P = 0.16$). These data indicate that NMDAR activation is
required for CR acquisition, glutamate receptor trafficking, and the conversion of silent synapses into active ones during in vitro conditioning.

**GluR4, but not GluR1-3, protein expression is increased during conditioning**

To assess whether AMPA receptor subunits underwent protein synthesis during early and late stages of conditioning, Western blot analysis was performed (Fig. 6). Both GluR1 and GluR2/3 AMPAR protein levels remained unchanged in preparations conditioned for two or five sessions compared with the pseudoconditioned controls (Fig. 6A-C; $F(2,9) = 0.1$, $P = 0.94$ for GluR1; $F(2,9) = 0.1$, $P = 0.91$ for GluR2/3). In contrast, protein levels for GluR4 AMPARs increased throughout conditioning. Preparations conditioned for either two or five pairing sessions showed significantly greater GluR4 protein compared to control (Fig. 6A,D; $F(2,9) = 5.9$, $P = 0.02$). Levels of NR1 protein did not reveal any changes in expression throughout conditioning (Fig. 6A,E; $F(2,3) = 0.12$, $P = 0.89$). Therefore, of the glutamate receptor subunits examined, only GluR4 protein synthesis is selectively increased during in vitro conditioning.

**Selective AMPAR subunit trafficking within the PSD fraction**

Subcellular fractionation of turtle brain stems that were conditioned or pseudoconditioned was performed to confirm selective AMPAR subunit trafficking within the PSD. Crude PSD fractions were isolated as demonstrated in Fig. 7A. These showed increasingly higher amounts of PSD-95 during the purification procedure with minimal contamination from presynaptic components as shown using synaptophysin as a marker. Immunoblotting for GluR1 AMPAR subunits showed they were present in high concentrations in the PSD fraction after one session of conditioning but declined to low levels later in conditioning after two and five pairing sessions.
(Fig. 7B). The levels of GluR2/3 subunits were unchanged by the conditioning procedures. In contrast, GluR4 subunits gradually increased after one, two and five sessions of conditioning (Fig. 7B). Quantitative data confirm that levels of GluR1 in the PSD fraction are highest early in conditioning and decline as conditioning proceeds while GluR4 levels gradually increase with further conditioning (Fig. 7C). These findings support our immunocytochemical observations that non-GluR4 AMPARs are trafficked into the PSD early in conditioning followed by insertion of GluR4-containing AMPARs in the later stages of conditioning.

DISCUSSION

Evidence for activity-dependent activation of silent synapses

Among the first studies to show that excitatory synapses may contain NMDARs in the absence of AMPARs were electrophysiological observations taken from neonatal hippocampal neurons. Using pharmacological current separation in developing CA1 hippocampal neurons, the variance of AMPAR-mediated currents was consistently observed to be larger than that generated by NMDARs suggesting that AMPARs were either absent or nonfunctional in a proportion of synapses (Kullmann, 1994). These electrophysiological observations were further supported by anatomical studies demonstrating excitatory synapses lacking postsynaptic AMPARs using immunogold electron microscopy of AMPARs and NMDARs (Nusser et al. 1998; Petralia et al. 1999) and by direct observation of nascent synapses that contained NMDAR punctate immunostaining but not AMPAR staining (Gomperts et al. 1998). The hypothesis that induction of LTP at physiologically silent synapses is the result of rapid delivery of synaptic AMPARs and the appearance of AMPAR-mediated responses (Isaac et al. 1995; Liao et al. 1995) is now widely accepted and several groups have obtained direct support for this concept.
using immunostained hippocampal neurons and fluorescence microscopy to visualize activity-dependent glutamate receptor trafficking (Liao et al. 1999; Lissin et al. 1999; Shi et al. 1999; Antonova et al. 2001). Criticism has been leveled against the AMPAR-silent synapse hypothesis, however, particularly regarding whether such synapses are prevalent in adult tissue. The primary concern is that the presence of silent synapses may be an age-dependent observation (see Groc et al. 2006 for a review). AMPAR signaling is thought to be in a particularly labile state in early stages of development that reaches a more stable condition with maturity. Supporting this view, the gradual acquisition of synaptic AMPARs during normal development of the hippocampus has been documented in anatomical studies (Nusser et al. 1998; Petralia et al. 1999). Moreover, surface recruitment of AMPARs during LTP in adult CA1 neurons did not occur as has been reported for juvenile tissue (Grosshans et al. 2002). Indeed, the majority of reports of silent synapses come from developing or immature tissue which, for a variety of technical reasons, better supports certain experimental approaches compared to adults. On the other hand, studies confirm the presence of silent synapses in adult tissue, albeit at levels ranging below 25% of the sample (Nusser et al. 1998; Petralia et al. 1999; Takumi et al. 1999; Racca et al. 2000).

