SYNAPTIC PLASTICITY INDUCED THROUGH CP-AMPARs IS DEPENDENT ON THE ERK/MAPK SIGNALLING CASCADE

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy.

Graduate Department of Physiology

University of Toronto

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Abstract

Recent literature has shown that AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors lacking the GluR2 subunit (thus Ca$^{2+}$ permeable) are widely expressed in the CNS, especially in interneurons and glia, where they contribute to synaptic transmission and plasticity. Studies have also indicated that calcium permeable AMPARs (CP-AMPARs) are expressed and participate in synaptic regulation in principal neurons, including hippocampal pyramidal neurons. Furthermore, CP-AMPARs and their resultant Ca$^{2+}$ influx are implicated in various pathophysiological conditions such as ischemia and seizures. However, the synaptic events activated by Ca$^{2+}$ influx through CP-AMPARs remain unknown. I took advantage of genetically altered mice without (GluR2-/-) or with reduced GluR2 (GluR2+/_), thus allowing the expression and detailed analysis of synaptic CP-AMPARs in hippocampal pyramidal neurons. Utilizing electrophysiological techniques, I demonstrated that these receptors were capable of inducing numerous forms of long-term potentiation (referred to as CP-
AMPAR-dependent LTP) through a number of different induction protocols, including high-frequency stimulation (HFS) and theta-burst stimulation (TBS). This included a previously undemonstrated form of protein-synthesis dependent late-LTP (L-LTP) at CA1 synapses that is NMDA-receptor (NMDAR) independent. This form of plasticity was completely blocked by the selective CP-AMPAR inhibitor IEM-1460. Surprisingly, calcium/calmodulin-dependent kinase II (CaMKII), the key protein kinase that is indispensable for NMDAR-dependent LTP at CA1 synapses appeared to be not required for the induction of CP-AMPAR-dependent LTP due to the lack of effect of two separate pharmacological inhibitors (KN-62 and staurosporine) on this form of potentiation. Both KN-62 and staurosporine strongly inhibited NMDAR dependent LTP in control studies. In contrast, inhibitors for the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade (PD98059 and U0126) significantly attenuated this CP-AMPAR-dependent LTP. Additional studies with knockout mice revealed that the ERK/MAPK signalling cascade is likely acting through p-21 activated kinase 1 (or PAK1, a Rho-GTPase associated kinase) dependent mechanisms. These results suggest that distinct synaptic signalling underlies GluR2-lacking CP-AMPAR-dependent LTP, and reinforces the recent notions that CP-AMPARs are important facilitators of synaptic plasticity in the brain.
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A. Introduction

A.1 Memory

Memory can be described as the retention of newly acquired information. It is a process that is of paramount importance in our everyday lives, being directly involved in the day-to-day interactions with the objects and people around us. Additionally, the skills and abilities required to perform all professional occupations are also highly dependent on retention. The major mechanisms underlying memory are reliant on several processes, the first of which is attention. The vast amount of data that an individual faces in a single day is immense. To potentially retain any of this, the first step would be to isolate and concentrate on a specific line of information. This could depend on a particular person’s interests or needs, and can vary according to the situation or environment concerned. For example, a student focusing on a lecture that he/she is attending, or paying attention to a song on the radio while driving. This aspect of memory acquisition can be either voluntary or involuntary. Once a particular facet of the inbound information has been filtered out, the next step is to encode it (Giovannini, 2006; Winters et al., 2008; Rugg et al., 2008). This involves the transition of this sensory input into a form that can be stored in the brain. This process is variable depending on the type of encoding that is taking place. This could be acoustic (what it sounds like), visual (what it looks like), tactile (what it feels like), or semantic (what is actually means or represents). The way data is coded is highly important in the types of recall it requires. For instance, the visual encoding of a mouse makes it easier to depict it in drawings. However, this type of encoding alone would make it harder to define what a mouse is. After information has
been coded according to a particular format, it is then stored in the brain. The potential processes underlying this retention will be presented in greater detail later, but can basically be divided into short-term and long-term storage or memory (Lynch, 2004; Cowan, 2008). This reservoir of information is retained for later when it is needed or called upon. The retrieval of this data is dependent on a number of factors. Primarily, the amount of time that elapses between storing a memory and recalling it is extremely crucial. Therefore, a memory located in short-term storage will only be able to be accessed within a small interval (often seconds or minutes), while long-term memories can be accessed even after extended periods (days or even years). Memories can routinely be transferred from short to long-term through a variety of methods. Frequently accessing a memory repetitively (rehearsal) can be a highly effectively technique in this regard. Therefore, a commonly used telephone number or e-mail address can become engrained into a more permanent form of storage. Additionally, the utilization of multiple forms of coding for a single topic can also greatly assist this process. Thus, the combination of acoustic, visual, tactile and semantic data on a particular piece of information provides a larger number of access points for memory to be retrieved. While all the steps mentioned above are critically important for memory to occur, retrieval is the only means by which memory can be measured.

A.1.1 Types of Memory

Memory can be further divided into short-term (or working) memory and long-term memory. Short-term memory was found to last between 3 to 20 seconds, and on average comprises of around 7 pieces of individual information (Peterson and Peterson, 1959).
Additional data beyond this limit usually takes the position of or replaces previous information stored in short-term memory. The molecular processes that underlie the transition of short-term to long-term memory remain largely unknown, however some researchers believe that long-term potentiation or LTP (discussed below) could be a possible explanation. Long-term memory can last decades, and is divided into (see Diagram 1) explicit (or declarative) and implicit (or procedural) memories (Anderson, 1976). Explicit memory depends on the cognisant retrieval of information. It consists of episodic and semantic forms of memory. Episodic memories are those associated with experiences, such as recollection of times, places and particular events. On the other hand, semantic memories are those linked with “text-book learning”, such as basic understanding and meanings, without corresponding to any precise experiences. The vast majority of research with regards to explicit memories has concentrated on a region of the brain known as the hippocampus. This structure is located in the temporal lobe, and consists of the CA (Cornu Ammonis) fields, the dentate gyrus, and the subicular complex. The hippocampus has been a principal area of interest in the search for a molecular correlate for learning and memory. Additionally, the layered arrangement of this structure, as well as the similar orientation of neurons in this region provides an invaluable resource for the investigation of synaptic transmission. Damage to the integrity of the hippocampus has revealed deficiencies of memory performance in both humans as well as animal models (Eichenbaum and Cohen, 2001). The occurrence of both anterograde amnesia (difficulty in the formation of new memories) as well as retrograde amnesia (affecting memories formed before the hippocampal injury) have been seen in these cases. Studies have provided contrasting results in terms of the
significance of the hippocampus in relation to episodic memories, with some investigations considering this region wholly important, while others suggest it is a temporary retention site that leads to the consolidation of memories in the neocortex (Deisseroth et al., 2004).
LONG-TERM MEMORY

EXPLICIT
(Conscious)

EPISODIC

IMPLICIT
(Unconscious)

SEMANTIC

Meanings of everyday words

Learning to swim

Remembering date of anniversary

Diagram 1. Long-term memory is largely dependent on the activity of the hippocampus, and can be divided into explicit or implicit memories. Explicit memory involves the conscious acquisition of information, and consists of episodic and semantic forms of memory. Episodic memories are those associated with personal experiences, such as weddings or birthdays. Semantic memories are related with conceptual learning, such as the specific meaning of a particular word.
A.2 Regulation of Synaptic Transmission

A.2.1 The Synapse

Most neurons consist of a cell body (or soma), branched projections that can accept inbound signalling (dendrites), and other types of branches that communicate signals away from the neurons (axons). A synapse is a junctional region where information is transmitted from one neuron to the other, an essential component of brain function. These structures are classified according to the region of neurotransmitter release (or the presynaptic membrane) and the region of binding to receptors (or the postsynaptic membrane). The presynaptic membrane is the site of neurotransmission, acting in accordance with the postsynaptic membrane, which is the receiving site. The most widespread form of synapses are axoaxonic (involving two axons), axosomatic (between an axon and the soma), and axodendritic (between an axon and a dendrite). The key steps involved in the modulation of synaptic transmission by synapses are outlined below.

A.2.2 Synaptic Transmission

Synaptic transmission is the process by which one neuron can send information or impulses to another through the actions of the synapse. In addition to organelles, the presynaptic membrane contains a myriad of membrane-bound structures called synaptic vesicles, which average around 40-50 nanometers in size. These vesicles contain neurotransmitters (such as glutamate, GABA, acetylcholine, adrenaline, noradrenaline and dopamine), biochemicals which carry the communicatory signals across a synapse.
Neurons can produce waves of depolarization known as action potentials. Action potentials are usually initiated by excitatory postsynaptic potentials or EPSPs from a presynaptic neuron in a direction away from the cell body. Action potentials result in an inward entry of calcium ions through the opening of calcium channels. These ions are important for the release of neurotransmitter molecules from the vesicles into the synaptic cleft (the spacing between the individual nerve cells). In a recycling process, the remnants of old vesicles can incorporate themselves into the presynaptic membrane while newer vesicles can arise from an adjoining region. After traversing through the cleft, the neurotransmitters reach the postsynaptic membrane where they bind to ionotropic receptors (discussed below) that are complimentary for them. The resulting consequences of this process are reflective of the particular receptors involved, and can lead to a depolarization of the postsynaptic site, an alteration in a cell’s membrane potential shifting it towards the positive, which is dependent on the actions of sodium or calcium channels. Alternatively, a hyperpolarization can also occur, which is a membrane potential shift to the negative that requires chloride or potassium channels. If this stimulatory effect leads to a depolarization, an EPSP can be recorded at the level of the postsynaptic membrane. Conversely, if the postsynaptic site undergoes hyperpolarization, an inhibitory postsynaptic potential (IPSP) can be seen. For the successful continuation of neurotransmission, synaptic vesicles have to be regenerated efficiently in the presynaptic membrane. This occurs through the process of endocytosis, after which the vesicles are loaded with specific neurotransmitters through the aid of neurotransmitter transporters (such as the excitatory amino acid transporters or EAAT in the case of the glutamate).
A.2.3 Receptors involved in synaptic transmission and plasticity

Glutamate is the major excitatory neurotransmitter in the brain. The receptors for this factor are classified according to the mechanisms they utilize to achieve activation of ion channels, i.e., direct or indirect. Ionotropic glutamate receptors (such as NMDA, AMPA and kainate receptors) directly form ligand-gated ion channels, while metabotropic receptors indirectly facilitate the activity of ion channels through their association with G-proteins.

A.2.3.1 NMDA Receptors

Plasticity in the majority of the hippocampal regions (excluding the mossy-fiber CA3 synapses) as well as multiple other regions of the brain was found to be dependent on NMDA (N-methyl-D-aspartate) receptor activation. NMDA receptors (or NMDARs) consist of heteromeric assemblies of the NR1 subunit in addition to one or more NR2 subunits (A-D). These subunits come together in the endoplasmic reticulum (ER) to form channels (Monyer et al., 1994; McIlhinney et al.; Ozawa et al., 1998). The NR1 gene is profoundly represented in all areas of the brain and was shown to be important for cerebral development and endurance (Li et al., 1994). The products of NR2A-NR2D accumulate with NR1 resulting in the alteration of channel properties. Neurons can modify their own response-induced excitability by dynamically altering the density of receptors in their postsynaptic membrane. NMDARs are added to the membrane by a process called exocytosis, and removed from it by a process called endocytosis. The exocytosis of NMDARs is endorsed by Protein kinase C (PKC) by two distinct mechanisms that can be either “fast” or “slow” in relation to response time:
(a) The phosphorylation of NR-1 by PKC intensifies the amount of NMDARs on the surface of COS7 cells over a period of 2-3 hours (Scott et al., 2001).

(b) NMDARs are transported within a period of minutes to the cell surface of dendritic shafts and spines through SNARE (soluble NSF attachment protein receptors) dependent exocytosis (Lan et al., 2001).

The endocytosis of NMDARs differs according to the maturity of synapses. The NR1-NR2A receptor complex predominate at the mature synapses, while the NR1-NR2B receptors dominate at the immature synapses. NR2A and NR2B both bind to AP2, an adapter protein integral to clathrin-dependent endocytosis. NR2A undergoes dephosphorylation of the tyrosine residues on its C-terminal tail whereas the internalization of NR2B is determinant to its relation to postsynaptic density protein 95 (PSD 95).

The role of NMDARs in long-lasting plasticity has been well documented. In the CA1 region of the hippocampus, they act as “coincidence detectors”, being only activated once the postsynaptic cell has been depolarized beyond a threshold level. While their activation is not the sole determining factor resulting in LTP, it is a crucial process that ultimately elevates the intracellular calcium concentration for plasticity to occur. The tetanic stimulation of the Schaffer collaterals in the CA1 region of the adult rat hippocampus was shown to induce LTP and demonstrated that early postnatal excitatory synapses possessed solely NMDARs (silent synapses) and only later recruited AMPARs (see below) as development progressed (Petralia et al., 1999). Given the established
importance of the hippocampus in memory retention, the disruption of spatial learning tasks in the presence of the NMDA antagonist AP5 as well as genetic manipulations of NMDARs further solidified the case for LTP as the cellular substrate for learning and recall (Morris et al., 1986; Amaral and Roesler, 2008; Lee and Silva, 2009).

**A.2.3.2 AMPA receptors**

AMPA receptors (AMPARs) are the principle mediators of fast excitatory synaptic transmission in the mammalian CNS. This is due to the fact that under normal physiological conditions, NMDARs are blocked by extracellular Mg$^{2+}$ and are therefore inactive even if they are bound to released glutamate. In addition, changes in AMPARs (either receptor density or channels) are required for the expression of various forms of synaptic plasticity, including LTP (see below). AMPARs are heterotetrameric complexes comprised of four subunits, GluR1 to GluR4 (or recently renamed GluA1 to GluA4). AMPARs are particularly important for the mediation of fast excitatory transmission in the synapses. All AMPAR subunits contain around 900 amino acids, and have a molecular weight of 105 kDa. The N-terminal segment and glutamine binding protein are homologous to the bacterial leucine-isoleucine-valine binding protein (LIVBP) and the ligand-binding domain (LBD) respectively. The C-terminus (carboxy-terminal tail) of the AMPAR subunits is intracellular, and varies structurally between them. GluR1, GluR4, and GluR2L (the long splice form of GluR2) have a long carboxy-terminal tail with a high degree of homology, while GluR2, GluR3 and GluR4C (the short splice form of GluR4) have shorter carboxy-terminal tails. The C-terminus of AMPARs is the site of extensive and specific protein interactions and phosphorylation, with different sets of proteins being involved with different AMPAR subunits, resulting in distinct regulation.
of receptor functionality and trafficking (see below). The subunit composition of the AMPARs determine whether their synaptic insertion is activity dependent (as in receptors formed from long tail subunits such as GluR1/2 and GluR2/4) or continuous (as in the case of receptors formed with short carboxy-terminal tails, such as GluR2/3) (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 2001). It has been reported that AMPARs are phosphorylated on a minimum of 12 different sites (Roche et al., 1996; Carvalho et al., 1999; Chung et al., 2000) and this phosphorylation is important for their role in synaptic plasticity (Fukunaga et al., 1993; Barria et al., 1997; Lee et al., 2000). A large number of proteins interact with AMPARs. Each of the four subunits join with neuronal activity-regulated pentraxin (NARP) and stargazing, the latter going on to interact with postsynaptic density 95kDa (PSD 95), a member of the membrane associated guanylate kinase (MAGUK) family. The GluR2 subunit has been associated with glutamate-receptor-interacting protein (GRIP) 1 and 2, protein interacting with C kinase (PICK1) and N-ethylmaleimide sensitive factor (NSF). The GluR1 subunit attaches to synapse associated protein (SAP 97) and protein 4.1 (4.1N). The insertion of AMPARs is thought to depend on the interaction of NSF with the SNARE complex through the assistance of SNAP (soluble NSF attachment proteins). AMPAR removal is determined either directly through endocytosis, or indirectly with the receptor shifting away from the synapse and undergoing endocytosis later through a clathrin-dependent mechanism (Lüscher and Frerking, 2001). Electrophysiological studies concerning the CA1 region of the hippocampus have shown that AMPARs are swiftly regulated in the synapses (Durand et al., 1996; Liao et al., 1995). These studies also showed that silent synapses were able to acquire AMPARs once the cell was depolarized for the activation of NMDARs. This
indicates that the activation of these silent synapses through the recruitment of AMPARs could be a very important step for the occurrence of NMDAR-dependent LTP.

