Published in final edited form as:

Eur J Neurosci. 2010 July ; 32(2): 269–277. doi:10.1111/j.1460-9568.2010.07339.x.

AMPA Receptor Trafficking and Learning

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Abstract

In the last several years it has become clear that AMPA type glutamate neurotransmitter receptors are rapidly transported into and out of synapses to strengthen or weaken their function. The remarkable dynamics of AMPA receptor (AMPA) synaptic localization provides a compelling mechanism for understanding the cellular basis of learning and memory, as well as disease states involving cognitive dysfunction. Here, we summarize the evidence for AMPAR trafficking as a mechanism underlying a variety of learned responses derived from both behavioral and cellular studies. Evidence is also reviewed supporting synaptic dysfunction related to impaired AMPAR trafficking as a mechanism underlying learning and memory deficits in Alzheimer's disease. We conclude that emerging data support the concept of multistage AMPAR trafficking during learning and that a broad approach to include examination of all the AMPAR subunits will provide a more complete view of the mechanisms underlying multiple forms of learning.

Keywords

fear conditioning; spatial learning; classical conditioning; eyeblink; Alzheimer's disease

Introduction

Glutamatergic synapses comprise the primary excitatory connections in brain. Two main types of ionotropic glutamate neurotransmitter receptors have been extensively studied, AMPA receptors (AMPA) and NMDA receptors (NMDARs). AMPARs are comprised of heterotetrameric complexes made up of at least two of four subunits designated GluR1-4 (Wenthold et al., 1996; recently renamed GluA1-A4, Collingridge et al., 2009). The different subunits are able to confer specific physiological properties to AMPAR channel function such as kinetics, conductance and permeability. Most notably, GluR2-containing AMPARs are impermeable to divalent cations while those lacking the GluR2 subunit are calcium (Ca^{2+}) permeable (Isaac et al., 2007). Each subunit is selectively regulated by phosphorylation which determines interactions with specific scaffolding partners and synaptic localization. AMPARs have been shown to underlie activity-dependent changes in excitatory synaptic function during different forms of learning. In the past several years, it has become clear that AMPARs are rapidly transported into and out of synapses to strengthen or weaken their action depending on the appropriate behavioral response. In some cases, trafficking of receptors can occur in a matter of minutes (Lissin et al., 1999; Shi et al., 1999; Yang et al., 2008). The remarkable dynamics of synaptic neurotransmitter receptor localization and its importance to mechanisms underlying learning, as well as to some disease states involving cognitive dysfunction, has only recently been recognized. Cellular mechanisms of AMPAR trafficking during synaptic plasticity are reviewed elsewhere in this

issue. Here, we will focus on evidence for AMPAR trafficking underlying learned behavioral responses and cognitive dysfunction associated with disease states such as Alzheimer's disease.

Pavlovian Fear Conditioning and Synaptic Incorporation of GluR1-Containing AMPARs

Pavlovian fear conditioning is a form of associative learning that occurs when a predictive relationship between a neutral conditioned stimulus (CS), usually a tone or specific environmental context, and a noxious unconditioned stimulus (US) such as a footshock is established. After only a few pairings, a robust learned fear response, typically a defensive behavior such as freezing in rodents, is produced by the CS alone that can persist for many days. The amygdala is a crucial site of synaptic modification that underlies this form of learning (Rodrigues et al., 2004). Enhanced neurotransmission at synapses that process information about the CS in the lateral nucleus of the amygdala (LA), a key area involved in the formation of fear memories, is mediated by activation of a number of signal transduction pathways including calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), and the mitogen-activated protein kinases (MAPKs). It has been difficult to define in greater detail the molecular mechanisms responsible for this form of learning in part because of the complexity of the neuronal pathways involved. The LA is thought to receive parallel information about the CS from both the auditory thalamus and cortex and shows AMPAR-mediated long-term potentiation (LTP) in both thalamo-amygdala and cortico-amygdala synaptic pathways. Recent studies, however, have seen considerable advancement in the elucidation of fear learning. Using a viral-vector approach to deliver recombinant glutamate receptors to "tag" synapses undergoing modification during fear conditioning, the Malinow laboratory (Rumpel et al., 2005) took advantage of GFP-tagged GluR1 homomeric AMPARs expressed in the LA that could be identified by their greater rectification to current injection compared to endogenous AMPARs. After fear conditioning, synaptic transmission between the thalamus and the amygdala was observed to have significantly greater rectification compared to subjects that received unpaired control stimulation indicating that the recombinant receptors were driven into thalamo-amygdala synapses during fear learning. These findings were consistent with the observation that transfection of neurons in the amygdala with a second vector containing a GluR1 construct that prevented synaptic incorporation resulted in a significant reduction in late phase LTP and fear conditioning. Therefore, the interference in GluR1 synaptic insertion and subsequent LTP in the amygdala corresponded with impairment of fear learning. These findings were largely confirmed and expanded on by Humeau et al. (2007) using GluR1 and GluR3 knockout mice. In that study, using a stimulation protocol somewhat similar to Rumpel et al. (2005) by pairing synaptic stimulation with postsynaptic depolarization, induction of thalamo-amygdala LTP in LA neurons was significantly impaired in GluR1^{-/-} but not in GluR3^{-/-} mice. Behaviorally, the GluR1^{-/-} mice failed to show any acquisition of tone-evoked or context-dependent fear conditioning. The GluR3^{-/-} mice, interestingly, exhibited delayed fear learning even though thalamo-amygdala LTP was similar to wildtype. Those mice, however, demonstrated reduced cortico-amygdala LTP while the GluR1^{-/-} mice were deficient in both thalamo- and cortico-amygdala LTP. These data are consistent with the idea that either cortical or thalamic projections to LA support synaptic plasticity and learning and that the loss of one pathway can be compensated for by the other. The reduction in cortically-induced LTP and delayed conditioning observed in GluR3^{-/-} mice having intact thalamic and impaired cortical projections may be due to a loss of the total number of AMPARs in LA projection neurons or to a loss of GluR3 subunits specifically. These studies strongly support the conclusion that GluR1-containing AMPARs are critical for fear learning in the amygdala.