In our in vitro preparation, evidence suggested that synaptic inputs from the auditory nerve onto abducens motor neurons were initially silent in naive, mature tissue. Inputs from both the trigeminal and auditory nerves are direct and converge onto the abducens motor neurons in apposition to the somata and proximal dendrites (Keifer and Mokin 2004). Based on previous electrophysiological studies, we proposed that trigeminal nerve synapses conveying the US contain AMPARs as these inputs onto abducens motor neurons were blocked by CNQX (Keifer 1993). Auditory nerve synapses that convey the CS contained both AMPARs and NMDARs
(Keifer 2001). This conclusion was based on the finding that weak stimulation of the auditory nerve failed to evoke a startle-like response recorded in the abducens nerve whereas a strong stimulus was required to evoke a reflex. This high threshold startle-like response was blocked by CNQX and therefore involved AMPARs, however, a decrement in its amplitude resulted after AP-5 treatment indicating an NMDAR-mediated component. One interpretation of these findings is that NMDARs were localized within auditory nerve synaptic junctions and produced no response to a weak stimulus while AMPARs were located further away, perhaps extrasynaptically, and were only activated by a strong stimulus. With this arrangement auditory nerve synapses onto abducens motor neurons in naive preparations are silent to weak stimuli. The present study shows additional evidence for this conclusion. In naive preparations, a proportion of synapses contain NMDARs without appreciable levels of AMPARs indicating the presence of silent synapses (42% of the sample). As conditioning proceeds in its earliest stages, GluR1-3-containing AMPARs are inserted into NMDAR-containing synaptic sites by the first pairing session without increasing their overall numbers or protein synthesis. Therefore, these GluR1-3 subunits appear to originate from pre-existing sources, perhaps by lateral diffusion from their extrasynaptic location (Ashby et al. 2006). This is consistent with our previous immunocytochemical study that found abducens motor neurons were strongly immunopositive for GluR1 and GluR2/3, and weakly immunoreactive for GluR4 AMPAR subunits in naive turtle brain (Keifer and Carr 2000). In later stages of conditioning, synaptic GluR1-3 subunits decline followed by insertion of GluR4-containing AMPARs into NR1-immunopositive synapses and this is associated with significantly increased protein synthesis and number of GluR4 subunits. Consistent with these observations, analysis of the PSD fraction shows high levels of GluR1 early in conditioning followed by increased GluR4 subunits later. GluR2/3 subunits remain
unchanged consistent with hypotheses that these subunits are constitutively cycled into and out of the synaptic membrane (Malinow and Malenka 2002). To further confirm these findings, isolation and analysis specifically of the auditory nerve inputs that convey CS information to the abducens motor neurons will be required to obtain a complete picture of receptor trafficking at these synapses during conditioning. Overall, our observations support the model for in vitro classical conditioning proposed by Keifer (2001) in which silent auditory synapses gradually become activated by acquiring existing extrasynaptic or intracellular nonGluR4-containing AMPARs. Accumulation of such nonGluR4-containing AMPARs results in sufficient depolarization of auditory synapses by the CS to release NMDARs from their Mg$^{2+}$ block and allow Ca$^{2+}$ to enter the abducens motor neurons which in turn would activate signal transduction cascades such as the mitogen-activated protein kinases (MAPKs) shown to underlie conditioning (Keifer 2003; Keifer et al. 2007). This process ultimately results in enhanced synthesis and insertion of GluR4-containing AMPARs into auditory synapses which is hypothesized to mediate expression of CRs.