**A.2.3.3 Kainate receptors**

Kainate receptors are a family of ionotropic receptors that are encoded by the gene families GluR5-7 and KA-1 and KA-2. They have shown to be involved in two main functions:

(a) Role in excitatory postsynaptic current/potential (EPSC/P): This has been described extensively in synapses at the levels of the hippocampus (Castillo et al., 1997), retina (DeVries and Schwartz, 1999), spinal cord (Li et al., 1999), amygdala (Li and Rogawski, 1998), and cortex (Kidd and Isaac, 1999) respectively. Most of these studies indicated that the kainate mediated EPSC (EPSC KA) has a far smaller peak amplitude and slower decay kinetics in comparison to AMPAR mediated EPSC (EPSC AMPA) in the same region. Earlier experiments in the hippocampus have indicated that EPSC KA and EPSC AMPA worked in parallel to facilitate glutamate release (Castillo et al., 1997). Further studies in other regions, such as the retina (DeVries and Schwartz, 1999) and cortex (Kidd and Isaac, 1999), showed that the existence of EPSC KA or EPSC AMPA independently of each other was also possible. One investigation also suggested that kainite receptors may play a role in synaptic plasticity, where mossy-fiber LTP was attenuated by LY382884, a selective antagonist of the GluR5 subunit (Bortolotto et al., 1999), a form of potentiation that was linked to a Ca\(^{2+}\) cascade (Lauri et al., 2003).
(b) Role as presynaptic modulators: The presynaptic role of kainate receptors has been examined in a number of studies, largely through affecting transmitter release from nerve terminals. (Rodríguez-Moreno et al., 1997; Clarke et al., 1997; Perkinton and Sihra, 1999)

A.2.3.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors are activated through an indirect metabotropic process. These belong to the group C family of G-protein coupled receptors and form complexes with glutamate or other specific agonists to activate G-proteins that trigger a variety of signalling pathways. Several types of metabotropic glutamate receptors (mGluRs) have been identified or cloned, and have since been classified into groups according to their structure and physiological activity (Shigemoto et al., 1997; Kew and Kemp, 2005; Kim et al., 2008). Group I comprises of mGluR1 and mGluR5, and can be stimulated by the amino-acid analog L-quisqualic acid (Chu and Hablitz, 2000; Bates et al., 2002). This group of mGluRs is particularly important for the activation of PKC through the release of calcium by stimulating phosphoinositide, as well as the generation of diacylglycerol (Nishizuka, 1988). By regulating sodium and potassium channels, group I mGluRs are mainly associated with an increase in presynaptic glutamate release, as well as an overall increase in excitation and conductance (Chu and Hablitz, 2000). Alternatively, evidence has also shown that they can inhibit glutamate release and increase the amplitude of inhibitory postsynaptic potentials (Endoh, 2004). Group II metabotropic receptors consist of mGluR2 and mGluR3, while Group III contains mGluR4, mGluR6, mGluR7 and mGluR8. These latter two groups inhibit the formation
of cyclic adenosine monophosphate (cAMP) through the inactivation of adenylyl cyclase, and have largely been associated with a role in presynaptic inhibition (Capogna, 2004; Endoh, 2004; Price et al., 2005). A number of studies have been conducted concerning the involvement of mGluRs in LTP, including at the level of the neocortex, hippocampus, striatum and nucleus accumbens (Anwyl, 2009). In the CA1 region of the hippocampus, the first subunit that was explored in this regard was mGluR5. Studies showed that the NMDAR mediated component of LTP was blocked in mGluR5 knockout mice, while AMPAR-mediated synaptic transmission was unaffected (Lu et al., 1997; Jia et al., 1998). However, another study showed that LY341495, a metabotropic glutamate receptor antagonist that inhibits all known receptors at a concentration of 100 µM, did not have any effect on LTP in the CA1 and CA3 regions of the hippocampus (Fitzjohn et al., 1998). The expression of another well known form of synaptic plasticity, LTD or long-term depression, was also seen to be inducible in the hippocampal CA1 region of neonatal rats through the activation of L-type voltage-gated calcium channels and/or metabotropic glutamate receptors (Bolshakov and Siegelbaum, 1994).

A.3 Synaptic Plasticity and LTP

The elucidation of the underlying mechanisms of memory has been a major goal of research for over a century. Early notions that memories were dependent on the formation of new neurons were dismissed when it was noted that the aging human brain did not develop a significant amount of new neurons. The Spanish neuroanatomist Cajal was the first to consider the neuron as a solitary unit, complexes of which made up the nervous system. He demonstrated that though these tiny cells came within close proximity of each
other, there was actually no physical contact between them. Cajal proposed that the storage of information was highly dependent on alternations in synaptic strength between existing neurons (y Cajal et al., 1909). The American psychologist and behaviourist Karl Lashley tried to unsuccessfully search for a single memory locus, in studies which suggested that the storage of memories may rather be spread throughout the cortex (Lashley, 1950). Donald Hebb introduced the Hebbian theory in 1949 which corroborated Cajal’s earlier work, stating that synaptic efficacy is strengthened when the presynaptic cell has a repeated and persistent stimulatory effect on the postsynaptic cell, adding a lasting stability to the circuit. This postulate formed the basis of future experimentation with regards to synaptic plasticity, eventually leading to the discovery of LTP by Bliss and Lomo in 1973. They reported that the administration of multiple trains of high-frequency stimulation (HFS) in the perforant path led to an enduring enhancement in the level of synaptic transmission in the dentate gyrus of rabbit models (Bliss and Lomo, 1973). This important finding was followed by a flurry of interest in this area, which looked to illustrate whether LTP was indeed the much sought after candidate for the molecular basis for memory (see Diagram 2). In this regards, three basic characteristics were attributed to LTP (Bliss and Collingridge, 1993):

(a) Input specificity: LTP induced at one synapse does not propagate to surrounding synapses, therefore, being input specific.

(b) Associativity: refers to the simultaneous synaptic strengthening that is observed in at least two adjoining pathways despite the fact that one of the pathways may be more weakly stimulated in comparison to the other.
Cooperativity: The collaborative weak stimulation of multiple pathways leading to a synapse will result in LTP in a similar manner to the strong stimulation of a single pathway to a synapse.

LTP was also found to correspondingly associate with the two basic sub-types of memory, short-term memory and long-term memory. Short-term retention can last for a period of hours, and does not lead to changes in relation to gene expression and new protein synthesis, consistent with the early phase of LTP (E-LTP). On the other hand, long-term memory can last for several days or years, and is highly dependent on de novo protein synthesis and changes in the level of gene expression, corresponding with the late phase of LTP (L-LTP). Over the last several decades, extensive experimental results on both E-LTP and L-LTP in relation to learning and recall have supported the theories that these and other forms of synaptic plasticity underlie the formation of at least certain types of memory formation. Therefore, both E-LTP and L-LTP continue to be a subject of extensive molecular and genetic analyses. Further discussion on the mechanisms of E-LTP and L-LTP is provided in the sections below.

A.3.1 Early-LTP (E-LTP)

LTP can be described as a long-lasting enhancement in the synaptic efficacy between neurons. It is thought to underlie major processes attributed to the CNS, including learning and memory. During synaptic transmission, the neurotransmitter glutamate is released from the presynaptic site into the synaptic cleft. After crossing the synaptic cleft, glutamate eventually reaches the postsynaptic membrane. Here, the glutamate
binds to AMPARs, which are the main components responsible for fast excitatory transmission in the brain. This causes the influx of sodium ions into the postsynaptic region, resulting in an excitatory postsynaptic potential (EPSP). The size of the EPSP is dependent on depolarization. Therefore, the act of applying a continuous barrage of repeated stimuli at a high frequency leads to EPSP summation. In this situation, a torrent of multiple EPSPs arrive at the postsynaptic membrane before the prior ones can decay, leading to a large increase in the overall depolarization. In the hippocampal CA1 region, which is the most studied region in regards to synaptic plasticity, this depolarization leads to the removal of the magnesium ion blockade of NMDARs to allow the initiation of the early phase of LTP (sometimes referred to as E-LTP). The unblocking of these receptors causes an influx of calcium into postsynaptic site, which in turns activates one of several signalling cascades (Lynch, 2004), including calcium/calmodulin-dependent kinase II (CaMKII), and protein kinase C (PKC). The activation of these pathways has the net effect of facilitating the actions of AMPARs as well as increasing their relative numbers at the level of the postsynaptic membrane. The additional amounts of transmission-mediating AMPARs provided by LTP-producing stimuli results in a cumulative enlargement of synaptic transmission and efficacy, the consequence of which is an increase in the amplitude of EPSPs.

**A.3.2 Late-LTP (L-LTP)**

L-LTP represents a more persistent increase in the efficacy of neurotransmission. Although the induction this form of plasticity requires similar stimuli (usually stronger in nature) as that of E-LTP, L-LTP is differentiated from the former by consisting of two
distinct components. The first component of this plasticity requires the formation of new proteins, while the second needs both protein synthesis as well as gene transcription. These changes are induced by the persistent activation of signalling cascades, in particular ERK/MAPK. The ERK/MAPK signalling cascade initiates the activation of transcription factors (Lynch, 2004) such as cAMP response element binding protein (CREB). This factor binds to cAMP response elements (CRE) to produce an increase in the transcription and expression of genes. While the exact proteins synthesized during this form of synaptic plasticity are largely unknown, recent research has suggested the involvement of protein kinase M zeta (PKMζ). This kinase is an isoform of PKC, with its own self-regulating catalytic domain. The elicitation of L-LTP leads to the formation of PKMζ, which improves synaptic transmission through an increment in the quantity of postsynaptic AMPARs (Sacktor, 2008). In contrast to E-LTP, L-LTP induced experimentally can last for several hours or even days.
Depolarization of neuron due to EPSP summation leads to the opening of NMDA receptors, which allows calcium to influx into the postsynaptic spine. Calcium binds to calmodulin, activating the CaMKII pathway. CaMKII phosphorylates AMPA receptors, resulting in increased channel conductance and/or elevated synaptic levels of AMPARs. Activation of additional signaling pathways leads to gene transcription and new protein synthesis. Diagram 2. Major mechanisms involved in hippocampal NMDAR-dependent LTP. LTP is an enhancement in the communicative efficacy between neurons. The neurotransmitter glutamate is released from the presynaptic membrane, traverses the synaptic cleft, and activates postsynaptic AMPARs, resulting in EPSP summation. This increase in depolarization leads to the removal of the magnesium ion blockade of NMDA receptors, resulting in an influx of calcium into the postsynaptic site. This results in the coupling of calcium with calmodulin, activating several signaling cascades, including the CaMKII pathway. The activation of these pathways enhances the delivery of synaptic AMPARs and/or channel conductance, and may lead to the formation of new proteins and gene expression (during the late-LTP phase), actions which both facilitate synaptic transmission and efficacy.
A.3.3 Role of AMPARs in trafficking during NMDAR-dependent synaptic plasticity

It has been speculated that the strength of a synapse is largely determined by the number of ion channels it possesses (Debanne et al., 2003). Most studies dealing with synaptic plasticity and LTP in particular have focused on the CA1 region of the hippocampus. Here, it was seen that LTP induction was wholly dependent on NMDARs, where the use of AP5 (an NMDAR antagonist) blocked the elicitation of plasticity (Collingridge et al., 1983). The activation of these receptors leads to their opening when coupled with glutamate (ligand-regulation) as well as depolarization of the postsynaptic membrane (voltage-regulation), ultimately resulting in an influx of calcium ions into the postsynaptic spine (a highly specialized dendritic protrusion where most excitatory synapses are formed). This NMDAR mediated Ca\(^{2+}\) influx is responsible for a signal transduction complex that is crucial for the establishment, amendment, and termination of synaptic function (Mori and Mishina, 1995). While a number of signalling pathways have been implicated in the induction of hippocampal NMDAR-induced LTP, it is widely agreed that the insertion of AMPARs into the postsynaptic membrane is the net result (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Ziff, 2007; Kessels and Malinow, 2009). LTP induction in cultured hippocampal preparations was shown to result in the expression of GluR1-GFP in spines (Shi et al., 1999; Hayashi et al., 2000). This translocatory effect of the GluR1 subunit is also transferred to complexes it is present in, with both GluR1/2 and GluR2/3 receptors being expressed at the surface following the elicitation of synaptic plasticity (Shi et al., 2001; Williams et al., 2007). AMPARs have also been shown to be stored in intracellular compartments, where they can either insert
back at the level of the synapses (in a state of recycling), or degrade (Ehlers, 2000; Passafaro et al., 2001). The translocation of AMPARs seems to be highly dependent on membrane fusion, where the inhibition of SNARE activity suppressed NMDAR-dependent LTP (Lledo et al., 1998). A possible regulator of trafficking also appears to be the PDZ-ligand of the GluR1 subunit (Hayashi et al., 2000; Kim et al., 2001; Piccini and Malinow, 2002), though there is contrary data on the subject with regards to synaptic plasticity (Kim et al., 2005). Another anchor of synaptic proteins that had been widely investigated is PSD 95. Expression of PSD 95 in hippocampal cultures led to the occlusion of LTP (Gerges et al., 2004; Ehrlich and Malinow, 2004) through the increase of GluR1 levels at the synapse. Therefore, there has been considerable evidence implicating AMPAR trafficking in the field of NMDAR induced synaptic plasticity.

A.3.4. GluR2 knockout mice as a model for the study of plasticity induced by CP-AMPARs

The vast majority of research in the hippocampus (with relation to learning and memory) has involved the study of LTP of pyramidal cells in the CA1 region (Collingridge et al., 1983). These studies have concentrated on the importance of calcium influx through NMDARs in the induction of this form of plasticity. However, in recent years, studies have revealed that certain pyramidal neurons in the hippocampus can contain CP-AMPARs that may have important physiological significance (Pruss et al., 1991; Lerma et al., 1994; Wenthold et al., 1996; Toomim and Millington, 1998; Yin et al., 1999). Of particular importance is their proposed role in different forms of synaptic plasticity. Recent studies have implicated CP-AMPARs in playing a critical role in the
classical NMDAR-dependent form of LTP (Plant et al., 2006), suggesting that calcium influx from NMDARs leads to the insertion of these AMPARs at the synapse, and that calcium influx from these latter receptors may be required for the consolidation of LTP. While the importance of CP-AMPARs in NMDAR-dependent forms of potentiation is not a focus of this thesis, the synaptic events that are initiated by these receptors remain unknown. Further investigation into this area has been limited due to restricted access to these cells by traditional electrophysiological techniques. Studies have also focused on another type of functionally important group of cells in the hippocampus. These interneurons are present in all the layers of the hippocampus, and through the aid of modeling studies were initially proposed to be play a role in plasticity that is vital to learning and memory (Grunze et al., 1996). The deficiency of the GluR2 subunit in certain interneuronal AMPARs allows the presence of both calcium permeable and calcium impermeable AMPARs in these cells (Leranth et al., 1996). These findings in both pyramidal cells and interneurons may be of particular interest as the dynamic regulation of calcium permeability of AMPARs has been implicated in a distinct form of plasticity in cerebellar stellate cells (Liu and Cull-Candy, 2000). Unfortunately, actual experimental data with regards to the mechanisms underlying these types of CP-AMPAR-dependent plasticity has been scarce due to a variety of complicating factors. A primary problem has been the initiation and identification of LTP occurring solely in these cells. The use of tetanic stimulation (one of the most common induction protocols) can lead to LTP being passively propagated from excitatory synapses at pyramidal cell synapses to interneurons, giving the false assumption that LTP is taking place independently in the latter cells (McBain and Maccafferri, 1997). Therefore, the few
studies looking at the presence of this LTP have involved complex procedures for both the identification as well as stimulation of these cells (Perez et al., 2001; Lamsa et al., 2007). Investigations have also suggested that the underlying mechanisms of plasticity elicited through CP-AMPARs may be significantly different from the classical NMDAR-dependent forms of LTP. One surprising discovery was that two of the fundamental components of the calcium-signalling cascade (CaMKII and calcineurin) essential for traditional forms of LTP are conspicuously absent in certain interneurons (Sík et al., 1998). Furthermore, potentiation induced at certain interneuronal synapses was actually seen to be occluded by postsynaptic depolarization, reflecting an anti-Hebbian nature (Kullmann and Lamsa, 2008). Plasticity through CP-AMPARs has been speculated to be “a common form of plasticity that can regulate both synaptic strength and calcium signalling at excitatory synapses” (Gardner et al., 2005). Additionally, in pathophysiological conditions, the increased calcium permeability of AMPARs has been associated with cell death in both global ischemia (Nellgård and Wieloch, 1992) and status epilepticus (Brusa et al., 1995). Taking these factors into consideration, the study of synaptic plasticity induced through CP-AMPARs in the hippocampus holds a significant interest in this field of research.