A more recent study (Matsuo et al., 2008) reported contextual fear conditioning requiring the hippocampus was associated with recruitment of GluR1-containing AMPARs into dendritic spines of CA1 hippocampal neurons. Transgenic mice expressing GluR1 subunits labeled with green fluorescent protein (GFP-GluR1) under control of a *c-fos* promoter to induce activity-dependent expression regulated by the doxycycline system were generated. Twenty-four hours after training, fear conditioned mice showed significantly enhanced recruitment of GFP-GluR1 preferentially to mushroom spines but not to other spine types. This effect, however, was transient and declined to control levels by 72 hours after conditioning. This finding might indicate that GluR1 AMPAR subunits were replaced by receptors containing other subunits as suggested by the authors. In contrast, 72 hours after fear training and presentation of extinction trials that reduced fear responses to control levels, GFP-GluR1 immunopositive mushroom spines were greatly increased. This result appears to be inconsistent with GluR1 synaptic insertion supporting learning. However, extinction is generally considered to be new learning (that is, learning not to respond) and may reflect this newly acquired behavioral response. The observation of GFP-GluR1 recruitment into spines following extinction was suggested to be prolonged retention of GluR1 in spines after the initial training but may in fact represent reinsertion of GluR1 associated with the new extinction learning (Keifer et al., 2008). It is also noteworthy that the first appearance of GFP-GluR1 in the dendrites of CA1 neurons occurs well after the behavioral training, the earliest time point being 6 hours afterward. Therefore, the time course of expression of GFP-GluR1 immunopositive spines in hippocampal neurons does not appear to closely match what would be expected if insertion of these AMPARs were responsible for the learning. However, synaptic incorporation of native GluR1-containing AMPARs may occur during the earliest stages of learning followed later by the *c-fos* induced GFP-GluR1 AMPARs. Apart from timing issues, these data indicate that incorporation of GluR1-containing AMPARs specifically into mushroom type spines in CA1 occurs during contextual fear conditioning. Also consistent with GluR1 recruitment during learning, both fear conditioning (Yeh et al., 2006) and one-trial inhibitory avoidance training (Whitlock et al., 2006) resulted in increased expression of GluR1 subunits. In inhibitory avoidance training (Whitlock et al., 2006), rodents learn to associate the dark side of a chamber with a foot shock and therefore avoid the dark side in successive trials. After such training, rats showed significantly increased levels of GluR1 and GluR2 AMPAR subunit protein in addition to elevated GluR1 phosphorylation at Ser831, but not Ser845, in CA1 hippocampal synaptoneurosome fractions. GluR1 subunits are phosphorylated at Ser831 by CaMKII and protein kinase C (PKC), and at Ser845 by PKA, which regulate synaptic incorporation (Esteban et al., 2003; Derkach et al., 2007). Taking this study a step further by using multiple electrode recording arrays these authors also found enhanced CA1 Schaffer collateral field EPSPs (fEPSPs) indicative of LTP after training. However, the time course of AMPAR expression and phosphorylation did not correspond well with the training-induced potentiation of fEPSPs. The levels of both AMPAR protein and phosphorylation declined to control values after only an hour after training while LTP in CA1 neurons was recorded for at least 3 hours. While data from that study support the idea that aversive conditioning induces LTP in CA1, there is weak corroboration with GluR1 AMPAR expression and phosphorylation in this case. These findings might be explained if synaptic incorporation of GluR1-containing AMPARs occurs in the early stages of learning and are replaced later by other AMPAR subunits or receptor types. A similar mechanism might also explain the findings of Matsuo et al. (2008). Multistage AMPAR trafficking such as this has been proposed elsewhere (Sun et al., 2005; Oh et al., 2006; Derkach et al., 2007; Yang et al., 2008; Zheng and Keifer, 2009a) and is discussed below.