**Presynaptic mechanisms of unsilencing**

Functional AMPARs and NMDARs may be present in synapses that appear to be silent due to either a low probability of glutamate release into the synaptic cleft or the remoteness of the release site from the receptors (Gasparini et al. 2000). To directly visualize presynaptic mechanisms acting during LTP, optical quantal analysis of transmission at individual mossy fiber synapses using confocal microscopy and intracellular fluorescent Ca$^{2+}$ indicators was performed (Reid et al. 2004). The results indicated that LTP was associated with both an increased probability of transmitter release and recruitment of new release sites. Furthermore,
Kim et al. (2003) showed a rapid activation of silent presynaptic terminals by filling of pre-existing empty varicosities with synaptic vesicles during long-term facilitation in Aplysia.

Although these studies do not minimize AMPAR insertion for recruiting active postsynaptic sites during LTP, they emphasize the role of presynaptic mechanisms in contributing to synaptic efficacy. An increase in the level of synaptophysin punctate staining observed in the present study after conditioning suggests that presynaptic modifications also take place during in vitro classical conditioning (Mokin and Keifer 2004; Mokin et al. 2006; Keifer et al. 2007). A caveat of the present study is that all synapses were sampled for receptor localization in early and late stages of conditioning. If new synapses are formed during in vitro conditioning as the data suggest, it is possible that GluR4-containing AMPARs are selectively recruited to those synapses (that also contain NR1) and do not necessarily replace GluR1-3. This study addresses an overall assessment of receptor modification in this preparation and future studies will address the role of newly formed synapses, if they occur, in conditioning. One potential candidate for the regulation of coordinated pre- and postsynaptic modifications is brain-derived neurotrophic factor (BDNF). Evidence suggests that BDNF is involved in both pre- and postsynaptic mechanisms underlying induction and maintenance of LTP, however, the exact molecular events are yet to be determined (Bramham and Messaoudi 2006). Our preliminary data show that BDNF-TrkB is involved in the early stages of in vitro classical conditioning. Incubation of preparations with TrkB antibodies prior to conditioning blocks the acquisition of abducens CRs while TrkA or TrkC antibodies do not (Li and Keifer, unpublished observations). Whether BDNF has a pre- and/or postsynaptic role in in vitro classical conditioning in this preparation remains to be elucidated.