The GluR2 subunit is the dominating subunit during AMPAR assembly, and the presence of this subunit causes heteromeric AMPARs to be calcium impermeable due to RNA editing at the Q/R site (see Diagram 3). Past studies in the Jia laboratory have revealed that the loss of this subunit in mice results in higher calcium permeabilities (9-fold) and enhanced hippocampal LTP (2-fold) (Jia et al., 1996). GluR2-lacking AMPARs have unique biophysical properties that greatly facilitate the ability to study these
receptors. Unlike GluR2-containing AMPARs, these calcium permeable receptors display faster deactivation kinetics, an increased single channel conductance, as well as an inwardly rectifying current-voltage relationship due to a voltage-dependent block by intracellular polyamines at positive membrane potentials (Jonas et al., 1994; Bowie and Mayer, 1995; Geiger et al., 1995). These include polyamine compounds such as spermine, polyamine-containing toxins extracted from wasp and spider venoms, and dicationic adamantane derivatives such as IEM-1460. Studies comparing CP-AMPARs with NMDARs have revealed that the former receptors have a relatively lower permeability to calcium. CP-AMPAR complexes consisting of GluR1 homomers displayed a $P_{Ca}/P_{Na,Cs,K}$ ratio of 2.34, while NR1-1a/NR2A and NR1-1a/NR2B NMDAR complexes demonstrated ratios between 3.1-17 (Burnashev et al., 1995; Dingledine et al., 1999). Through the utilization of the GluR2 knockout model, I plan to conduct electrophysiological and biochemical studies to investigate synaptic plasticity that is induced specifically through calcium influx from GluR2-lacking AMPARs at the level of the synapse.
Diagram 3. Importance of the GluR2 subunit. The GluR2 subunit is the dominant AMPAR subunit during receptor assembly. Post-transcriptional editing of the GluR2 mRNA alters an amino-acid in the TMD2 region from glutamine (Q) to arginine (R). The occurrence of this Q/R editing determines the relative calcium permeability of an AMPAR. TMD = Transmembrane Domain.
B. Design and goals of the present study

B.1 Study rationale

Research in the field of synaptic plasticity has focused on regions of the brain where principal neurons largely possess calcium-impermeable AMPARs with GluR2-containing complexes. However, studies have also revealed the ability of GluR2-lacking CP-AMPARs to potentially induce LTP in the amygdala and hippocampus (Mahanty and Sah, 1998; Lei and McBain, 2002). Interneurons in the hippocampal CA3 region (stratum radiatum and lucidum) displayed a unique form of LTD induced through CP-AMPARs (Laezza et al., 1999; Pelkey et al., 2005; Toth et al., 2000). Additionally, mossy fiber-interneuron synapses demonstrated parallel forms of LTD elicited from both NMDARs or CP-AMPARs that relied on Ca\(^{2+}\) influx (Lei and McBain, 2002), potentially advocating that both types of calcium permeable receptors could work correspondingly in support of the promotion of synaptic plasticity. In cerebellar stellate cells, Ca\(^{2+}\) influx from these receptors led to a novel form of plasticity where CP-AMPARs are replaced by GluR2-containing receptors through the actions of PICK1, GRIP and NSF (Liu and Cull-Candy, 2000, 2005). The interaction between neurons and NG2 glial cells was also seen to produce a form of LTP that was susceptible to the CP-AMPAR antagonist philanthotoxin-433 (Ge et al., 2006). Lastly, despite the relative scarceness of GluR2-lacking AMPARs in CA1 pyramidal neurons, a number of labs have reported that the temporary insertion of these receptors at the synapse following the application of NMDAR-dependent induction protocols (Plant et al., 2006; Lu et al., 2007; Guire et al.,
2008) is essential for the fulfillment of this form of potentiation (though other laboratories dispute these results; see Gray et al., 2007; Adesnik and Nicoll, 2007).

In addition to the above processes, the actions of CP-AMPARs have also been implicated in a number of pathophysiological conditions (Cull-Candy et al., 2006). Ischemic conditions result in a lessening of the expression of GluR2, and therefore an increase in AMPAR Ca\(^{2+}\) influx that facilitates mechanisms that ultimately lead to cell death (Tanaka et al., 2000; Liu et al., 2004). These studies showed that viral transfection of either calcium permeable or calcium impermeable AMPARs under these conditions was detrimental or protective to neurons respectively. CP-AMPARs were also implicated in the incidence of amyotrophic lateral sclerosis (ALS), a major neurological disorder. The expression of GluR2-lacking AMPARs was also seen to facilitate the migration and proliferation of glioblastoma tumor cells, a form of malignancy that is resistant to surgical interventions (Ishiuchi et al., 2002). Furthermore, mechanisms essential for the occurrence of addiction to various substances are thought to be mediated by these receptors (Carlezon and Nestler, 2002; Bellone and Lüscher, 2006).

Whilst the above reports have clearly established the importance of CP-AMPARs, several key issues remain unanswered. Firstly, while the presence of GluR2-lacking receptors has been deemed indispensable to numerous regulatory processes, the significance of the Ca\(^{2+}\) influx initiated by CP-AMPARs and its possible activation of signalling cascades has not been explored. The presence of calcium has been demonstrated to be crucial to NMDAR-dependent LTP and other forms of plasticity (Malenka and Nicoll, 1999; Lynch, 2004). However, it has yet to be ascertained whether Ca\(^{2+}\) influx through GluR2-lacking AMPARs can similarly instigate essential kinase
systems to fulfill physiological functions. Therefore, I plan to use the GluR2 knockout model to investigate whether these receptors are solely capable of altering synaptic efficacy and plasticity through the activity of protein cascades. In addition to possibly inducing potentiation, CP-AMPARs have also been suggested to be involved in the consolidation of NMDAR-dependent LTP (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008). While this assertion is not going to be debated during the course of this thesis, the additional attainment of CP-AMPARs seen in the above circumstances may suggest the ability of these receptors to recruit unique signalling mechanisms that are beyond the scope of NMDARs. Indeed, structural and physiological differences between AMPARs and NMDARs could result in differential effects of calcium signalling on synaptic plasticity (Wang et al., 2002; Santos et al., 2009; Kessels and Malinow, 2009). Therefore, plasticity induced through CP-AMPARs could potentially involve pathways that are distinct from the classical NMDAR-dependent forms of potentiation. To explore this possibility, I intend to use kinase inhibitors that have been previously proven to be effective in the attenuation of NMDAR-dependent LTP to test whether these effectors are also involved in CP-AMPAR-dependent forms of plasticity.

Another characteristic trademark of NMDARs is the ability to induce L-LTP, a form of plasticity synonymous with long-term memory (Lynch, 2004). The ability of other calcium permeable receptors to induce a similar form of potentiation has been largely disregarded, in spite of their significant numbers throughout the brain (Cull-Candy et al., 2006). Therefore, I plan to investigate the possible capability of CP-AMPARs to induce protein synthesis forms of potentiation that can last for a period of hours. This would
reveal the importance of these receptors in longer-lasting forms of plasticity, and therefore facilitating more deeply engrained forms of recall.

In divulging the mechanisms initiated following \( \text{Ca}^{2+} \) influx through CP-AMPARs, I hope to outline for the first time the processes and components (such as kinase signalling cascades and new protein formation) that underlie the functional role of these receptors throughout the brain. The discovery of these pathways may help provide a more detailed perspective on the physiological and pathological conditions mentioned previously, therefore assisting research in the field of CP-AMPARs in the numerous regions that they are found.
B.2 Main Hypothesis and study approach

In this section, I will outline my intended experimental plans towards approaching the investigation of plasticity induced through CP-AMPARs. The major outlying hypothesis of the study is that “CP-AMPARs are capable of inducing multiple forms of synaptic plasticity through signalling mechanisms that are distinct from those of NMDAR-dependent LTP”. The study will be divided into four sub-hypotheses that will be discussed in detail in the following paragraphs.

B.2.1 Sub-hypothesis 1: CP-AMPARs are capable of inducing multiple forms of LTP through the utilization of different induction protocols.

B.2.1.1 Rationale and Objectives:

A handful of past studies have tried to employ intricate techniques to elucidate LTP through CP-AMPARs, facing complexities in the identification of cells that contain a significant number of them at the synapse (Lamsa et al., 2007), as well as the restricted use of induction protocols to eliminate secondary plasticity occurring through involvement of other cells (Perez et al., 2001). However, these studies have a number of limitations. Previous models only allowed the elicitation of CP-AMPAR-induced LTP through the use of whole-cell or perforated-patch recordings (Perez et al., 2001; Lamsa et al., 2007). The GluR2 knockout model allows the use of common inductive stimulation protocols (that have been previously established in NMDAR-dependent forms of potentiation) for the study of CP-AMPAR-dependent plasticity from a population of intact cells. Previous studies also involved whole-cell recordings using brain slices obtained from immature animals (less than 6 weeks of age) as slices obtained
from older animals can be more difficult to patch. Since more than one laboratory has reported changes in signalling pathways during the occurrence of LTP due to aging (Yasuda et al., 2003), it would be difficult to ascertain the kinase systems that are involved in CP-AMPAR-induced LTP in adult mice under these previous experimental designs. Therefore, electrophysiological studies of CP-AMPAR-induced LTP need to be conducted on brain slices from adult animals for better understanding of this plasticity. The GluR2 knockout model allows the examination of CP-AMPAR-dependent plasticity at various stages of development and aging without the need of any experimental modification or a difference in the level of expertise required. To examine whether CP-AMPARs have the versatility to induce multiple forms of potentiation, I will utilize the following protocols during field EPSP studies:

(a) High-frequency stimulation (HFS): HFS is the most commonly used and classical form of LTP stimulation. For LTP studies, HFS is usually delivered in “trains” at a frequency of 100 Hz following a stable baseline period. Normally, one or two trains are applied for non-protein synthesis dependent forms of LTP.

(b) Theta-burst stimulation (TBS): TBS was a type of induction protocol found to mimic the endogenous rhythms of the brain, and is therefore considered more physiologically relevant.
B.2.2 Sub-hypothesis 2: CP-AMPARs at the level of the synapse are self-sufficient, in that they can exclusively induce plasticity without the aid of other calcium sources.

B.2.2.1 Rationale and Objectives:

Calcium influx has been shown to be a critical factor in the initialization of synaptic plasticity. Other than CP-AMPARs, other types of receptors and channels in the hippocampus are also calcium permeable. These include NMDARs and L-type calcium channels, which could theoretically play at least a partial role in CP-AMPAR-dependent plasticity.

To address the exclusivity of CP-AMPARs in the induction of CP-AMPAR-dependent forms of plasticity, I would need to utilize a specific pharmacological inhibitor of these receptors. IEM-1460 is one such inhibitor that has been utilized previously in past studies to inhibit the activity of CP-AMPARs (Gray et al., 2007). The administration of this inhibitor should therefore completely inhibit all forms of CP-AMPAR-dependent plasticity induced in GluR2 mutant animals.

B.2.3 Sub-hypothesis 3: CP-AMPARs are capable of inducing long-lasting forms of plasticity (L-LTP).

B.2.3.1 Rationale and Objectives:

Another major interest in the field of learning and memory in recent years has been the subject of L-LTP. This form of plasticity conforms with the biochemical processes associated with longer-lasting forms of memory, such as gene transcription and protein
synthesis. The study of L-LTP in research has almost entirely been devoted to the ability of NMDARs to induce this type of plasticity, with the possibility of other calcium permeable receptors being able to generate L-LTP being largely ignored. Previous models investigating plasticity induced through CP-AMPARs could only facilitate recordings for short periods for time, and the possible role of these receptors in L-LTP (which can last for several hours) could not be investigated. L-LTP studies are normally performed by applying multiple trains (three or more) of HFS at a frequency of 100 Hz in the hippocampal CA1 region. The GluR2 knockout model is capable of performing such extended recordings at all age groups. If the usage of these stronger stimulation protocols does indeed present potentiation induced through CP-AMPARs, further tests would be required to confirm whether this form of plasticity conforms to the traditional classification of L-LTP. This would require the use of a suitable protein synthesis inhibitor (such as anisomycin) to verify that this form of potentiation requires de novo protein synthesis. Additional studies with the CP-AMPAR antagonist IEM-1460 would also be needed to determine the exclusivity of these receptors in the induction of CP-AMPAR-dependent L-LTP.

**B.2.4 Sub-hypothesis 4:** Plasticity induced through CP-AMPARs may involve distinct signalling pathways.

**B.2.4.1 Rationale and Objectives:**

Although calcium influx from CP-AMPARs may produce potentiation of similar magnitude and appearance as that of NMDAR dependent forms of plasticity, the mechanisms underlying this lesser known form of plasticity may be unique. The
determination of downstream effectors involved in NMDAR-dependent forms of plasticity in the hippocampus has been a major part of research in relation to synaptic plasticity. A number of key signalling molecules have so far been identified (including CaMKII and PKC). These studies have relied on the use of pharmacological inhibitors that block their activity during the course of plasticity. The application of these individual drugs can require several minutes to hours. Past studies concerning plasticity induced through CP-AMPARs under whole-cell conditions were limited due to the inability of perfusing inhibitors at length during the baseline period (prior to the application of plasticity-inducing protocols). Additionally, biochemical studies to examine changes in protein phosphorylation levels in response to receptor stimulation were also not possible. In this regard, the GluR2 knockout model can be successfully utilized for pharmacological LTP studies for a period of hours, allowing the specific verification of factors involved during either the inductive or maintenance phases of plasticity. Biochemical investigations specifically targeting the downstream components involved following CP-AMPAR stimulation can also be accomplished without major experimental complications. In my pursuit of the underlying signalling factors involved in CP-AMPAR-dependent induced plasticity, I will be using past results concerning NMDAR-dependent forms of potentiation as a template on which to base my search (see Diagram 4). This is due to the fact that the latter form of plasticity is the most widely established in research today. However, due to differences between the structure and kinetics of these two types of receptors (Wang et al., 2002; Santos et al., 2009; Kessels and Malinow, 2009), I would predict that there is a strong possibility that they may trigger different and diverse signalling cascades. Additionally, the fact that NMDARs have shown to recruit
GluR2-lacking AMPARs to the synapse (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008) to fulfill their function could possibly suggest that the supplementary need for CP-AMPARs may be for the activation of alternative biochemical signalling pathways. To confirm this hypothesis, all studies performed with kinase system inhibitors in CP-AMPAR-induced plasticity will be accompanied by control experiments with the same drugs in NMDAR-dependent forms of potentiation.
Diagram 4. Experimental plan of action. Following NMDAR-dependent calcium influx, numerous kinase pathways have been demonstrated to play a role in plasticity induced in the hippocampal CA1 region, including CaMKII and ERK/MAPK. Therefore, by utilizing the well-explored field of NMDAR-dependent plasticity as a template, I will investigate possible signalling factors activated following calcium influx during the occurrence of CP-AMPAR-induced synaptic plasticity.
C. Materials and Methods

C.1 GluR2 knockout mice

GluR2 knockout mice were created and bred as previously described (Jia et al., 1996; Meng et al., 2003). For genotyping of GluR2 knockout mice, a standard protocol for Taqman PCR was used. A common reverse primer 5’-TCGCCCATTTTCCCATATAC-3’ and forward primers 5’-GGTTGGTCACTCACCTGCTT-3’ and 5’-TCGCCCATTTTCCCATATAC-3’ were used to detect wild-type allele and the neomycin resistance cassette in knockout mice respectively. All studies with mutant animals (GluR2-/- or GluR2+/+) were performed alongside wild-type littermates (GluR2 +/+) for controls. Experimental protocols were approved by The Hospital for Sick Children Animal Care Committee.