It is well known, particularly among college undergraduates, that low levels of stress or fear enhance learning, but what about AMPAR trafficking? Natural stress or incubation of hippocampal slices in the stress hormone norepinephrine (NE) induced transient

phosphorylation of GluR1 at Ser831 and Ser845 in mice (Hu et al., 2007). Induction of LTP and synaptic incorporation of GFP-GluR1 was also facilitated by NE application, however, NE by itself did not induce either GluR1 trafficking or LTP. Mice treated with epinephrine showed better contextual fear learning compared to saline-treated controls, and significantly, this effect was not apparent in GluR1 double phosphomutant mice in which both Ser831 and Ser845 were replaced by alanines thereby rendering the sites resistant to phosphorylation. Instead, epinephrine-treated mutant mice showed levels of training similar to normal controls suggesting that epinephrine is not important during normal non-stressed learning. These results are revealing and seem consistent with those of others on spatial learning (Zamanillo et al., 1999; Reisel et al., 2002; Lee et al., 2003; see below) that some forms of learning are not adversely affected by elimination of GluR1 itself or its phosphorylation sites at Ser831/Ser845. Taken together, these studies indicate that GluR1-containing AMPARs are recruited into synapses to support learned behavior such as fear conditioning. The evidence for a precise correlation between the synaptic insertion of GluR1, subsequent induction of LTP, and acquisition and retention of learned responses requires further study of the timing of these events to firmly establish them as a coherent mechanism of learning. Currently, the findings suggest that action of neuromodulatory factors or synaptic incorporation of other AMPAR subunits also participate in aspects of these learned behaviors.

Spatial Learning and AMPAR Trafficking

The importance of GluR1 AMPAR subunit synaptic incorporation is also documented in studies of spatial learning and memory, but as for fear conditioning, GluR1 action alone is not likely to be the full story. In one notable early study using GluR1^{-/-} adult mice, hippocampal CA1 LTP in response to tetanization could not be evoked but acquisition of spatial learning during the Morris water maze task was readily obtained (Zamanillo et al., 1999). This study suggested an inconsistency between GluR1-mediated LTP and spatial learning. Further analysis revealed greater complexity in behavioral responsiveness of GluR1^{-/-} mice. Impaired performance on spatial working memory tasks but normal learning of spatial reference tasks was reported (Reisel et al., 2002). For example, spatial working memory was tested on a trial to trial basis when the mice were rewarded for selecting the arm of a T-maze that was not experienced during the previous forced sample run (that is, the alternate arm; Fig. 1). Spatial reference memory was tested using the Morris water maze in which the association between spatial cues and the location of a hidden platform was required. The GluR1^{-/-} mice performed poorly in the working memory task but very well, similar to wildtypes, in the spatial reference task. The high level of performance of the retained behaviors in this study was subsequently impaired following hippocampal lesions, indicating they required the hippocampus but not one form of NMDAR-dependent CA1 LTP as shown earlier (Zamanillo et al., 1999). These data were generally interpreted to indicate that various hippocampal-dependent forms of learning have different underlying molecular mechanisms, some requiring GluR1-mediated LTP and others not. These behavioral observations from GluR1^{-/-} mice showing poor performance using working memory but good spatial reference memory likely reflects the differential role of these subunits in short-term and long-term memory processes (Fig. 1). The behavioral deficits correspond with impaired early LTP induced by a theta-burst-pairing protocol but intact late-phase, GluR1-independent, LTP in hippocampal neurons of GluR1^{-/-} mice (Hoffman et al., 2002). Early LTP may contribute to short-term memory tasks requiring GluR1 subunits such as working memory, whereas those that are acquired over a period of days, such as the watermaze, may be related more to GluR1-independent late-phase LTP. The recent study of Sanderson et al. (2009; see also, Schmitt et al., 2003) using GluR1^{-/-} mice tested in a novelty preference task showed significantly impaired short-term but enhanced long-term spatial memory, interpreted as evidence for separate processes that compete with each other.

These findings may have implications for AMPAR trafficking models as applied to spatial learning (Fig. 1). The flexibility and rapid onset required for short-term memory processes may require the immediate responsiveness of GluR1 trafficking, while the gradual acquisition characteristic of long-term, GluR1-independent, memory likely utilizes mechanisms involving other AMPAR subunits. It remains to be seen the extent to which AMPAR trafficking mechanisms underlying the two memory processes act independently or cooperatively. Behavioral studies such as these demonstrating significant learning and retention capabilities of GluR1^{-/-} mice raise the likelihood that AMPARs containing subunits other than GluR1 are key players in spatial learning, or at the very least, can compensate for the loss of GluR1.