Synaptic mechanisms underlying vertebrate associative learning
In Pavlovian fear conditioning the amygdala is a crucial site of synaptic modification that underlies this form of learning (Maren and Quirk 2004). Enhanced neurotransmission at synapses that process information about the CS in the lateral nucleus of the amygdala (LA), a key component involved in the formation of fear memories, is mediated by activation of NMDARs (Miserendino et al. 1990; Rodrigues et al. 2001). This activates amygdalar signal transduction pathways including Ca\(^{2+}\)-calmodulin-dependent protein kinase II (Rodrigues et al. 2004) and the MAPKs (Schafe et al. 2000). Similar to LTP, GluR1-containing AMPARs are driven into thalamo-amygdala synapses after fear learning in LA neurons (Rumpel et al. 2005). The cellular mechanisms that underlie eyeblink classical conditioning are not yet as well characterized as for fear conditioning. Eyeblink conditioning is NMDAR-mediated (Servatius and Shors 1996) and blockade of cerebellar AMPARs with CNQX has been shown to impair the acquisition and expression of CRs in behaving rabbits (Attwell et al. 1999). A central role for second messenger systems is also implicated and evidence suggests involvement of the MAPKs (Zhen et al. 2001). Eyeblink conditioning appears to be associated with modifications in AMPARs. Quantitative autoradiography has shown a conditioning-related increase in \(^{3}H\)-AMPAR binding, but not NMDAR binding, of hippocampal neurons after conditioning (Tocco et al. 1991). Other plasticity mechanisms are implicated in eyeblink conditioning including synaptogenesis (Geinisman et al. 2001; Kleim et al. 2002) and enhanced neuronal intrinsic excitability (Moyer et al. 1996). Using an in vitro model of classical conditioning, our studies have furthered an understanding of synaptic mechanisms that underlie this form of learning. In the present study, we show selective AMPAR trafficking serves to initially activate previously silent synapses which are then modified by a second wave of AMPAR insertion required to generate the CRs. Data suggests this process involves rapid synaptic insertion of existing
GluR1-3-containing AMPARs followed by replacement of these subunits with GluR4-containing AMPARs, a step that is associated with protein synthesis. Synaptic GluR4 subunit insertion has been shown to be NMDAR-mediated (Keifer 2001; Keifer and Clark 2003), is associated with the IEG-induced protein Arc through interactions with the actin cytoskeleton (Mokin et al. 2006), and is mediated by the MAPK signal transduction pathways (Keifer et al. 2007).

Interestingly, ERK MAPK is activated early in conditioning during CR acquisition whereas p38 MAPK is activated later during CR expression. Clearly, it will be important to establish whether ERK is selectively involved in GluR1-3 synaptic trafficking while p38 mediates delivery of GluR4 subunits. Taken together, these studies indicate emerging parallels in synaptic plasticity mechanisms among studies of different models of associative learning.

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FIGURE LEGENDS

FIG. 1. Acquisition curves of abducens nerve CRs from the preparations examined using immunocytochemistry. (A) Abducens nerve recordings from a preparation that exhibited conditioning in the second pairing session. In the first pairing session (*upper trace*) CRs were not expressed and only the UR was evoked by the stimuli. During the second pairing session (*lower trace*) a CR (*arrow*) was recorded as a burst discharge in the abducens nerve followed by
Acquisition curves are shown from preparations that were conditioned (filled circles) or pseudoconditioned (open circles) for one (Cond 1), two (Cond 2), or five sessions (Cond 5). Means ± SD are plotted.

FIG. 2. Confocal images of abducens motor neurons illustrating increased delivery of GluR1-3 AMPARs to silent synapses during early stages of conditioning. Images from abducens motor neurons of punctate staining for synaptophysin (green), GluR1-3 AMPAR subunits (red), and NR1 NMDAR subunits (blue), are shown from a preparation pseudoconditioned for one session (A), and preparations conditioned for one (B) or five (C) pairing sessions. Higher magnification images from the selected areas are shown below. (A) A neuron from a pseudoconditioned preparation shows a high proportion of silent synapses indicated by colocalization of synaptophysin with NR1 while GluR1-3 subunits are absent (arrows). (B) After one session of conditioning, there is enhanced colocalization of synaptophysin with both GluR1-3 and NR1 subunits indicating active synapses (arrowheads). (C) After five sessions of conditioning synapses lacking GluR1-3 AMPAR subunits are again observed (arrows). Scale bars = 20 µm, upper panels; 2 µm, lower panels.

FIG. 3. Confocal images of abducens motor neurons illustrating increased delivery of GluR4 AMPARs to silent synapses during late stages of conditioning. Examples of punctate staining for synaptophysin (green), GluR4 AMPAR subunits (red) and NR1 NMDAR subunits (blue) are shown from a preparation pseudoconditioned for one session (A) and those conditioned for one (B) or five (C) pairing sessions. (A) After one session of pseudoconditioning, there were a
number of colocalized puncta for synaptophysin and NR1 in which GluR4 subunits were absent (arrows). (*B*) After one session of conditioning, GluR4 subunits remained absent from regions of colocalized staining for synaptophysin and NR1. (*C*) There was a significant increase in colocalization of punctate staining for synaptophysin, NR1 and GluR4 after five sessions of conditioning (arrowheads). Scale bars = 20 μm, upper panels; 2 μm, lower panels.