C.2 Acute hippocampal slices

The use of freshly obtained brain slices has become the technique of choice in regards to the investigation of synaptic mechanisms. This includes the specific study of electrophysiological, biochemical and morphological aspects of one or more of the numerous brain structures. The prepared slices can be maintained in ACSF (Artificial Cerebral Spinal Fluid) for several hours in completely controlled conditions such as temperature, oxygenation conditioning, pH levels and pharmacological drug concentrations. This model provides the advantage of eliminating the use of anesthetics and other uncontrollable systemic homeostatic mechanisms while using in vivo models. The attainment of several slices from a single animal brain can allow for numerous
experiments to be performed under varying conditions, such as the utilization of a variety of pharmacological inhibitors. This is in contrast to in vivo models where different types of experimental settings would require multiple animals, resulting in an increased wastage of life. One of the most common forms of brain slice preparations is that of the hippocampus. The layered structure of the hippocampus makes it an ideal candidate for slicing, with individual synapses being available for experimental manipulation. The hippocampal CA regions consist of multiple layers or strata, including two superficial ones (stratum alveus and stratum oriens), an intermediate one containing the pyramidal cells (pyramidal layer or stratum pyramidale), and two deeper layers (the stratum radiatum and stratum lacunosum moleculare respectively). Within the layers, the hippocampus demonstrates an afferent set of three connected pathways known as the “trisynaptic circuit”. These are:

(a) Perforant path: provides the major input to the hippocampus, providing a connectional route from the entorhinal cortex to the dentate gyrus, CA fields, and the subiculum.

(b) Mossy Fiber pathway: which contains axons of granule cells that project to the pyramidal neurons of the CA3 region.

(c) Schaffer collateral pathway: consists of axons that project from the CA3 region to the CA1 hippocampal region. This pathway has been the subject of widespread studies with relation to synaptic plasticity and LTP in particular.
C.2.1 Preparation of hippocampal slices

The preparation of brain slices has been previously described (Asrar et al., 2009a, b). Wild-type, GluR2, PAK1 and GluR2PAK1 double knockout mice (3-6 months) were sacrificed using cervical dislocation, after which the brain was extracted quickly from the skull utilizing a curved spatula. The extracted brain was then transferred to ice-cold ACSF containing (in mM) 120 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 1.0 NaH$_2$PO$_4$, 26 NaHCO$_3$, 2.5 CaCl$_2$, and 11 D-glucose (where it was kept for a period of 1-2 minutes). Following this, the brain was then moved to a vibratome (752M Vibroslice, Campden Instruments, Lafayette, IN, U.S.A.) where hippocampal slices (400 µm) were sectioned and obtained in the presence of ice-cold ACSF. The slices were then allowed to recover in a submerged holding chamber bubbled with carbogen (95% O$_2$-5% CO$_2$). The time of recovery was at least 1 hour for recording experiments, and 4 hours for biochemical treatment experiments.

C.3 Extracellular fEPSP electrophysiological recordings

Field excitatory postsynaptic potential (or fEPSP) recordings consist of evoked potentials that are recorded from a group of cells extracellularly. This is often called a population response, and comprises of superimposed post-synaptic potentials induced in the dendrites (referred to as population excitatory post-synaptic potentials) as well as action potentials recorded from the axons or cell body (referred to as population spikes). The slope of the resulting fEPSP represents the magnitude of the current elicited, and is the most extensively used measurement marker in the field of synaptic plasticity. An
increase in the size or slope of the fEPSP, such as seen during LTP, indicates an increase in the strength of the synaptic responses mediated by AMPARs. The stimulus artifact that occurs before the fEPSP is representative of the current that is being applied, while the prefiber volley is directly indicative of the excitability of the fibers stimulated. In many electrophysiological studies, both fEPSP and whole-cell recordings are combined to achieve the objectives of the experiments. Extracellular fEPSP recordings is often the preferred technique of choice for the study of LTP for a number of reasons. Firstly, it allows plasticity to be induced and recorded through a variety of different induction protocols from intact cells. Since the fEPSPs are recorded extracellularly from a population of cells (allowing for greater stability), no time is consumed towards patching a single cell as in whole-cell patch clamp recordings. The use of adult mice (> 2 months) as experimental animals is also more common place in extracellular fEPSP recordings, since patching from mature mice is difficult. Extracellular field recordings in a single slice can also stay viable and stable for several hours at a time. This characteristic allows it to be the only technique that allows the study of electrophysiological L-LTP, which is thought to be a molecular analogue for long-term memory. Pharmacological tests with drugs can also be performed by perfusing them in the ACSF solution for extended periods. This allows the investigation of factors which are involved in both the inductory as well as maintenance phases of LTP.
Diagram 5. Experimental procedure for the induction of LTP in the hippocampus. Freshly isolated hippocampal slices were placed in the recording chamber and synaptic responses were evoked by bipolar tungsten electrodes placed in the CA1 area. The length of fEPSPs were measured prior to and following the induction of LTP by taking the slope of the rising phase between 5% and 60% of the peak response (as indicated by red lines). Application of either HFS (high-frequency stimulation) or TBS (theta-burst stimulation) following a baseline period of at least 20 minutes led to a long-lasting enhancement of the fEPSP slope known as LTP.
C.3.1 Procedure

After a recovery period of at least 1 hour in the submerged holding chamber, a single slice was then transferred to the recording chamber and submerged and superfused with 95% O₂-5% CO₂ saturated artificial cerebral spinal fluid (ACSF, 2 ml/min) at a temperature of 28°C. The ACSF contained (in mM) 120 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, 2.5 CaCl₂, and 11 D-glucose. For field EPSPs, the glass recording pipette (3 MΩ) was filled with ACSF solution.

Synaptic responses were evoked by bipolar tungsten electrodes placed 200–400 µm from the cell body layer in the CA1 area (see Diagram 5). fEPSPs were measured by taking the slope of the rising phase between 5% and 60% of the peak response. All data acquisition and analysis were done using pCLAMP 7 software (Axon instruments). After a stable baseline period, LTP was induced by high-frequency stimulation (HFS) using 2 trains of 100 Hz at 10 second intervals (with each train lasting 1s) or a TBS protocol (15 bursts of 4 pulses at 100Hz, with each burst being separated by 200 ms) while late-phase LTP (L-LTP) was induced by 4 trains of 100 Hz at 20 second intervals (with each train lasting 1s). Paired-pulse facilitation (PPF) was recorded prior to and following induction of LTP (with the first and second responses being separated by an interval of 50 ms). The ratio of the slope of the second response to the slope of the first response was subsequently calculated. All drugs for fEPSP recordings were purchased from Sigma Aldrich (Oakville, Canada), Tocris (Missouri, U.S.A.) and LC Laboratories (Massachusetts, U.S.A.). D,L-AP5 and IEM-1460 were dissolved in distilled water. Staurosporine, KN-62, PD98059, U0126, LY294002, wortmannin and anisomycin were dissolved in DMSO. For tests of inhibition of LTP, drugs were added to ACSF perfusate.
15-20 minutes before the LTP induction protocol was initiated (with staurosporine, KN-62, PD98059, U0126, LY294002, wortmannin and anisomycin having a maximum final concentration of 0.1% DMSO or less after being added to ACSF). For experiments concerning the maintenance phase of L-LTP, PD98059 was added to the ACSF perfusate 20-30 minutes post-induction. Vehicle treatments were performed with 0.1% DMSO and/or distilled water depending on the drug/drugs perfused. All pharmacological inhibition studies with mutant animals (GluR2/- or GluR2+/-) were performed alongside wild-type littermates (GluR2 +/-) for controls. All data was statistically evaluated by Student's *t*-test. Experimental protocols were approved by The Hospital for Sick Children Animal Care Committee.

### C.4 Slice treatment studies

Brain hippocampal slices were obtained from wild-type, GluR2, PAK1 and GluR2PAK1 double knockout mice as described above. After a recovery period of 4 hours in a submerged holding chamber bubbled with carbogen (95% O₂-5% CO₂), hippocampal slices were treated with the NMDAR antagonist D,L-AP5 (100 µm), the L-type calcium channel antagonist Nifedipine (20 µm), and in certain cases (where noted), the CP-AMPAR antagonist IEM-1460 (100 µm), for a period of 20-30 minutes. Following this, one slice was obtained (designated as the “0” sample) and stored in lysis buffer (containing 50 mM Tris pH 7.5, 0.15 M NaCl, 1% Triton-X-100, 5 mM EDTA, and 5 mM EGTA). AMPA (10 µm) was then administered. Further slice samples were then obtained at time intervals 1, 5 and 10 minutes post-treatment. Slice samples were then broken down using homogenizers and converted into protein lysates at cold temperatures (4°C). Protease and protein phosphatase inhibitors were added to the lysis
buffer to prevent degradation of the sample proteins as well as changes in phosphorylation levels. Following preparation, standard Western Blot techniques were utilized (described below). The levels of ERK/MAPK expression was measured using antibodies directed against its total and activated (phosphorylated) components. All treatment experiments in brain slices were conducted in the presence of D,L-AP5 and Nifedipine. All studies in knockout mice will be complemented by control experiments in wild-type mice.

C.4.1 Western Blot

Western Blot is an immunoblotting technique that allows the detection of a specific protein from the numerous proteins located in tissue homogenates. The method is dependent on the use of a probe (antibody) directed against the protein of interest. The proteins in a given sample are separated by size through the utilization of SDS-PAGE (PolyAcrylamide Gel Electrophoresis). Western blot techniques have become common place in research due to its ability to detect even small levels of protein in a given sample.

C.4.1.1 Procedure

The Western blotting technique which I utilized can be divided into four parts:

(a) Gel Electrophoresis: Protein expression in the slice homogenate are separated by molecular weight (measured in kiloDaltons, kDa) using this method (Bio-Rad, U.S.A.). Lysis buffer was prepared with 50 mM Tris pH 7.5, 0.15 M NaCl, 1% Triton-X-100, 5 mM EDTA, and 5 mM EGTA. Slice protein samples were prepared by adding lysis buffer with protein phosphatase inhibitors (1 mM
sodium orthovanadate and 1 mM sodium fluoride). These samples (15 µl) were loaded onto wells in the gel. One well was reserved for a marker or ladder, producing bands of known size to help identify my protein of interest (ERK/MAPK). The individual gels were prepared from a 20ml stock mix of polyacrylamide and consisted of 7.9 ml H₂O, 6.7 ml acrylamide mix, 5 ml 1.5 M Tris, 0.2 ml 10% SDS, 0.2 ml 10% ammonium persulfate and 0.008 ml TEMED (Sigma Aldrich, U.S.A). An electric current was used to shift negatively charged protein molecules to the positive end across the polyacrylamide gel. The resistance provided by the gel against this movement allows smaller proteins to migrate faster through it, while the larger weight proteins are displaced more passively, leading to a separation. The ERK/MAPK1/2 proteins are 44 kDa and 42 kDa respectively.

(b) Transfer: After a desirable amount is separation is obtained, the proteins are transferred onto a nitrocellulose membrane to make them more viable for detection by antibodies. These membranes have unique protein-binding abilities, and are able to “catch” proteins from the gel after an electric current is applied at 90 degrees. The success of the transfer procedure can be evaluated through the use of dyes, such as Ponceau S.

(c) Blocking: Once transfer of proteins onto the nitrocellulose membrane is completed, the next step is blocking non-specific interactions between the antibody and the membrane. This is achieved by placing the membrane in a
solution of non-fat dry milk and detergent (Tween 20). Proteins in the solution bind to areas not occupied by my protein of interest (ERK/MAPK), thus allowing the antibodies to specifically target these proteins when they are administered later.

(d) Visualization: Visualization of the ERK/MAPK1/2 protein requires several steps. Firstly, the primary antibody (p44/42 or phospho-p-42/44; Cell Signalling, U.S.A.) which is specific to the protein of interest is diluted in a buffer solution (Tris buffered solution or TBS) containing a small amount of detergent (tween). This solution is then incubated with the membrane overnight under gentle agitation (utilizing a platform shaker) at a temperature of 4ºC. The next day, the membrane is rinsed several times in TBS to remove any unbound primary antibody. It is then incubated for 2 hours with another buffer solution (TBS) containing non-fat dry milk and the secondary antibody. This antibody binds to the primary antibody, targeted to a particular species (goat anti-rabbit). In the final visualization stage, the western blot is incubated with a substrate that will luminesce when exposed to a reporter located on the secondary antibody. This is a horse radish peroxidase (HRP) chemiluminescent agent (Denville Scientific Inc., U.S.A.) that is required for protein detection. A CCD (charge-coupled device) camera setup is used to display dark bands representing ERK/MAPK protein levels after the membrane is exposed to the HRP detecting agent. These exposed bands are then analyzed with the aid of densitometry. Changes in the level of activated ERK/MAPK in correspondence to AMPA treatment was evaluated by
comparing the ratio of phosphorylated-ERK/MAPK with the total-ERK/MAPK levels of hippocampal slice protein samples at the relevant time intervals.

**C.5 Statistical Evaluation**

The statistical evaluation of both electrophysiology and slice treatment results were performed using Student’s $t$-test. This test compared the means of two groups of data (treated vs. untreated and/or wild-type vs. mutant studies). A p-value was calculated based on the probability of the likelihood of a false-positive occurrence. For comparison of the magnitude of E-LTP and L-LTP between different groups, the last 10 minutes of recordings were compared statistically. $n$ represents the number of hippocampal slices used in each experiment. Normally, one slice per mouse was used for experiments. When average data was plotted, data was normalized to the average of the baseline responses unless indicated otherwise. The representative traces were averages of four successive sweeps during recording. For slice treatment studies, the percentage change in the ratio of phosphorylated-ERK/MAPK with the total-ERK/MAPK levels of hippocampal slice protein samples were compared between baseline (pre-AMPA treatment) and post-AMPA treatment intervals (1, 5 and 10 mins) respectively by Student’s $t$-test to obtain a p-value.
D. Results

D.1 Early-LTP (E-LTP) Results

Recordings with specific inhibitors were conducted in both wild-type (control experiments) and GluR2 knockout mice (for testing plasticity induced through CP-AMPARs). My initial studies sought to confirm past studies in our laboratory indicating the involvement of CP-AMPARs in an NMDAR-independent form of synaptic plasticity. We have previously demonstrated that GluR2 mutants exhibit high Ca\(^{2+}\) permeability and inward rectification as well as an enhanced form of plasticity at CA1 synapses facilitated by Ca\(^{2+}\) influx through both NMDARs and CP-AMPARs (Jia et al., 1996; Meng et al., 2003). The utilization of an NMDAR antagonist such as D,L-AP5 allowed me to specifically isolate plasticity induced through CP-AMPARs, and thus investigate the molecular mechanisms underlying long-lasting synaptic increases induced by Ca\(^{2+}\) influx through these receptors. Therefore, in the present study, I used hippocampal slices prepared from these mice to investigate CP-AMPAR-induced synaptic plasticity by performing field recordings at the CA1 synapses. In wild-type animals, a brief high frequency stimulation (HFS, 2 trains of 100 Hz, with each train lasting 1 second) produced a long-lasting increase in field excitatory postsynaptic potentials (fEPSPs) that could be completely blocked by application of 100 µM D,L-AP5 (vehicle = 149 ± 5.1%; D,L-AP5 = 103 ± 1.6%; \(P < 0.001\)) during the induction phase, indicating that this form of E-LTP was completely NMDAR-dependent (Figure 1). In contrast, a significant
Figure 1. E-LTP in the hippocampal CA1 region of wild-type mice is dependent on NMDARs (A) The utilization of an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/-) mice displayed a robust enhancement of the synaptic response (vehicle treated = 149 ± 5.1%; n = 5) following a brief baseline period. In comparison, this form of potentiation was completely inhibited in the presence of the NMDAR antagonist D,L-AP5 (D,L-AP5 treated = 103 ± 1.6%; n = 5; \( P < 0.001 \)). This illustrated the dependence of the induction of synaptic plasticity in the wild-type CA1 region on the activation of NMDARs. (B) Comparison of the last 10 minutes of plasticity between D,L-AP5 treated and vehicle treated slices in wild-type animals. 100 µM D,L-AP5 was added to the ACSF perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 1.