Spatial learning is also associated with phosphorylation of GluR1 and regulation of trafficking. Lee et al. (2003), using the Ser831/Ser845 double phosphomutant mice, showed greatly impaired NMDAR-dependent LTD and reduced LTP. Therefore, the GluR1 phosphorylation state at the CaMKII/PKC and PKA sites have key roles in these forms of hippocampal synaptic plasticity. Interestingly, the double phosphomutant mice also demonstrated deficits in long-term retention during the Morris water maze in which the task is acquired normally and recalled when the animals are tested 2 to 4 hours after training, but impairment in retention was revealed when they were tested after longer delays of 8 and 24 hours. Since LTP was reduced in these mice and yet they showed retention when tested after 4 hours, there appears to be no clear correspondence between the deficits in LTP and their performance of the Morris water maze. It was argued in that study that a reduction in LTP might have produced an unstable and transient form of memory consolidation that did not exceed 4 hours. Unfortunately, the levels of native GluR1 phosphorylation in wildtype mice during the specific time points of acquisition and retention examined in this task were not analyzed to correlate with the behavioral findings from the phosphomutants. Mice with the same mutations also showed deficits in incentive learning (Crombag et al., 2008a,b). Learning was completely blocked in mice with the double mutation even though they could still acquire discrimination conditioning. In contrast, single mutant mice in which the PKA site was blocked at Ser845 showed no deficits in Pavlovian transfer learning or conditioned reinforcement, but blockade of the CaMKII/PKC site at Ser831 resulted in impairment in reinforcement learning, indicating this site was critical. Evidence suggests that there are complex interactions on trafficking between Ser831 and Ser845 and another phosphorylation site at Ser818, a substrate for PKC (Boehm et al., 2006), that complicate interpretation of these behavioral studies without closer examination of AMPARs directly.

Classical Conditioning of Defensive Reflexes in Aplysia

Similar to Pavlovian fear conditioning, classical conditioning of simple defensive reflexes is evoked when a neutral stimulus (CS) such as a tone predicts the arrival of an aversive sensory stimulus (US) that normally generates a reflexive response, typically a withdrawal movement or an eyeblink. After a number of pairings of the two stimuli a defensive behavioral response to the CS alone is produced. Because of its emotional quality and the extreme arousal state generated by it, fear learning occurs more rapidly than other types of conditioning. Nevertheless, generation of defensive responses to novel stimuli are adaptive and necessary for survival, therefore learning is also rapid and robust.

The defensive siphon and gill withdrawal reflex in the marine mollusc *Aplysia* was introduced as a model system for studies directed at elucidating the cellular mechanisms underlying associative learning many years ago (Carew et al., 1981; Walters et al., 1981). A great body of research since then has led to the generally accepted conclusion that classical conditioning in this model is accompanied by cellular changes involving LTP (Roberts and Glanzman, 2003). While the precise mechanisms underlying this form of associative

learning remain to be elucidated, it is thought that trafficking of AMPA-like receptors is related to at least some of the observed synaptic changes. Support for this idea comes largely from cellular analogs of conditioning using application of the neuromodulator serotonin (5-HT) to isolated motor neurons in cell cultures. Following tail-shocks used to evoke defensive withdrawal reflexes during conditioning, 5-HT is released and, in addition to presynaptic effects, enhances the responsiveness of postsynaptic motor neurons controlling behavior. 5-HT-induced facilitation of postsynaptic potentials generated in cultured motor neurons to brief puffs of glutamate was blocked by the AMPAR antagonist DNQX suggesting the 5-HT-induced enhancement was mediated by an increase in postsynaptic AMPARs (Chitwood et al., 2001). Moreover, 5-HT-induced facilitation was also inhibited by postsynaptic injection of botulinum toxin, a selective blocker of vesicle exocytosis, that would presumably suppress the synaptic incorporation of AMPARs into motor neuron synapses (Chitwood et al., 2001; Li et al., 2005). These studies were extended to a reduced preparation in which tail nerve shock-induced facilitation was shown to depend on increased levels of postsynaptic Ca^{2+} from intracellular stores and resulted in enhanced sensorimotor EPSPs mediated mainly by AMPARs but not NMDARs (Li et al., 2005). Moreover, postsynaptic loading with botulinum toxin inhibited dishabituation of the siphon withdrawal reflex, a 5-HT-dependent form of nonassociative learning. Together, these data provide strong evidence for the hypothesis proposed by the Glanzman laboratory (Li et al., 2005) that 5-HT-dependent synaptic enhancement during facilitation and dishabituation requires release of postsynaptic intracellular Ca^{2+} stores that induces subsequent exocytotic delivery and synaptic incorporation of AMPARs. While these data support the hypothesis for AMPAR trafficking during learning in *Aplysia*, they currently remain indirect. It appears that antibodies developed in mammals fail to identify AMPAR-like proteins in invertebrates so that they can not be used to directly visualize receptors using microscopy or used for Western blotting to determine changes in protein levels or phosphorylation state. Moreover, there is scarce information available about the sequence structure of putative AMPARs in *Aplysia*, and the number of functional subunits and splice variants remains to be described. There may be at least seven AMPAR-like subunits (*ApGluR1-7*) that show unique patterns of staining with *in situ* hybridization in the abdominal ganglia (Glanzman, 2007). Further progress on the role of AMPAR trafficking during learning in *Aplysia* will be hampered until some of this information can be obtained.