FIG. 4. Quantitative data showing the mean number of individual puncta for naïve, conditioned or pseudoconditioned, and AP-5 treated preparations. Total levels of punctate staining for GluR1-3 (*A*), NR1 (*B*), synaptophysin (*C*) and GluR4 (*D*) are plotted. There were no significant changes in the number of GluR1-3 or NR1 puncta across conditioned or pseudoconditioned training sessions. In contrast, the mean number of synaptophysin and GluR4 puncta was significantly increased after two and five sessions of conditioning. Punctate staining for the AP-5 treated group was not significantly different from the pseudoconditioned controls. *Open circles* represent pseudoconditioned preparations, *closed circles* represent conditioned preparations, *squares* indicate the AP-5 treated group, *N* indicates the naive group. Data are expressed as the mean ± SEM. *P* values are given in the text.

FIG. 5. Quantitative data of the mean number of colocalized puncta for each of the experimental conditions examined. (*A*) A significant increase in triple colocalization of GluR1-3-NR1-synaptophysin was observed early in conditioning compared to pseudoconditioning in sessions one and two but was not observed in later stages of conditioning in session five. (*B*) In contrast, colocalization of GluR4-NR1-synaptophysin was low initially and significantly increased after
two and five sessions of conditioning. The AP-5 treated group was not significantly different from the pseudoconditioned controls. \( P \) values are given in the text.

FIG. 6. Western blot analysis shows an increase in GluR4 protein synthesis after two and five sessions of conditioning but not in other glutamate receptor subunits. (A) Representative bands are shown from preparations pseudoconditioned for two sessions (\( Pseudo \ 2 \)) and those conditioned for two (\( Cond \ 2 \)) or five (\( Cond \ 5 \)) sessions. Actin was used as a loading control. No significant changes in the levels of GluR1 (\( B \)), GluR2/3 (\( C \)) or NR1 protein (\( E \)) were observed between the experimental groups. However, GluR4 protein (\( D \)) was significantly increased after two and five sessions of conditioning. \( P \) values are given in the text.

FIG. 7. Subcellular fractionation of turtle brain stems shows selective GluR1 and GluR4 AMPAR trafficking during different stages of conditioning. (A) Demonstration of isolation of the PSD fraction. Immunoblotting for PSD-95 from homogenates (\( H \)), synaptosomal (\( S \)) and PSD fractions show enhanced levels of crude postsynaptic membranes with minimal contamination with the presynaptic membrane as indicated by synaptophysin. (B) Immunoblotting of PSD fractions for AMPAR subunits shows that GluR1 is present after one session of conditioning (\( Cond \ 1 \)) and declines after further conditioning. GluR2/3 subunits are also present in the PSD fraction but they appear to be unaltered by the conditioning procedures. The levels of GluR4 in the PSD fraction gradually increase during conditioning for one, two (\( Cond \ 2 \)) and five sessions (\( Cond \ 5 \)). Actin loading controls are also shown. (C) Quantitative data of GluR1-4 subunit protein levels in the PSD fraction after conditioning for one (\( C1 \)), two (\( C2 \)), or five (\( C5 \)) pairing sessions normalized to levels during the same number of sessions of
pseudoconditioning which was 100 percent. Asterisks indicate $P < 0.05$ for GluR1 after one session of conditioning and GluR4 after five sessions of conditioning compared to pseudoconditioned values.
143x138mm (100 x 100 DPI)
A

GluR1-3 + NR1 + Syn

Mean Number Puncta /100 μm²

N 1 2 3 4 5

B

GluR4 + NR1 + Syn

N 1 2 3 4 5

* * * * *