A

- R2+/+ (vehicle)
- R2 +/+ (D,L-AP5)

B

* P < 0.001

Normalized means of last 10 mins of LTP (°)
Figure 2. High frequency stimulation (HFS) can induce E-LTP in GluR2 mutants in the presence of D,L-AP5 (A) In contrast to wild-type animals, the use of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated significant E-LTP (D,L-AP5 treated = 166 ± 8%; n = 6; P < 0.001) despite the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was induced through CP-AMPARs, and therefore NMDAR independent. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between baseline (pre-HFS) and stimulated periods (post-HFS). All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 2.

A

- R2 -/- (D,L-AP5)

B

- R2 -/- (baseline)
- R2 -/- (D,L-AP5, post-HFS)

*  

$P < 0.001$
amount of E-LTP was generated with the same induction protocol in GluR2-/- mice (166 ± 8%; \( P < 0.001 \)) despite the presence of 100 μM D,L-AP5 (Figure 2). One question that needed to be addressed was the possible differences in the involvement of presynaptic factors in the induction of this form of plasticity. A potential indicator of presynaptic involvement is the use of electrophysiological studies with paired-pulse facilitation (PPF), a short term form of plasticity. This experiment was carried out in both wild-type and GluR2 knockout mice slices prior to and following the induction of plasticity. However, PPF results did not reveal a significant difference between the two groups (\( P > 0.1 \)), suggesting no significant differences in the contribution of presynaptic factors in both NMDAR and AMPAR-induced forms of plasticity (Figure 3). To test whether this NMDAR-independent E-LTP could be induced by other stimulation protocols, I utilized theta burst stimulation (TBS), which is considered to be more physiologically relevant. As shown in Figure 4, this protocol generated a significant amount of E-LTP during extracellular (141 ± 3.1%; \( P < 0.001 \)) recordings in knockout slices in the presence of the NMDAR antagonist. These results confirm the existence of E-LTP induced through CP-AMPARs in the hippocampal slices obtained from adult GluR2 knockout mice, and form the basis of using these animals as a model for these particular studies. To exclude the possibility that other receptor subtypes (such as high voltage activated calcium channels) may play a role in the induction of CP-AMPAR-dependent plasticity, I decided to test whether this form of potentiation was susceptible to the selective CP-AMPAR inhibitor IEM-1460 (Gray et al., 2007; Buldakova et al., 2007). Administration of 100 μM IEM-1460 significantly reduced basal transmission in GluR2-/- slices (pre-treatment = 223 ± 10.7%; treated = 105 ± 1.8%; \( P < 0.001 \)) and completely blocked the subsequent
induction of CP-AMPAR-dependent E-LTP by 2 trains of 100 Hz (Figure 5) in the presence of D,L-AP5 (D,L-AP5 + IEM-1460 = 101 ± 4.7%). These results confirm that CP-AMPAR-dependent plasticity is induced exclusively through CP-AMPARs in the GluR2 knockout mouse model.

Figure 3. No differences in presynaptic function between NMDA and CP-AMPAR induced forms of E-LTP (A) Paired pulse facilitation (PPF) is a short term form of plasticity, where the ratio of two responses separated by a time interval (50 ms) is a indicator of changes in presynaptic involvement. Monitoring the PPF before and following the induction of E-LTP induced by a protocol of 2 trains of 100 Hz revealed no significant difference in the ratio between the two responses in both NMDAR induced and CP-AMPAR induced forms of E-LTP (P > 0.1). CP-AMPAR-dependent E-LTP recordings in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 3.
Figure 4. Theta-burst stimulation (TBS)-E-LTP can be induced in GluR2 mutants in the presence of D,L-AP5 (A) The use of a theta-burst stimulation (TBS) induction protocol that mimics the endogenous rhythms of the brain in the CA1 region of the hippocampus of GluR2-lacking (GluR2 /- ) mice yielded significant E-LTP (D,L-AP5 treated = 141 ± 3.1%, n = 6; \( P < 0.001 \)) in spite of the presence of the NMDAR antagonist D,L-AP5. This revealed the capability of CP-AMPARs to elicit plasticity through multiple stimulatory protocols. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between baseline (pre-TBS) and stimulated periods (post-TBS). CP-AMPAR-dependent E-LTP recordings in GluR2 mutants involved the addition of 100 \( \mu \)M D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 4.

A

- R2 -/- (D,L-AP5)

![Graph showing normalized field EPSP (%)]

Normalized field EPSP (%)

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<td>350</td>
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B

- R2 -/- (baseline)
- R2 -/- (D,L-AP5, post-TBS)

![Bar chart showing normalized means of last 10 mins of LTP (%)]

Normalized means of last 10 mins of LTP (%)

<table>
<thead>
<tr>
<th>R2 -/-</th>
<th>R2 -/-</th>
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</thead>
<tbody>
<tr>
<td>100</td>
<td>150</td>
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</table>

* P < 0.001
Figure 5. **CP-AMPAR-dependent E-LTP in GluR2-/- mice is induced exclusively through CP-AMPARs** (A) Administration of the CP-AMPAR antagonist IEM-1460 produced a significant reduction in the basal synaptic response in the hippocampal CA1 region of GluR2 -/- slices (IEM-1460 treated = 105 ± 1.8%; n = 6; P < 0.001) following a brief baseline period (pre-treatment = 223 ± 10.7%). The subsequent induction of E-LTP by 2 trains of 100 Hz was also completely inhibited in the presence of IEM-1460 (D,L-AP5 + IEM-1460 = 101 ± 4.7%). This experiment illustrated the importance and exclusivity of CP-AMPARs in the induction of NMDAR independent E-LTP in GluR2 knockout mice. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response in slices between (1) untreated, (2) IEM-1460 and D,L-AP5 treated and (3) IEM-1460 and D,L-AP5 treated post-HFS. CP-AMPAR-dependent E-LTP recordings in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 100 µM IEM-1460 was added to the ACSF perfusate 25 minutes prior to E-LTP induction in GluR2 -/- slices and was present throughout the entire recording period. Error bars represent SEM.
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<th>INHIBITOR</th>
<th>RESULTS</th>
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<td>Vehicle</td>
<td>Significant NMDAR-dependent E-LTP</td>
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<tr>
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<td>GluR2/-</td>
<td>Theta-burst stimulation</td>
<td>D,L-AP5</td>
<td>Significant CP-AMPAR-induced E-LTP</td>
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**Table 1.** Summary of LTP results with 2 trains of 100 Hz (HFS) and TBS.
D.1.1 Role of CaMKII signalling in E-LTP

My next goal was to determine whether the downstream role of calcium influx through AMPARs in E-LTP was similar to that seen in NMDAR-dependent forms of E-LTP. One factor that has consistently displayed a role in the latter form of E-LTP induced by calcium influx has been CaMKII. CaMKII is derived from a family of four homologous but distinct genes, with over 30 alternatively spliced isoforms described. It is a ubiquitous mediator of Ca$^{2+}$-linked signalling that phosphorylates a wide range of substrates to coordinate and regulate Ca$^{2+}$-mediated alterations in cellular function. The role of CaMKII in Ca$^{2+}$ signal transduction is shaped by its autoregulation, isoenzymic type and subcellular localization. CaMKII inhibitors have shown to block NMDAR-dependent forms of LTP both intracellularly (Malinow et al., 1989) and extracellularly (Ito et al., 1991). LTP is also impaired following genetic manipulation of CaMKII function (Silva et al., 1992). It was also seen that NMDAR-dependent forms of LTP were associated with a dendritic accumulation of active CaMKII (Fukunaga et al., 1996) together with phosphorylation of AMPARs (Barria et al., 1997). Lastly, CaMKII was found to mimic and occlude the induction of LTP (Lledo et al., 1995) and cause AMPAR insertion into synapses in a manner analogous to LTP (Hayashi et al., 2000).

Therefore, CaMKII is a key Ca$^{2+}$-activated protein kinase indispensable for the induction of NMDAR-dependent E-LTP. To test whether CaMKII also plays a role in CP-AMPAR-dependent E-LTP induced by 2 trains of 100 Hz, I first utilized the broad spectrum CaMKII inhibitor staurosporine (Rüegg and Burgess, 1989; Sanchez-Martinez et al., 2003). Administration of 100 nM staurosporine drastically reduced NMDAR-dependent E-LTP (Figure 6) in the wild-type animals (vehicle treated = 160 ± 6.9%;
staurosporine = 116 ± 7.5%; P = 0.002), but surprisingly had no effect on the amount of CP-AMPAR dependent E-LTP (Figure 7) induced in the presence of D,L-AP5 (D,L-AP5 + vehicle = 149 ± 6.4%; D,L-AP5 + staurosporine = 148 ± 3; P = 0.97). To confirm these findings, I performed further investigations by including the CaMKII specific inhibitor KN-62 in the perfusion solution. Accordingly, I found that KN-62 (15 µM) also had no effect on CP-AMPAR-induced E-LTP in GluR2/- mice. As shown in Figure 9, the magnitude of E-LTP was indistinguishable with or without KN-62 (D,L-AP5 + vehicle = 155 ± 4.2%; D,L-AP5 + KN-62 = 148 ± 6.8%; P = 0.42). The lack of KN-62 effect on CP-AMPAR-dependent E-LTP was not due to the ineffectiveness of the drug because it could effectively block NMDAR-dependent E-LTP (Figure 8) in wild-type animals (vehicle treated = 174 ± 4.8%; KN-62 = 102 ± 12.1%; P < 0.001) and also significantly inhibited the enhanced E-LTP in GluR2/- mice (Figure 10) in the absence of D,L-AP5 (vehicle treated = 221 ± 12.4%; KN-62 = 170 ± 13.4%; P = 0.02). These results indicate that CaMKII is not required for CP-AMPAR-dependent E-LTP, and suggest that a distinct synaptic signalling cascade must be operating during this form of E-LTP.
Figure 6. Staurosporine inhibits E-LTP in wild-type mice (A) The utilization of an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/+) mice displayed a significant enhancement of the synaptic response (vehicle treated = 160 ± 6.9%; n = 6) following a brief baseline period. This form of potentiation was strongly inhibited in the presence of the broad spectrum CaMKII inhibitor staurosporine (staurosporine treated = 116 ± 7.5%; n = 5; P < 0.01). This illustrated the dependence of the induction of synaptic plasticity in the wild-type CA1 region on the activity of CaMKII. (B) Comparison of the last 10 minutes of plasticity between staurosporine treated and vehicle treated slices in wild-type animals. 100 nM staurosporine was added to perfusate 15-20 minutes prior to E-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 6.

A

- ○ R2 +/+ (Staurosporine)
- ● R2 +/+ (vehicle)

B

- ■ R2 +/+ (vehicle)
- □ R2 +/+ (Staurosporine)

* $P < 0.001$
Figure 7. Staurosporine has no effect on CP-AMPAR-dependent E-LTP in GluR2 mutant mice (A) In contrast to wild-type animals, the use of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated significant E-LTP (D,L-AP5 + vehicle = 149 ± 6.4%; n = 5) despite the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was not susceptible to the administration of the broad spectrum CaMKII inhibitor staurosporine (D,L-AP5 + staurosporine treated = 148 ± 3%; n = 5; P = 0.97). This would suggest that the CaMKII does not play a role in synaptic plasticity induced by CP-AMPARs. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both staurosporine and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 100 nM staurosporine was added to perfusate 15-20 minutes prior to E-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 7.

A

- ○ R2 -/- (D.L-AP5 + Staurosporine)
- ● R2 -/- (D.L-AP5 + vehicle)

B

- ● R2 -/- (D.L-AP5 + vehicle)
- □ R2 -/- (D.L-AP5 + Staurosporine)

P = 0.97
Figure 8. KN-62 inhibits the induction of E-LTP in wild-type mice (A) Utilizing an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/+) mice revealed significant E-LTP (vehicle treated = 174 ± 4.8%; n = 5) following a brief baseline period. This form of potentiation was completely inhibited in the presence of the specific CaMKII inhibitor KN-62 (KN-62 treated = 102 ± 12.1%; n = 5; P < 0.001). This further demonstrated the dependence of synaptic plasticity induced in the wild-type hippocampal CA1 region on the activity of CaMKII. (B) Comparison of the last 10 minutes of plasticity between KN-62 treated and vehicle treated slices in wild-type animals. 15 µM KN-62 was added to ACSF perfusate 15-20 minutes prior to E-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 8.

A

- Circles: R2 +/+ (KN-62)
- Black dots: R2 +/+ (vehicle)

Normalized field EPSP (%) vs. Time (min)

B

- Grey: R2 +/+ (vehicle)
- White: R2 +/+ (KN-62)

* P < 0.001

Normalized means of last 10 mins of LTP (%)
Figure 9. KN-62 does not significantly alter CP-AMPAR-dependent E-LTP in GluR2 knockout mice (A) The use of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/-) mice demonstrated a significant augmentation of the synaptic response (D,L-AP5 + vehicle = 155 ± 4.2%; n = 5) in spite of the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was not significantly inhibited in the presence of the specific CaMKII inhibitor KN-62 (D,L-AP5 + KN-62 treated = 148 ± 6.8%; n = 5; P = 0.42). This would further imply that the activity of CaMKII is not required for the induction of synaptic plasticity elicited by CP-AMPARs. (B) Comparison of the last 10 minutes of potentiation seen between both KN-62 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 15 µM KN-62 was added to perfusate 15-20 minutes prior to E-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 9.

A

- O R2 -/- (D,L-AP5 + KN-62)
- • R2 -/- (D,L-AP5 + vehicle)

Normalized field EPSP (%)

Time (min)

B

- ■ R2 -/- (D,L-AP5 + vehicle)
- □ R2 -/- (D,L-AP5 + KN-62)

Normalized means of last 10 mins of LTP (%)

P = 0.4
Figure 10. KN-62 can inhibit the NMDAR induced component of E-LTP in GluR2 knockout mice (A) The utilization of an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/-) mice revealed a highly significant form of potentiation that was collectively induced from both NMDA and CP-AMPARs (vehicle treated = 221 ± 12.1%; n = 6) following a brief baseline period. This form of potentiation was strongly but not completely attenuated in the presence of the specific CaMKII inhibitor KN-62 (KN-62 treated = 170 ± 13.4%; n = 5; P < 0.05). These results would suggest that KN-62 inhibited only the NMDAR induced component of potentiation seen in GluR2 -/- mice, while having no significant effect on the E-LTP elicited by CP-AMPARs. (B) Comparison of the last 10 minutes of plasticity between KN-62 treated and vehicle treated slices in GluR2-lacking mutants. 15 µM KN-62 was added to perfusate 15-20 minutes prior to E-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 10.