In Vitro Neural Correlate of Eyeblink Classical Conditioning Requires GluR1 and GluR4

The cellular mechanisms that underlie eyeblink classical conditioning in behaving animals are not yet as well characterized as for fear conditioning. This is in part because studies have focused on identifying sites of potential plasticity that support conditioning involving feedback connections among the cerebellar cortex, cerebellar nuclei, red nucleus, inferior olive, and other regions. Eyeblink conditioning has been shown to be NMDAR-mediated (Servatius and Shors, 1996), involve cerebellar AMPARs because injection of CNQX impairs the acquisition and expression of CRs (Attwell et al., 1999), and may depend on a synaptic potentiation process similar to LTP (Madronal et al., 2007). Learning-related increased excitability of CA1 pyramidal neurons after trace eyeblink conditioning by reduced postburst afterhyperpolarization has also been extensively studied and may involve PKA-mediated signaling pathways (Oh et al., 2009). Like other forms of learning, behavioral eyeblink conditioning also appears to be associated with modifications in AMPARs. Quantitative autoradiography showed a conditioning-related increase in (^3H)-AMPA binding, but not NMDAR binding, of hippocampal neurons (Tocco et al., 1991). While the neural substrates for eyeblink conditioning are gradually being elucidated,

progress has been slow on detailing the molecular mechanisms responsible for CR acquisition and expression.

A great deal of progress on cellular mechanisms of learning and memory has been made using *in vitro* model systems. An *in vitro* model of vertebrate associative learning using a brainstem preparation from turtles that generates a neural analog of eyeblink classical conditioning was developed to examine cellular mechanisms of CR acquisition (Keifer, 2003, for a review). This preparation has the unique advantage that turtle brain tissue is highly resistant to anoxia allowing large portions to be maintained in a dish for many hours or even days and agents such as pharmacological compounds or small interfering RNAs (siRNAs) to be applied by incubation procedures. Initial studies of conditioning used an isolated brainstem-cerebellum preparation (Keifer et al., 1995). However, subsequent studies found that isolation of the pons alone without the cerebellum showed robust acquisition of CRs and that the cerebellum was involved in their appropriate timing (Anderson and Keifer, 1997, 1999). In place of using tone and airpuff stimuli as in behaving animals, paired stimulation of the auditory nerve (the “tone” CS) with the trigeminal nerve (the “airpuff” US) results in burst discharge in the ipsilateral abducens nerve, which controls blinking in this species, that is characteristic of conditioned eyeblink responses (Fig. 2A). (Since turtles do not have muscles of facial expression, the facial nerve does not contribute to blinking.) Once CS-US pairing begins, CRs are acquired rapidly in about one hour or by the beginning of the second pairing session (Fig. 2B). A pairing session consists of 50 paired CS-US presentations (lasting 25 minutes) followed by a 30 minute rest period in which there is no stimulation. Acquisition is followed by a period of asymptotic CR expression (Fig. 2B). Studies using this *in vitro* preparation support a two-stage model of AMPAR synaptic delivery in abducens motor neurons during acquisition of eyeblink conditioning that is the result of multiple signal transduction elements illustrated in Figure 1C (Zheng and Keifer, 2009a). During the first stage of *in vitro* classical conditioning in which GluR1 subunits are delivered to synapses (Early in Fig. 2C), PKA and the CaMKs (II and IV) are phosphorylated within 15 minutes of the onset of CS-US pairing (Fig. 2C; Zheng and Keifer, 2009a). PKA and CaMKIV activate CREB through known interactions at Ser133. There are undoubtedly multiple target genes for CREB during conditioning. In this system, a novel extracellularly secreted tolloid-like metalloproteinase (secreted turtle tolloid-like protein, tTLLs) is transiently expressed during *in vitro* conditioning and functions in the proteolytic conversion of the precursor of brain-derived neurotrophic factor (BDNF), proBDNF, into its active form, mature BDNF, which is required for synaptic delivery of AMPARs (Li and Keifer, 2008, 2009; Keifer et al., 2009). The requirement for tTLLs in conditioning was demonstrated by application of siRNAs to suppress expression that could be rescued by transfection with an siRNA-resistant form of the gene (Keifer et al., 2009). Both tTLLs mRNA and BDNF protein are expressed after CREB is activated and initial sequencing of the promotor region for tTLL suggests a potential CREB binding site (unpublished data). Evidence indicates that BDNF is a necessary and sufficient step for synaptic GluR1 and GluR4 incorporation because not only does application of BDNF alone induce activation of ERK and synaptic delivery of these subunits (Li and Keifer, 2008, 2009) but treatment of preparations with the PKA agonist Sp-cAMPs plus BDNF antibodies suppresses synaptic incorporation that can be induced by Sp-cAMPs alone (Zheng and Keifer, 2009a; unpublished). The intracellular activation of ERK by BDNF is postulated to proceed by signaling through the BDNF receptor tropomyosin-related kinase B (TrkB), as has been proposed in other systems (e.g., mouse hippocampus, Patterson et al., 2001; *Aplysia*, Sharma et al., 2006). Once activated, ERK induces delivery of GluR1-containing AMPARs that is rapid and does not involve protein synthesis but instead utilizes translocation of existing receptor proteins. Synaptic insertion of AMPARs containing GluR1 is thought to induce enough postsynaptic depolarization during the CS to activate NMDARs embedded in silent auditory nerve synapses on abducens motor neurons (Mokin et al., 2007)

and allow Ca^{2+} entry which triggers the synthesis and incorporation of GluR4 AMPAR subunits.