A

- R2 -/- (KN-62)
- R2 -/- (vehicle)

B

* 
$P < 0.05$

Normalized means of last 10 mins of LTP (%)
<table>
<thead>
<tr>
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<th>RESULTS</th>
</tr>
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<tr>
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</tr>
<tr>
<td>GluR2-/-</td>
<td>2 trains of 100Hz HFS</td>
<td>KN-62</td>
<td>Total E-LTP (2-fold) significantly reduced.</td>
</tr>
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**Table 2.** Summary of LTP results with CaMKII inhibitors.
D.1.2 Role of ERK/MAPK signalling in E-LTP

Subsequently, I decided to explore the involvement of other prospective downstream effectors involved in CP-AMPAR-induced E-LTP in my next set of studies. One of these was the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling cascade. This signalling pathway is widely expressed, and involved in a large number of processes including cell division. During the ERK or MAPK signalling cascade, Ras (a signal transduction protein) first acts on RAF (a serine/threonine-specific kinase), which in turn phosphorylates MEK (mitogen activated protein kinase kinase), which finally activates ERK/MAPK. The ERK/MAPK signalling cascade targets a variety of substrates, including signalling proteins (PLA$_2$ and RSK2), cytoskeletal proteins (MAP-2, Tau, and Arc), synaptosomal proteins (synapsin), and nuclear proteins (CREB, ATF, and ELK1) (Lynch, 2004). This pathway has been implicated in a number of activities, including viral infections and cancer. Numerous studies have implicated ERK/MAPK in several types of synaptic plasticity, including NMDAR-dependent (English and Sweatt, 1997) and NMDAR-independent (Kanterewicz et al., 2000) forms of LTP in the CA1 region of the hippocampus. ERK/MAPK was also found to be associated with plasticity in the amygdala (Schafe et al., 2000), dentate gyrus (English and Sweatt, 1997), and visual cortex (Di Cristo et al., 2001). These findings have been corroborated with behavioral testing in animals, particularly the importance of ERK/MAPK in spatial learning (Blum et al., 1999) as well as fear conditioning (Akirav et al., 2001). ERK/MAPK has several downstream substrates, out of which CREB has garnered the most attention with regards to synaptic plasticity (Davis et al., 2000).
My initial E-LTP studies in wild-type slices with the MEK inhibitor PD 98059 (50 µM) showed that the elicitation of NMDAR-dependent form of plasticity was significantly blocked in its presence (vehicle treated = 162 ± 7.4%; PD98059 = 107 ± 4.3%; P < 0.001; Figure 11). I also showed that the administration of this inhibitor significantly abolished CP-AMPAR-induced E-LTP in slices from GluR2 knockout mice (D,L-AP5 + vehicle = 150 ± 6.3%; D,L-AP5 + PD98059 = 100 ± 4.6%; P < 0.001; Figure 12). The involvement of the ERK/MAPK cascade in CP-AMPAR dependent plasticity was further demonstrated by the suppression of E-LTP in knockout slices (D,L-AP5 + vehicle = 150 ± 6.3%; D,L-AP5 + U0126 = 125 ± 6%; P = 0.02; Figure 13) by the PD98059-structurally unrelated MEK inhibitor U0126 (35 µM). Taking these results into account, I ascertained a clear involvement of the ERK/MAPK signalling pathway in this lesser known form of plasticity.
Figure 11. PD98059 inhibits the induction of E-LTP in wild-type mice (A) The administration of an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the hippocampal CA1 region of wild-type (GluR2 +/+) mice displayed a significant enhancement of the synaptic response (vehicle treated = 162 ± 7.4%; n = 5) following a brief baseline period. This form of potentiation was completely attenuated in the presence of the MEK (MAPKK or ERK/MAPK kinase) inhibitor PD98059 (PD98059 treated = 107 ± 4.3%; n = 6; \( P < 0.01 \)). This illustrated the dependence of the induction of synaptic plasticity in the wild-type CA1 region on the activity of the ERK/MAPK signalling cascade. (B) Comparison of the last 10 minutes of plasticity between PD98059 treated and vehicle treated slices in wild-type animals. 50 µM PD98059 was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 11.

A

- R2 +/+ (vehicle)
- R2 +/+ (PD98059)

B

- R2 +/+ (vehicle)
- R2 +/+ (PD98059)

* P < 0.001
Figure 12. PD98059 completely inhibits CP-AMPAR-dependent E-LTP in GluR2 knockout mice (A) In contrast to wild-type animals, the use of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated significant E-LTP (D,L-AP5 + vehicle = 150 ± 6.3%; n = 6) despite the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was completely susceptible to the administration of the MEK inhibitor PD98059 (D,L-AP5 + PD98059 treated = 100 ± 4.6%; n = 5; P < 0.001). This would suggest that the ERK/MAPK signalling cascade plays a crucial role in synaptic plasticity induced by CP-AMPARs. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both PD98059 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 50 µM PD98059 was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 12.

A

- R2-/-(D,L-AP5 + vehicle)
- R2-/-(D,L-AP5 + PD98059)

B

- R2 -/- (D,L-AP5 + vehicle)
- R2 -/- (D,L-AP5 + PD98059)

\[ * \]

\[ P < 0.001 \]
Figure 13. U0126 significantly inhibits CP-AMPAR-dependent E-LTP in GluR2 mutants (A) The usage of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2 knockout (GluR2 -/- ) mice demonstrated a robust E-LTP (D,L-AP5 + vehicle = 150 ± 6.3%; n = 6) in spite of the administration of the NMDAR antagonist D,L-AP5. This type of potentiation was strongly attenuated in the presence of the MEK inhibitor U0126 (D,L-AP5 + U0126 treated = 125 ± 6%; n = 5; \( P < 0.05 \)). These results provide further evidence that the ERK/MAPK signalling cascade has an important role in the induction of synaptic plasticity through CP-AMPARs. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both U0126 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 35 µM U0126 was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 13.

A
- R2 -/- (D,L-AP5 + vehicle)
- R2 -/- (D,L-AP5 + U0126)

B
- R2 -/- (D,L-AP5 + vehicle)
- R2 -/- (D,L-AP5 + U0126)

* $P < 0.05$
Table 3. Summary of LTP results with ERK/MAPK inhibitors.

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</tr>
<tr>
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<td>Significant CP-AMPAR-induced E-LTP</td>
</tr>
<tr>
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</tr>
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D.2 Late-LTP (L-LTP) Results: Requirement of ERK/MAPK in the induction of plasticity

To determine the persistence of LTP induced through CP-AMPARs, I delivered multiple trains of HFS (4 trains of 100 Hz at 20 second intervals), which are commonly used to induce a long-lasting (or a late phase) LTP (L-LTP), and recorded fEPSPs for an extended period of time (up to 2 hours; Figure 14). Utilizing this protocol, a CP-AMPAR dependent L-LTP (Figure 15) was prominent in D,L-AP5-perfused GluR2-/− slices (228 ± 20%; \( P < 0.001 \)). One potential problem with the above experiment was that all the AMPARs in GluR2-/− mice lack the GluR2 subunit, which may rarely occur under normal physiological or pathological conditions. GluR2-/− mice may also suffer developmental compensations that could lead to changes in neuronal signalling processes. Therefore, in addition to GluR2-/− animals, I utilized GluR2+/− (heterozygous) mice, where the level of total GluR2 protein is reduced and both GluR2-containing and GluR2-lacking AMPARs are expressed at CA1 synapses. In addition, GluR2+/− mice are completely indistinguishable from the wild-type animals in growth and behavioral responses as opposed to GluR2-/− mice, which have multiple deficits (Jia et al., 1996).

As shown in Figure 16, long-lasting L-LTP was also clearly generated in GluR2+/− mice in the presence of 100 µM D,L-AP5 (149 ± 6.2%; \( P < 0.001 \)). This form of CP-AMPAR-dependent L-LTP was also completely inhibited by the administration of the CP-AMPAR antagonist IEM-1460 (100 µM) in GluR2+/− slices (D,L-AP5 = 157 ± 10.1%; D,L-AP5 + IEM-1460 = 102 ± 3.7%; \( P < 0.001 \); Figure 17). Giving weight to the notion that CP-AMPARs play a direct role in classical hippocampal forms of plasticity, I found no significant presynaptic changes following L-LTP induction through both NMDARs and
AMPARs during PPF studies ($P > 0.5$; Figure 18). This CP-AMPAR-induced L-LTP shared the characteristic dependence of longer-lasting forms of plasticity on the formation of new proteins (Lynch, 2004), where plasticity induced in both wild-type (vehicle treated = 186 ± 12.1%; anisomycin = 133 ± 7.3%; $P = 0.006$; Figure 19) and GluR2+/− slices (D,L-AP5 + vehicle = 163 ± 10.8%; D,L-AP5 + anisomycin = 106 ± 7.3%; $P = 0.002$; Figure 20) was significantly reduced under the administration of the protein synthesis inhibitor anisomycin (25 μM). These results indicate that CP-AMPARs can induce various types of long-lasting synaptic plasticity at CA1 synapses, including a previously undemonstrated form of protein synthesis-dependent L-LTP that is NMDAR-independent.
Figure 14. Late-LTP in the hippocampal CA1 region of wild-type mice is dependent on NMDARs (A) Utilizing an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/-) mice exhibited a significant augmentation of the synaptic response (vehicle treated = 186 ± 5.3%; n = 5) following a brief baseline period. In comparison, this form of potentiation was completely inhibited by the administration of the NMDAR antagonist D,L-AP5 (D,L-AP5 treated = 110 ± 3.2%; n = 5; P < 0.001). This demonstrated the dependence of late-long term potentiation (L-LTP) in the wild-type CA1 region on the activation of NMDARs. (B) Comparison of the last 10 minutes of plasticity between D,L-AP5 treated and vehicle treated slices in wild-type animals. 100 µM D,L-AP5 was added to the ACSF perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 15. HFS can induce CP-AMPAR-dependent L-LTP in GluR2/- mice in the presence of D,L-AP5 (A) The utilization of an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated robust L-LTP (D,L-AP5 treated = 228± 20%; n = 5; P < 0.001) despite the presence of the NMDAR antagonist D,L-AP5. This demonstrated the capability of CP-AMPARs to induce longer-lasting forms of plasticity in the hippocampus. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between baseline (pre-HFS) and stimulated periods (post-HFS). All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 15.

A

- **R2 -/- (D,L-AP5)**

B

- **R2 -/- (baseline)**
- **R2 -/- (D,L-AP5)**

*P < 0.001*
Figure 16. CP-AMPAR-dependent L-LTP can be induced in GluR2+/- mice in the presence of D,L-AP5 (A) Following the administration an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/- ) mice, a significant L-LTP (D,L-AP5 treated = 149± 6.2%; n = 5; P < 0.001) was observed even though the NMDAR antagonist D,L-AP5 was present in the ACSF perfusate. This further exhibited the ability of CP-AMPARs to induce plasticity that can last for several hours in the hippocampal region. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between baseline (pre-HFS) and stimulated periods (post-HFS). CP-AMPAR-dependent L-LTP recordings in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 16.

A

B
Figure 17. CP-AMPAR-dependent L-LTP in GluR2+/- mice is induced specifically through CP-AMPARs (A) The utilization of an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/-) mice demonstrated robust L-LTP (D,L-AP5 treated = 157± 10.1%; n = 5) despite the presence of the NMDAR antagonist D,L-AP5. This form of plasticity was completely inhibited in the presence of the CP-AMPAR antagonist IEM-1460 (D,L-AP5 + IEM-1460 treated = 102± 3.7%; n = 5; P < 0.001). This demonstrated that the long-lasting form of potentiation seen in these mutant mice is induced exclusively through CP-AMPARs. (B) Comparison of the last 10 minutes of plasticity between both IEM-1460 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 100 µM IEM-1460 was added to the ACSF perfusate 15-20 minutes prior to L-LTP induction in GluR2 +/- slices up until 5 minutes post-induction. Error bars represent SEM.
Figure 17.

A

- R2 +/- (D,L-AP5)
- R2 +/- (D,L-AP5 + IEM-1460)

B

- R2 +/- (D,L-AP5)
- R2 +/- (D,L-AP5 + IEM-1460)

normalized means of last 10 mins of LTP (%)
Figure 18. No differences in presynaptic function between NMDA and CP-AMPAR induced forms of L-LTP (A) Paired pulse facilitation (PPF) is a short term form of plasticity, where the ratio of two responses separated by a time interval (50 ms) is a indicator of changes in presynaptic involvement. Monitoring the PPF before and after the induction of L-LTP induced by a protocol of 4 trains of 100 Hz revealed no significant difference in the ratio between the two responses in both NMDAR induced and CP-AMPAR induced forms of L-LTP ($P > 0.5$). All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 18.
Figure 19. L-LTP induced in wild-type mice is dependent on new protein synthesis

(A) The use of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/+ ) mice revealed a long-lasting form of L-LTP (vehicle treated = 186 ± 12.1%; n = 5) following a brief baseline period. This form of potentiation was significantly inhibited by the administration of the protein synthesis inhibitor anisomycin (anisomycin treated = 133 ± 7.3%; n = 5; \( P < 0.01 \)). This demonstrated the characteristic dependence of L-LTP on the formation of new proteins for its persistence. (B) Comparison of the last 10 minutes of plasticity between anisomycin treated and vehicle treated slices in wild-type animals. 25 \( \mu \)M anisomycin was added to perfusate 15-20 minutes prior to L-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 19.

A

- R2 +/+ (vehicle)
- R2+/+ (Anisomycin)

B

*P < 0.001

Normalized means of last 10 mins of LTP (%)
Figure 20. CP-AMPAR-dependent L-LTP induced in GluR2 mutants requires new protein synthesis (A) Utilizing an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/- ) mice demonstrated robust L-LTP (D,L-AP5 treated = 163± 10.8%; n = 5) despite the presence of the NMDAR antagonist D,L-AP5. This form of potentiation was completely inhibited in the presence of anisomycin (D,L-AP5 + anisomycin treated = 106± 7.3%; n = 5; P < 0.01), revealing the reliance of CP-AMPAR-dependent L-LTP on de novo protein synthesis. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both anisomycin and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 25 µM anisomycin was added to perfusate 15-20 minutes prior to L-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 20.

A

- R2+/- (D,L-AP5 + vehicle)
- R2+/- (D,L-AP5 + Anisomycin)

B

- R2 +/- (D,L-AP5 + vehicle)
- R2 +/- (D,L-AP5 + Anisomycin)

* $P < 0.001$
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<tr>
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<td>100Hz HFS</td>
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**Table 4.** Summary of L-LTP results with 4 trains of 100Hz (HFS).
D.2.1 Role of ERK/MAPK pathway in L-LTP

To further elucidate the role of the ERK/MAPK signalling cascade in CP-AMPAR dependent plasticity, I tested whether the MEK inhibitor PD98059 (50 µM) would attenuate potentiation when perfused during the maintenance phase of CP-AMPAR dependent L-LTP induced by 4 trains of 100 Hz. I first demonstrated that the administration of this inhibitor prior to and during the induction of L-LTP in wild-type slices significantly inhibited potentiation (vehicle treated = 192 ± 11.2%; PD98059 = 137 ± 6.7%; \( P = 0.002 \); Figure 21), while having no effect when introduced during the maintenance phase of this NMDAR-dependent plasticity (vehicle treated = 206 ± 12.5%; PD98059 = 204 ± 12.7%; \( P = 0.9 \); Figure 22). In a similar manner, the presence of PD98059 in GluR2+/−slices significantly blocked CP-AMPAR dependent L-LTP in the inductory (D,L-AP5+vehicle in induction phase = 171 ± 10%; D,L-AP5 + PD98059 in induction phase = 129 ± 6.3%; \( P = 0.009 \); Figure 23) but not the maintenance phase (D,L-AP5 + vehicle in maintenance phase = 155 ± 6.3%; D,L-AP5 + PD98059 in maintenance phase = 154 ± 5.5%; \( P = 0.83 \); Figure 24) of this form of potentiation. These results suggest that the ERK/MAPK signalling cascade is an essential component in the induction but not maintenance of both NMDAR- and CP-AMPAR dependent forms of plasticity.
Figure 21. PD98059 inhibits L-LTP in wild-type when administered during the induction phase (A) The administration of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/+) mice revealed a long-lasting form of L-LTP (vehicle treated = 192 ± 11.2%; n = 5) following a brief baseline period. This longer-lasting form of potentiation was significantly inhibited by the administration of the MEK inhibitor PD98059 during the induction phase (PD98059 treated = 137 ± 6.7%; n = 6; P < 0.01). This demonstrated the dependence of the induction of L-LTP on the ERK/MAPK signalling cascade. (B) Comparison of the last 10 minutes of plasticity between PD98059 treated (induction phase) and vehicle treated slices in wild-type animals. For MAPK cascade L-LTP induction studies, 50 µM PD98059 was added to ACSF perfusate 15-20 minutes prior to induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 21.

A

- R2 +/+ (vehicle in induction phase)
- R2 +/+ (PD98059 in induction phase)

B

R2 +/+ (vehicle in induction phase)
R2 +/+ (PD98059 in induction phase)

* $P < 0.005$
Figure 22. PD98059 does not affect L-LTP induced in wild-type mice when administered during the maintenance phase. (A) Utilizing an induction protocol of 4 trains of 100 Hz in the hippocampal CA1 region of wild-type (GluR2 +/+) mice revealed a protein synthesis dependent form of L-LTP (vehicle treated = 206 ± 12.5%; n = 5) following a brief baseline period. This enhanced potentiation was not significantly altered in the presence of the MEK inhibitor PD98059 when administered during the maintenance phase (PD98059 treated = 204 ± 12.7%; n = 6; P = 0.9). This provided evidence for the lack of involvement of the ERK/MAPK signalling cascade in the maintenance phase of plasticity in wild-type animals. (B) Comparison of the last 10 minutes of plasticity between PD98059 treated (maintenance phase) and vehicle treated slices in wild-type animals. For MAPK cascade L-LTP maintenance studies, 50 µM PD98059 was added to the ACSF perfusate 20-30 minutes post-induction. Error bars represent SEM.
Figure 22.

A

- R2 +/+ (vehicle in maintenance phase)
- R2 +/+ (PD98059 in maintenance phase)

B

- R2 +/+ (vehicle in maintenance phase)
- R2 +/+ (PD98059 in maintenance phase)

P = 0.9
Figure 23. PD98059 attenuates CP-AMPAR-dependent L-LTP in GluR2 mutant mice when administered during the induction phase (A) The use of an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/- ) mice demonstrated a significant augmentation of the synaptic response (D,L-AP5 + vehicle = 171 ± 10%; n = 5) in spite of the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was significantly inhibited in the presence of the specific MEK inhibitor PD98059 (D,L-AP5 + PD98059 treated = 129 ± 6.3%; n = 5; P < 0.01) when administered during the induction phase of CP-AMPAR dependent L-LTP. These results would suggest that the late-phase of CP-AMPAR-dependent plasticity also requires the ERK/MAPK signalling cascade for its induction in a similar manner to early LTP recordings. (B) Comparison of the last 10 minutes of potentiation seen between both PD98059 (induction phase) and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. For MAPK cascade L-LTP induction studies, 50 µM PD98059 was added to ACSF perfusate 15-20 minutes prior to induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 23.

A

- R2 +/- (D,L-AP5 + vehicle in induction phase)
- R2 +/- (D,L-AP5 + PD98059 in induction phase)

B

- R2 +/- (D,L-AP5 + vehicle in induction phase)
- R2 +/- (D,L-AP5 + PD98059 in induction phase)

* P < 0.01
Figure 24. PD98059 does not significantly alter CP-AMPAR-dependent L-LTP induced in GluR2 mutant mice when administered during the maintenance phase

(A) Utilizing an induction protocol of 4 trains of 100 Hz in the hippocampal CA1 region of GluR2-less (GluR2 +/- ) mice demonstrated a robust L-LTP (D,L-AP5 + vehicle = 155 ± 6.3%; n = 5) despite the presence of the NMDAR antagonist D,L-AP5. This form of plasticity was not significantly affected by the specific MEK inhibitor PD98059 (D,L-AP5 + PD98059 treated = 154 ± 5.5%; n = 5; P = 0.83) when administered during the maintenance phase of CP-AMPAR dependent L-LTP. This demonstrated that the late-phase of CP-AMPAR-dependent plasticity does not require the activity of the ERK/MAPK signalling cascade during its maintenance phase, in a manner similar to NMDAR dependent forms of L-LTP. (B) Comparison of the last 10 minutes of potentiation seen between both PD98059 (maintenance phase) and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. For MAPK cascade L-LTP maintenance studies, 50 µM PD98059 was added to the ACSF perfusate 20-30 minutes post-induction. Error bars represent SEM.
Figure 24.

A

- R2 +/- (D,L-AP5 in induction phase, vehicle in maintenance phase)
- R2 +/- (D,L-AP5 in induction phase, PD98059 in maintenance phase)

B

- R2 +/- (D,L-AP5 in induction phase, vehicle in maintenance phase)
- R2 +/- (D,L-AP5 in induction phase, PD98059 in maintenance phase)

P = 0.83
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**Table 5.** Summary of L-LTP results with ERK/MAPK inhibitors.
D.2.2 Biochemical evidence: The ERK/MAPK cascade can be activated by CP-AMPARs in the hippocampal CA1 region

My electrophysiological results presented so far are consistent with my hypothesis that ERK/MAPK activation is critically involved in CP-AMPAR-induced E-LTP and L-LTP. To provide further biochemical evidence that CP-AMPARs are capable of activating the ERK/MAPK cascade, I performed AMPA treatment studies on freshly isolated hippocampal slices from both mutant and wild-type animals in the presence of 100 µM D,L-AP5 (NMDA antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist). Subsequently, the treated slice samples underwent Western blot techniques for the evaluation of activated (phosphorylated) and totals levels of ERK/MAPK protein. My first set of experiments revealed that the level of phosphorylated ERK/MAPK (p-ERK/MAPK) was significantly increased 5 minutes after 10 µM AMPA treatment in GluR2-/- slices (phospho/total ERK/MAPK= 134% +/- 10; \( P = 0.04 \); Figure 26). This was in contrast to AMPA treatment in wild-type slices, where p-ERK/MAPK was not significantly altered at any time interval post-treatment (\( P > 0.2 \); Figure 25, 27). The increase in p-ERK/MAPK in GluR2-/- slices following AMPA treatment was susceptible to the CP-AMPAR antagonist IEM-1460, where the drug reduced activated ERK/MAPK to basal levels (phospho/total ERK/MAPK = 102% +/- 5; \( P > 0.8 \); Figure 28). These results indicate that CP-AMPARs rather than Ca2+-impermeable AMPARs induce activation of the ERK/MAPK signalling cascade.
Figure 25. AMPA treatment does not activate ERK/MAPK in wild-type mice.

Freshly obtained acute hippocampal slices from wild-type mice were treated with AMPA. (A) Representative ERK1/2 protein bands obtained from wild-type hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Densitometry analysis of protein bands acquired from western blotting revealed no significant increase in the level of phosphorylated-ERK/MAPK (p-ERK/MAPK) at any time interval following treatment ($P > 0.05$). These results suggest that AMPA treatment alone does not activate the ERK/MAPK signalling cascade in wild-type slices. All biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 25.

A

B

ERK ½ (phospho/total)
Figure 26. ERK/MAPK is activated following AMPA treatment in GluR2-lacking mice. Acute hippocampal slices from GluR2-lacking (GluR2 -/-) mice were treated with AMPA. (A) Representative ERK1/2 protein bands obtained from GluR2-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Subsequent densitometric analysis of bands acquired from western blotting techniques divulged a significant increase in the level of p-ERK/MAPK at 5 minutes following treatment (134 ± 10%; n = 4; P < 0.05). These studies demonstrated that AMPA treatment can initiate the ERK/MAPK signalling cascade in GluR2 -/- slices, probably through the activation of CP-AMPARs. Biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes before the addition of 10 µM AMPA. Error bars represent SEM.
Figure 26.

A

B

---

ERK 1/2 (phospho/total)

Time (min)

p-ERK
Total-ERK

0               1              5             10
(mins)

R2^-/- (n=4)

p= 0.04

*
Figure 27. p-ERK/MAPK is significantly increased in GluR2-/- mutants in comparison to wild-type mice in response to AMPA treatment. (A) Representative ERK1/2 protein bands obtained from GluR2-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Comparison of the level of p-ERK/MAPK in wild-type (from Figure 25) versus GluR2 -/- (from Figure 26) slices revealed a significant difference 5 minutes post-AMPA treatment (AMPA in GluR2-/- = 134 ± 10%; n = 4; AMPA in wild-type = 94 ± 5.5%; n = 4; P < 0.005). Biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 27.

A

B

p-ERK
Total-ERK

(p-ERK)
(Total-ERK)

(mins)

R2+/+(n=4)
R2-/- (n=4)

p = 0.003

ERK1/2 (phospho/total %)

0 1 5 10

Time (min)
Figure 28. The ERK/MAPK signalling cascade is activated by CP-AMPARs in GluR2-/- mice. Freshly obtained acute hippocampal slices from GluR2-/- mice were treated with AMPA and the CP-AMPAR antagonist IEM-1460. (A) Representative ERK1/2 protein bands obtained from GluR2-/- hippocampal slice samples at different time intervals in response to AMPA treatment in the presence of IEM-1460. (B) Densitometry analysis of protein bands acquired from western blotting revealed no significant increase in the level of p-ERK/MAPK at any time interval following AMPA treatment ($P > 0.05$) in the presence of IEM-1460. This was in contrast to the increase in p-ERK/MAPK that was seen in slices 5 minutes post-AMPA treatment when IEM-1460 was not present (AMPA in GluR2-/- = 134 ± 10%; n = 4; AMPA + IEM-1460 in GluR2-/- = 102 ± 5.4%; n = 4; $P < 0.05$). These results suggest that the AMPA activation of the ERK/MAPK signalling cascade is initiated through the action of CP-AMPARs. Biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist), 20 µM Nifedipine (L-type calcium channel antagonist) and 100 µM IEM-1460 which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 28.

A

p-ERK
Total-ERK

0               1              5               10
(mins)

B

R2 -/- (n=4)
R2-/- IEM 1460 (n=4)

p= 0.013
D.3 Late-LTP Results: Role of PAK1 signalling

In my search for potential activators of the ERK/MAPK signalling pathway during CP-AMPAR dependent plasticity, I performed additional L-LTP studies on Rho-associated serine/threonine protein kinase PAK1 (or p21-activated kinase 1) knockout mice. Previous studies have shown that PAK1 phosphorylates MEK1 on Ser298 (Frost et al., 1997) as well as Raf-1 (Coles and Shaw, 2002) during the activation of ERK/MAPK, suggesting that PAK1 may act as a potential upstream activator of the ERK/MAPK cascade. My studies revealed that PAK1 knockout mice exhibited a deficiency of NMDAR-dependent L-LTP induced by 4 trains of 100Hz at 20 second intervals (Figure 29) in comparison to wild-type controls (138% +/- 5 in PAK1 knockout slices compared to 206% +/- 11 in wild-type slices). The attenuation of L-LTP in PAK1 knockout mice was statistically similar to that seen in wild-type slices that I previously treated with the ERK/MAPK cascade inhibitor PD-98059 (Figure 21, 40), strongly suggesting that PAK1 and ERK/MAPK act through similar signalling pathways during plasticity. This notion was further reinforced when the administration of PD-98059 in PAK1 knockout slices was shown to not significantly attenuate the already deficient L-LTP seen in these mice (138% +/- 5 in untreated PAK1 knockout slices compared to 133% +/- 11 in PAK1 knockout slices treated with PD 98059; Figure 30). This was in contrast to the further attenuation of previously deficient L-LTP (Figure 31) by the same inhibitor in ROCK2 knockout mice, a factor that is known to interact with the Rho-GTPase but not ERK/MAPK signalling cascade (170% +/- 8 in untreated ROCK2 knockout slices compared to 143% +/- 6 in ROCK2 knockout slices treated with PD 98059; Figure 32). To test for the involvement of PAK in CP-AMPAR-dependent forms of L-LTP, I
generated and analysed double knockout mice deficient in both GluR2 and PAK1 (developed through cross-breeding) in additional electrophysiological studies. CP-AMPAR dependent L-LTP induced by 4 trains of 100Hz at 20 second intervals in the presence of D,L-AP5 revealed a significantly lower level of plasticity (Figure 33) in GluR2-/−PAK1-/− slices compared to GluR2-/− slices (228% +/- 19 in GluR2-/− slices compared to 139% +/- 8 in GluR2-/−PAK1-/− slices; $P = 0.02$). Additionally, CP-AMPAR dependent L-LTP was also substantially reduced in GluR2+/−PAK1-/− slices (Figure 34) compared to GluR2+/− slices alone (163% +/- 11 in GluR2+/− slices compared to 123% +/- 5 in GluR2+/−PAK1-/− slices; $P = 0.009$). The deficient CP-AMPAR-dependent L-LTP seen in GluR2+/−PAK1-/− mice was statistically similar to the level of plasticity seen in GluR2+/− slices treated with PD98059 (Figure 35; $P = 0.47$). Taken together, these results suggest that PAK1 regulates the activity of the ERK/MAPK signalling pathway during synaptic plasticity induced through CP-AMPARs.
Figure 29. Late-LTP induced in the hippocampus of PAK1-/- mutants is largely attenuated in comparison to wild-type mice (A) Utilizing an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type mice exhibited a significant augmentation of the synaptic response (206 ± 10.9%; n = 5) following a brief baseline period. In comparison, this form of potentiation was strongly inhibited when administered in PAK1-/- mutant mice (138 ± 4.8%; n = 6; \( P < 0.001 \)). These results suggest the importance of PAK1 signalling in NMDAR dependent forms of plasticity. (B) Comparison of the last 10 minutes of plasticity between PAK1-/- mutant and wild-type animal slices. Error bars represent SEM.
Figure 29.

A

- Pak1+/+ (n=5)
- Pak1-/- (n=6)

B

Pak1 +/+ (n=5)
Pak1 -/- (n=6)

* P < 0.001
Figure 30. The deficient L-LTP seen in PAK1-/- mutants is not further susceptible to PD98059 (A) The use of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of PAK1-/- mice revealed an attenuated form of L-LTP (138 ± 4.8%; n = 6) following a brief baseline period. This form of potentiation was not further inhibited by the administration of the MEK inhibitor PD98059 (133 ± 10.6%; n = 6; P = 0.68). This indicated that the L-LTP deficiency seen in PAK1-/- slices is dependent on the ERK/MAPK signalling cascade. (B) Comparison of the last 10 minutes of plasticity between PD98059 treated and vehicle treated slices in PAK1-/- animals. 50 μM PD98059 was added to ACSF perfusate 15-20 minutes prior to induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 31. The induction of L-LTP induced in the hippocampal CA1 region of ROCK2-/- mutants is significantly reduced in comparison to wild-type mice (A) The usage of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the hippocampus of wild-type mice exhibited robust L-LTP (184 ± 5.5%; n = 9) following a brief baseline period. In comparison, this form of potentiation was significantly attenuated when administered in ROCK2-/- mutant mice (159 ± 7.7%; n = 9; $P < 0.05$). These results indicate that ROCK2 signalling plays a role in NMDAR dependent forms of plasticity. (B) Comparison of the last 10 minutes of plasticity between ROCK2-/- mutant and wild-type animal slices. Error bars represent SEM.
Figure 31.

A

- Rock2+/+ (n=9)
- Rock2-/- (n=9)

Normalized field Eps (%) over time (min).

B

Rock2+/+(n=9) vs. Rock2-/- (n=9) comparison.

* P < 0.05

Normalized means of last 10 mins of LTP (%)
Figure 32. The attenuated form of L-LTP seen in ROCK2-/- mutants is further vulnerable to the MEK inhibitor PD98059 (A) The use of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of ROCK2-/- mice revealed a reduced form of L-LTP (170 ± 8.3%; n = 6) following a brief baseline period. This form of potentiation was additionally inhibited by the administration of the MEK inhibitor PD98059 (143 ± 5.9%; n = 4; *P* < 0.05). These results suggest that the deficiency in L-LTP seen in ROCK2-/- mice is not dependent on the activity of the ERK/MAPK signalling cascade. (B) Comparison of the last 10 minutes of plasticity between PD98059 treated and vehicle treated slices in ROCK2-/- animals. 50 µM PD98059 was added to ACSF perfusate 15-20 minutes prior to induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 32.