The second stage of conditioning-related AMPAR delivery involves NMDAR-mediated synthesis and synaptic replacement of GluR1 by GluR4-containing AMPARs that are hypothesized to support the generation of CRs (Late in Fig. 2C; Mokin et al., 2007; Zheng and Keifer, 2009a). Transient synaptic incorporation of AMPARs is supported by observations from LTP in which Ca^{2+} -permeable GluR2-lacking AMPAR subunits are delivered during the induction phase and later replaced by GluR2-containing AMPARs for maintenance (Plant et al., 2006). In conditioning, GluR2/3 subunits are thought to be constitutively cycled into and out of synapses (Mokin et al., 2007) while GluR1 and GluR4 subunit trafficking is conditioning-dependent. More direct evidence that GluR1 is replaced by GluR4 subunits in the same synapses is required to support this model. GluR4 subunits may be targeted specifically during this form of learning rather than GluR1 because studies indicate they are well represented in the cranial nerve nuclei (Petrálie and Wenthold, 1992; Keifer and Carr, 2000) and contribute to rapidly decaying fast synaptic currents that are prominent in the auditory system (Mosbacher et al., 1994). Confocal imaging studies using the NMDAR antagonist AP-5 showed that synaptic incorporation of GluR4 during conditioning, but not GluR1, was NMDAR-dependent and resulted in blockade of CR acquisition (Li and Keifer, 2009; Zheng and Keifer, 2009a). Furthermore, while the PKA activator Sp-cAMPs induced synaptic incorporation of both GluR1 and GluR4, only GluR4 was suppressed by coapplication with AP-5 (Zheng and Keifer, 2009a). Therefore, synaptic delivery of GluR4 subunits is an NMDAR-dependent process while GluR1 is not. Data also suggest that GluR4 subunits may be trafficked through interactions involving the immediate-early gene activity-regulated cytoskeleton associated protein (Arc or Arg3.1) that immunoprecipitates with GluR4 and whose expression is also NMDAR-dependent (Mokin et al., 2006; Keifer et al., 2008). Pharmacological evidence suggests that CaMKII is involved in GluR4 trafficking as well as Ca^{2+} -dependent and independent isoforms of PKC (Zheng and Keifer, 2008, 2009a). The coordinated actions of these kinases with ERK are hypothesized to result in GluR4 synaptic insertion and expression of CRs during conditioning.

Multistage models of AMPAR trafficking during LTP have been proposed and there appear to be several important similarities and differences between those and classical conditioning as illustrated in Table 1. For hippocampal LTP, as well as dopamine facilitated LTP, PKA signaling is thought to phosphorylate and mobilize GluR1-containing AMPARs to the surface membrane of extrasynaptic sites, an NMDAR-independent step that serves to “prime” synapses (Sun et al., 2005, 2008; Oh et al., 2006; Derkach et al., 2007; Yang et al., 2008). This is followed by a second step in which NMDAR-dependent Ca^{2+} influx induces activation of signaling pathways including CaMKII and PKC (Hayashi et al., 2000; Boehm et al., 2006) resulting in translocation of GluR1 by lateral diffusion into synapses to strengthen them. The proposal for *in vitro* classical conditioning involves GluR1-containing AMPARs already present near the synapse that are incorporated into silent synapses, a step that does not require NMDARs but is dependent on PKA (Keifer, 2001; Zheng and Keifer, 2008, 2009a). This initial step is similar to LTP (see Table 1) in that mobilization of GluR1 in LTP requires PKA but not NMDAR signaling, however, during *in vitro* conditioning GluR1 is inserted into synaptic sites rather than extrasynaptically as is the case for LTP. Conditioning requires a second NMDAR-dependent step involving synaptic incorporation of AMPARs, like LTP, but in the case of conditioning GluR1-containing AMPARs are hypothesized to be replaced by those containing GluR4 subunits. NMDAR-mediated Ca^{2+} entry activates CaMKII and PKC that induce the synthesis and synaptic delivery of GluR4-containing AMPARs. Previous work (Zhu et al., 2000; Esteban et al., 2003), primarily using postnatal hippocampal slice cultures, found that spontaneous synaptic activity resulted in

delivery of GluR4-containing AMPARs that was AP-5 sensitive and required PKA. In contrast to those findings, GluR4 subunits during conditioning are delivered in response to paired, but not unpaired, stimuli, is NMDAR-mediated and occurs after the PKA-dependent step. The phosphorylation site Ser842 on GluR4 subunits is targeted by PKA, CaMKII and PKC (Carvalho et al., 1999) and therefore it would not be surprising if these signaling cascades regulated GluR4 trafficking differently in response to specific stimulation protocols. Fear conditioning is thought to involve LTP-like synaptic changes and therefore shares many of the same molecular mechanisms such as synaptic incorporation of GluR1-containing AMPARs (Table 1; Rodrigues et al., 2004). Specific stages of AMPAR trafficking during fear learning have so far not been identified. While many questions remain with regard to the function of specific signal transduction cascades, findings from a variety of model systems are converging on the concept that there is multistage trafficking of AMPARs to synapses during synaptic plasticity and learning.

Synaptic AMPAR Trafficking and Cognitive Dysfunction in Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a progressive decline in cognitive function and is characterized pathologically by the presence of senile plaques and intracellular neurofibrillary tangles. Amyloid beta ($A\beta$) peptides are the major constituent of senile plaques in AD brain and have long been implicated in playing an important role in the pathogenesis of the disease (Shankar and Walsh, 2009). Recent data suggest that $A\beta$ peptides, especially the soluble oligomeric form, contribute significantly to dysfunction of synapses that underlie cognitive decline and possibly the very early stages of AD before plaques develop and neuronal cell death occurs.

Postsynaptic glutamate receptor trafficking may be a prime target in AD. Studies showed decreases in GluR1, GluR2, and GluR2/3 AMPAR subunit levels in vulnerable brain areas of AD patients, such as CA1, the subiculum, and entorhinal cortex (Ikonomovic et al., 1995, 1997). The reduction of AMPARs in AD was mimicked in transgenic mouse models that overexpress amyloid precursor protein (APP) and by *in vitro* soluble $A\beta$ treatment (Almeida et al., 2005; Chang et al., 2006; Hsieh et al., 2006; Ting et al., 2007). Cultured neurons from APP mutant transgenic mice (Tg2576) showed significantly reduced surface expression of GluR1-containing AMPARs that was paralleled by exogenous application of $A\beta$ to wild-type neurons (Almeida et al., 2005), and might be mediated, at least in part, through a reduction in CaMKII (Gu et al., 2009). Double knockin mice carrying mutated human APP and presenilin-1 genes exhibited a reduction in AMPAR number and AMPAR-mediated synaptic currents in CA1 that strongly corresponded with increased age, $A\beta$ levels and plaque deposition (Chang et al., 2006). Moreover, the double knockin mice showed age-related deficits in LTP, LTD, and memory performance. Working memory and spatial learning assessed with the Morris water maze appeared normal, but performance of a more complex spatial learning task was significantly impaired in the older mice with greater plaque deposition (Chang et al., 2006). The decline in synaptic AMPAR number induced by $A\beta$ may be achieved through an endocytotic and/or apoptotic pathway (Hsieh et al., 2006; Lu et al., 2006). Hsieh et al. (2006) elegantly demonstrated that $A\beta$ overexpression resulted in AMPAR endocytosis, depression of synaptic transmission, and dendritic spine loss. Interestingly, $A\beta$ -induced synaptic depression was mediated by p38 MAPK and calcineurin, similar to mechanisms required for LTD. Furthermore, they showed that the effects of $A\beta$ were blocked by introducing a mutation into GluR2 that weakens clathrin-mediated endocytotic mechanisms. These studies and others provide strong evidence that, like mechanisms for LTD, $A\beta$ drives AMPAR endocytosis resulting in structural and functional synaptic deficits. Other research has found that activation of proteases including the

caspses by A β induces suppression of AMPAR currents and neuronal apoptosis. The caspses, as well as the calpains and cathepsins, play an important role in initiation of cellular apoptotic events and are implicated in neurodegeneration associated with AD. Activation of caspase-1 by A β resulted in impairment of LTP in CA1 hippocampal slices (Minogue et al., 2003) and caspase-1 inhibition enhanced AMPAR-mediated synaptic currents and LTP (Lu et al., 2006). Therefore, caspase-1 appears to be a negative regulator of LTP and AMPAR function. Chan et al. (1999) showed that cultured hippocampal neurons exposed to A β induced caspase activation and degradation of AMPAR, but not NMDAR, subunits thereby resulting in decreased levels of full-length AMPARs. Brain tissue from AD patients also showed enhanced levels of caspase activation and reduced levels of full-length AMPAR subunits. Consistent with these findings, caspase-mediated proteolytic cleavage of AMPARs induced by glutamate and Ca²⁺ influx suppressed AMPAR currents in hippocampal slices (Lu et al., 2006). On the contrary, a recent report by Harris et al. (2010) showed that caspase cleavage of APP is not required for synaptic dysfunction and deficits in learning and memory in APP transgenic mice. The decline in AMPAR number and synaptic function induced by A β through endocytosis is a plausible mechanism for events that occur in the very early stages of AD. However, AMPAR reduction through apoptotic mechanisms likely does not explain the earliest events in synaptic dysfunction leading to AD.

Evidence suggests that BDNF plays a critical role in regulating expression and synaptic delivery of AMPAR subunits (Caldeira et al., 2007; Li and Keifer, 2008, 2009; Keifer et al., 2009), and that BDNF is dramatically reduced in AD and neural tissue exposed to A β (Peng et al., 2005; Garzon and Fahnestock, 2007). In mild cognitively impaired (MCI) and AD patient groups, BDNF was reduced by 34% and 62%, respectively (Peng et al., 2005). The major BDNF transcripts were also found to be dramatically decreased in the AD brain (Garzon and Fahnestock, 2007). Importantly, the decline in levels of BDNF may be manifest in pre-clinical stages of AD (Peng et al., 2005). On the other hand, BDNF expression is implicated as a marker for high cognitive function in the normally aging brain (Komulainen et al., 2008). To confirm the importance of BDNF in cognitive function and learning, BDNF gene delivery in a transgenic mouse model of AD was found to reverse synapse loss, partially restore synaptic markers, and improve spatial learning and memory (Nagahara et al., 2009). BDNF has been shown to have a critical role in synaptic AMPAR delivery. Application of BDNF resulted in upregulation of AMPAR subunit expression through TrkB receptors and promoted synaptic delivery of GluR1 in cultured organotypic hippocampal slices (Caldeira et al., 2007). Likewise, application BDNF induced synaptic delivery of GluR1- and GluR4-containing AMPARs through TrkB- and ERK-mediated mechanisms in the *in vitro* turtle brainstem (Li & Keifer, 2008, 2009). Moreover, application of BDNF to brain slices from BDNF knockout mice resulted in conversion of silent synapses into AMPAR-containing synapses (Itami et al., 2003). Studies of *in vitro* classical conditioning in turtles further showed that application of oligomeric A β , but not the less toxic fibrillar form, significantly reduced BDNF protein levels, GluR1 and GluR4 AMPAR synaptic incorporation, and acquisition of conditioning. Delivery of these AMPAR subunits could be rescued by coapplication of oligomeric A β with BDNF (Zheng and Keifer, 2009b). Together, these data suggest that an A β -induced reduction in BDNF results in suppression of AMPAR trafficking to synapses that results in synaptic dysfunction, impairment of synaptic plasticity, and cognitive decline. Treatments to enhance AMPAR function may be therapeutically beneficial for treating cognitive deficits. Application of the AMPAR potentiator LY451395 administered for 8 weeks, however, showed no significant effect on cognitive function in patients with mild to moderate AD (Chappell et al., 2007). Possible reasons for a lack of clinical efficacy may involve the toxicologically limited doses and short duration of treatment. More work will need to be done in the future to understand the relation between AMPAR trafficking and AD.

Conclusion

There is convincing evidence for AMPAR trafficking to synapses during a variety of learned responses. While there is strong evidence of a role for GluR1, the details and timing of its trafficking during learning require further study. Other AMPAR subunits have to some degree been overlooked but are likely to be involved in the learning process. Evidence is also building that synaptic dysfunction resulting from impaired AMPAR trafficking mechanisms underlies some disease states that affect learning and cognitive function. Emerging data support the concept of multistage AMPAR trafficking during learning. Further studies will reveal the interplay among the trafficking of different AMPAR subunits and their specific roles in multiple forms of learning.

Acknowledgments

We thank Dr. Brian Burrell for comments on the manuscript. Supported by NIH grants NS051187 and P20 RR015567 which is designated as a Center of Biomedical Research Excellence (COBRE) to J.K.

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Figure 1.

Primary findings of spatial learning studies on $\text{GluR1}^{-/-}$ mice. Spatial working memory deficits in $\text{GluR1}^{-/-}$ mice can be demonstrated using a T-maze (left) in which mice are forced to run down one arm (sample run) and are subsequently required to choose the alternate arm to obtain a reward (choice run). This short-term memory task requires the subject to remember the previously unsampled arm, which changes unpredictably, on a trial-by-trial basis. $\text{GluR1}^{-/-}$ mice show profound performance deficits in this task indicating it is GluR1-dependent. In these mice, GluR1 AMPAR subunits are not available to be inserted into synaptic sites. In contrast, the same mice perform a spatial reference memory task (right) as well as wildtypes. This form of long-term memory can be examined using the Morris water maze in which mice gradually learn to find a submerged platform in an opaque pool of water based on spatial cues. The high level of performance of $\text{GluR1}^{-/-}$ mice on these types of tasks indicates they are likely mediated, at least in part, by synaptic trafficking of AMPARs containing GluR2-4 subunits. Both forms of learning require the hippocampus and are NMDAR-dependent. N, NMDARs; A, AMPARs.

Figure 2.

Summary of *in vitro* eyeblink classical conditioning. (A) Abducens nerve recordings showing burst discharge characteristic of a UR alone (top record) taken at the beginning of paired stimulation and an example of a CR (lower record, *arrow*) followed by a UR taken later in conditioning. (B) A typical acquisition curve for *in vitro* conditioning (Cond) showing few CRs during the first pairing session and significant acquisition of CRs by the second session. This phase of rapid acquisition is followed by an asymptotic phase of CR expression during subsequent pairing sessions. Unpaired stimuli during pseudoconditioning (Ps) results in no CRs. (C) Summary of the signaling pathways and AMPAR trafficking proposed for *in vitro* eyeblink classical conditioning. The early NMDAR-independent phase of conditioning is thought to occur during the first pairing session, before CRs are expressed, and involves synaptic incorporation of existing GluR1 AMPAR subunits to unsilence auditory nerve synapses. The later NMDAR-dependent phase occurs during the second pairing session and involves NMDAR-mediated synthesis and synaptic insertion of GluR4-containing AMPARs that are hypothesized to underlie the CRs. See text for details. GluR1, yellow ovals; GluR4, green ovals; pink rectangles, NMDARs.