A

- Rock2-/- (vehicle)
- Rock2-/- (PD98059)

Normalized field EPSP (%)

Time (min)

B

- Rock2-/- (vehicle)
- Rock2-/- (PD98059)

* P < 0.05

Normalized means of last 10 mins of LTP (%)

Rock2 +/-  
Rock2 -/-
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Table 6. Summary of NMDAR-dependent L-LTP results in PAK1-/- and ROCK2-/- mice.
Figure 33. CP-AMPAR-dependent L-LTP induced in GluR2-/-PAK1-/- double knockout mice is significantly lower than that elicited in GluR2-/- mice (A) Utilizing an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated robust L-LTP (D,L-AP5 treated = 228 ± 19.4%; n = 5) in spite of the presence of the NMDAR antagonist D,L-AP5. The use of the same protocol produced a markedly reduced level of CP-AMPAR-dependent L-LTP in GluR2-/-PAK1-/- double knockout mice (D,L-AP5 treated = 139 ± 8.3%; n = 3; P < 0.05). These results suggest PAK1 plays a crucial role in the initiation of CP-AMPAR-dependent plasticity, possibly in an ERK/MAPK-dependent manner. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between D,L-AP5 treated slices in GluR2 -/- animals versus D,L-AP5 treated slices in GluR2-/-PAK1-/- mutants. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 33.

A

- R2/- (D,L-AP5)
- R2/-PAK1/- (D,L-AP5)

B

- R2/- (D,L-AP5)
- R2/-PAK1/- (D,L-AP5)

*  

P < 0.05
Figure 34. L-LTP induced through CP-AMPARs is reduced in GluR2+/−-PAK1−/−mutant mice in comparison to GluR2+/− animals (A) Administering an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/-) mice demonstrated a vigorous augmentation of the basal synaptic response (D,L-AP5 treated = 163 ± 10.8%; n = 5) despite the presence of the antagonist D,L-AP5. This was in contrast to a vastly attenuated form of CP-AMPAR-dependent L-LTP seen in GluR2+/−-PAK1−/− mutants under the same experimental conditions (D,L-AP5 treated = 123 ± 4.5%; n = 5; P < 0.01). This would further indicate that CP-AMPAR-induced forms of plasticity require PAK1, potentially through mechanisms that rely on the ERK/MAPK signalling cascade. (B) Comparison of the last 10 minutes of potentiation between D,L-AP5 treated slices in GluR2 +/- animals versus D,L-AP5 treated slices in GluR2+/−-PAK1−/− mutants. CP-AMPAR-dependent L-LTP field recordings involved the addition of 100 μM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 35. The deficient CP-AMPAR-dependent L-LTP seen in GluR2+/-/PAK1-/- is similar to that produced in GluR2 +/- mice in the presence of PD98059 (A) Using an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2 +/- mice revealed a significantly diminished form of CP-AMPAR-induced L-LTP in the presence of the MEK antagonist PD98059 (D,L-AP5 + PD98059 treated = 129 ± 6.3%; n = 5). This reduced level of plasticity was statistically similar to that observed in GluR2+/-/PAK1-/- utilizing the same induction protocol (D,L-AP5 treated = 123 ± 4.5%; n = 5; P = 0.47). This would suggest that PAK1 and ERK/MAPK act in a linear signalling cascade during the implementation of CP-AMPAR-dependent forms of potentiation. (B) Comparison of the last 10 minutes of potentiation between D,L-AP5 and PD98059 treated slices in GluR2 +/- animals versus D,L-AP5 treated slices in GluR2+/-/PAK1-/- mutants. All field EPSP recordings of CP-AMPAR-dependent L-LTP involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 50 µM PD98059 was added to ACSF perfusate 15-20 minutes prior to induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 35.

A

- R2+/+ (D,L-AP5 + PD98059 in induction phase)
- R2+/−-Pak1−/− (D,L-AP5 in induction phase)

B

- R2+/+ (D,L-AP5 + PD98059)
- R2+/−-Pak1−/− (D,L-AP5)

P = 0.47
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<tr>
<td>GluR2-/</td>
<td>4 trains of 100Hz HFS</td>
<td>D,L-AP5</td>
<td>Robust CP-AMPAR-induced L-LTP</td>
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<tr>
<td>GluR2/-</td>
<td>4 trains of 100Hz HFS</td>
<td>D,L-AP5</td>
<td>CP-AMPAR-induced L-LTP attenuated</td>
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<tr>
<td>PAK1/-</td>
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<tr>
<td>GluR2+/</td>
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<td>D,L-AP5</td>
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<td>GluR2+/</td>
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Table 7. Summary of CP-AMPAR-dependent L-LTP results in GluR2/-PAK1/- and GluR2+/PAK1/- mice.
D.3.1 Biochemical evidence: ERK/MAPK activation by CP-AMPARs is dependent on PAK1

To determine whether PAK1 is involved the activation of p-ERK/MAPK, I performed further treatment experiments with PAK1-/- and GluR2-/-PAK1-/- knockout mice. In contrast to GluR2-/- mice, GluR2-/-PAK1-/- double knockout mice did not show a significant increase in the level of p-ERK/MAPK following 10 µM AMPA treatment (phospho/total ERK/MAPK = 94% +/- 7; P > 0.5; Figure 38, 39). Similar to wild-type mice, PAK1 mutants also did not reveal an increase in the level of p-ERK/MAPK following AMPA treatment (phospho/total ERK/MAPK = 99% +/- 6; P > 0.9; Figure 36, 37). In conjunction with the previous electrophysiological experiments, these results together suggest that CP-AMPARs activate ERK/MAPK during synaptic plasticity through PAK1-dependent pathways.
Figure 36. PAK1-/- mice do not reveal an increased level of p-ERK/MAPK following treatment by AMPA. Acutely obtained hippocampal slices from PAK1-/- mice were treated with AMPA. (A) Representative ERK1/2 protein bands obtained from PAK1-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Subsequent densitometric analysis of bands acquired from western blotting techniques revealed no significant increase in the level of p-ERK/MAPK at any time interval following treatment ($P > 0.05$). These results suggest that AMPA treatment alone does not activate the ERK/MAPK signalling cascade in slices from PAK1-/- mice. All biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 36.

A

B

p-ERK
Total-ERK

0               1              5              10 (mins)

Time (min)

ERK 1/2 (phospho/total)

Pak1+/− (n=4)

90
100
110
120
130
140
150

0      1      5      10

Time (min)
Figure 37. Both wild-type and PAK1-/- mice display a similar lack of ERK/MAPK activity in reply to AMPA treatment. (A) Representative ERK1/2 protein bands obtained from PAK1-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Comparison of the level of p-ERK/MAPK in wild-type versus PAK1-/- slices (from Figure 36) revealed no significant difference at any time interval post-AMPA treatment (AMPA in wild-type at 5 minutes = 94 ± 5.5%; n = 4; AMPA in PAK1-/- at 5 minutes = 99 ± 6.4%; n = 4; P > 0.5). Biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 37.

A

B

ERK 1/2 (phospho/total %)

Time (min)

p > 0.2
Figure 38. GluR2-/-PAK1-/- knockout mice do not reveal an elevation in phosphorylated ERK/MAPK in response to AMPA treatment. Freshly obtained acute hippocampal slices from GluR2-/-PAK1-/- double knockout mice were treated with AMPA. (A) Representative ERK1/2 protein bands obtained from GluR2-/-PAK1-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Densitometry analysis of protein bands acquired from western blotting revealed no significant increase in the level of phosphorylated-ERK/MAPK (p-ERK/MAPK) at any time interval following treatment ($P > 0.05$). These results suggest that AMPA treatment alone does not initiate the ERK/MAPK signalling cascade in slices obtained from GluR2-/-PAK1-/- double knockout mice. All biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 38.

A

B

R2−/−Pak1−/− (n=5)
Figure 39. p-ERK/MAPK is significantly decreased in GluR2-/-PAK1-/- mutants in comparison to GluR2-/- mice in response to AMPA treatment. (A) Representative ERK1/2 protein bands obtained from GluR2-/-PAK1-/- hippocampal slice samples at different time intervals in response to AMPA treatment. (B) Comparison of the level of p-ERK/MAPK in GluR2-/- versus GluR2-/-PAK1-/- double knockout (from Figure 38) slices revealed a significant difference at 5 minutes post-AMPA treatment (AMPA in GluR2-/- = 134 ± 10%; n = 4; AMPA in GluR2-/-PAK1-/- = 94 ± 6.7%; n = 5; P < 0.005). Biochemical treatment studies were performed in the presence of 100 µM D,L-AP5 (NMDAR antagonist) and 20 µM Nifedipine (L-type calcium channel antagonist) which were added 30 minutes prior to the addition of 10 µM AMPA. Error bars represent SEM.
Figure 39.

A

![Western Blot](image)

p-ERK

Total-ERK

0 1 5 10 (mins)

B

![Bar Graph](image)

R2+/+ (n=4)

R2+/−Pak1+/− (n=5)

p = 0.003

ERK 1/2 (phospho/total %)

0 1 5 10 Time (min)
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<th>MOUSE</th>
<th>Treatment</th>
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<tr>
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<td>AMPA</td>
<td>D,L-AP5, Nifedipine</td>
<td>p-ERK levels unchanged</td>
</tr>
<tr>
<td>GluR2-/-</td>
<td>AMPA</td>
<td>D,L-AP5, Nifedipine</td>
<td>p-ERK significantly increased 5 mins post-treatment</td>
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<tr>
<td>GluR2-/-</td>
<td>AMPA</td>
<td>D,L-AP5, Nifedipine, IEM-1460</td>
<td>p-ERK levels unchanged</td>
</tr>
<tr>
<td>PAK1-/-</td>
<td>AMPA</td>
<td>D,L-AP5, Nifedipine</td>
<td>p-ERK levels unchanged</td>
</tr>
<tr>
<td>GluR2-/- PAK1-/-</td>
<td>AMPA</td>
<td>D,L-AP5, Nifedipine</td>
<td>p-ERK levels unchanged</td>
</tr>
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Table 8. Summary of biochemical slice treatment studies with AMPA in wild-type, GluR2-/-, PAK1-/- and GluR2-/-PAK1-/- mice.
E. Summary of major results

1) CP-AMPARs are self-sufficient for the induction of multiple forms of E-LTP in the hippocampal CA1 region of GluR2 knockout mice despite the presence of D,L-AP5 (NMDAR antagonist). This form of plasticity is entirely dependent on calcium influx from CP-AMPARs as it is sensitive to the antagonist IEM-1460. The combination of D,L-AP5 and Staurosporine/KN-62 (CaMKII inhibitors) did not block CP-AMPAR-dependent E-LTP in GluR2 knockout mice, and the results were not significantly different from slices where D,L-AP5 was administrated alone (p-value > 0.1). This indicates that CaMKII does not play a significant role in this form of plasticity.

2) CP-AMPAR-dependent L-LTP was also inducible through CP-AMPARs in GluR2 mutant mice, a form of potentiation that is dependent on new protein synthesis, as in the case of classical NMDAR-dependent forms of plasticity.

3) The administration of PD 98059 and the structurally unrelated inhibitor U0126 (ERK/MAPK cascade inhibitors) blocked CP-AMPAR-induced E-LTP and L-LTP in GluR2 knockout and heterozygous mice. This implicates the involvement of the ERK/MAPK signalling cascade in plasticity evoked through CP-AMPARs.
4) CP-AMPAR-dependent L-LTP induced in GluR2-/-PAK1-/- and GluR2+/-PAK1-/- double knockout mice was significantly reduced in comparison to GluR2-/- and GluR2 +/- mice respectively. Taken in conjunction with L-LTP studies in PAK1-/- mice utilizing the ERK/MAPK inhibitor, this suggests that PAK1 plays a role in the activation of the ERK/MAPK cascade during plasticity.

5) Hippocampal slice treatment studies with AMPA in GluR2PAK1 double knockout mice reveals that p-ERK/MAPK is not significantly increased in these animals, in contrast to GluR2-/- mutants. This would suggest that calcium influx through CP-AMPARs leads to the activation of the ERK/MAPK signalling cascade in a PAK1-dependent manner.
F. Discussion

F.1 Importance of CP-AMPARs

The AMPA subtype glutamate receptors are the principal mediators of the fast excitatory synaptic transmission in the mammalian CNS, and are crucial for the expression of various forms of long lasting synaptic plasticity, including NMDAR-induced LTP (Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003). AMPARs are heteromeric complexes assembled from four distinct subunits (GluR1-4), of which GluR2 is of particular interest as it dictates a number of important biophysical and biochemical properties (Hestrin, 1993; Jonas et al., 1994; Bowie and Mayer, 1995; Geiger et al., 1995). In particular, AMPARs that lack edited GluR2 are Ca\(^{2+}\) permeable with higher conductance and inwardly rectifying I/V relationships.

While these GluR2-deficient CP-AMPARs are a minority in comparison to GluR2-containing AMPARs, they are widely expressed in the CNS (including aspiny neurons such as interneurons, stellate and glial cells) where they can facilitate synaptic transmission and changes in synaptic efficacy (Isaac et al., 2007) as well as induce different forms of synaptic plasticity, including NMDAR-independent LTP (Gu et al., 1996; Mahanty and Sah, 1998; Rozov and Burnashev, 1999; Liu and Cull-Candy, 2000; Kullmann and Lamsa, 2007; Liu and Zukin, 2007). Subunit composition switching from GluR2-lacking to GluR2-containing AMPARs was demonstrated as essential to plasticity in cerebellar stellate cells (Liu and Cull-Candy, 2000) and the ventral tegmental area (Bellone and Lüscher, 2005). CP-AMPARs were also shown to mediate the induction of LTP at hippocampal neuron-glia synapses (Ge et al., 2006). At interneuron
synapses, CP-AMPARs are believed to play an important role in a unique form of anti-Hebbian LTP (Lamsa et al., 2007).

Mounting evidence from recent studies indicate that GluR2-lacking CP-AMPARs are also expressed in cortical and hippocampal pyramidal neurons (Isaac et al., 2007). At early developing hippocampal mossy fiber-pyramidal synapses, the loss of CP-AMPARs underlies a depolarization-induced form of LTD (Ho et al., 2007). Furthermore, mossy fiber-interneuron synapses were shown to demonstrate concomitant forms of LTD from either NMDARs or CP-AMPARs that were dependent on $\text{Ca}^{2+}$ influx (Lei and McBain, 2002), suggesting that both types of calcium permeable receptors could work in parallel to collectively contribute to synaptic plasticity in regions where they coexist. In particular relevance to the present study is the finding that GluR2-lacking CP-AMPARs are transiently recruited to CA1 synapses by LTP-inducing stimulations where they are involved in the consolidation of this NMDAR-dependent LTP (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008). The expression of GluR2-lacking CP-AMPARs and the resultant $\text{Ca}^{2+}$ influx are also associated with a number of pathophysiological states, including ischemia, epileptic seizures and drug addiction (Pellegrini-Giampietro et al., 1997; Bellone and Lüscher, 2006; Liu and Zukin, 2007; Mameli et al., 2007; Conrad et al., 2008).

Despite the significance of CP-AMPARs in synaptic regulation and pathology, the molecular processes activated by $\text{Ca}^{2+}$ influx through these receptors were unknown. This was mainly due to complications of identifying cellular systems that contained CP-AMPARs, isolating plasticity induced exclusively through these receptors, and the inability to use pharmacological inhibitors in past experimental models. In this study, I
utilized genetically altered mice lacking GluR2 (GluR2-/-) or having a reduced level of GluR2 (GluR2+/-) to present evidence that a distinct synaptic signalling underlies CP-AMPAR-dependent LTP. Using a combination of genetic, electrophysiological and biochemical techniques, I demonstrated that these receptors are capable of inducing several forms of long-lasting enhancements in synaptic strength that required the ERK/MAPK signalling cascade. Unexpectedly, CaMKII, a crucial regulator of NMDAR-dependent LTP, was not required for this type of plasticity. These results indicate that a distinct synaptic signalling process underlies CP-AMPAR-induced synaptic plasticity.

**F.2 Characterisation and versatility of plasticity induced through CP-AMPARs**

During the course of my study, I demonstrated that a number of induction paradigms were able to induce CP-AMPAR-dependent plasticity. These included TBS, which is often used to induce NMDAR-dependent LTP at resting membrane potentials, suggesting that CP-AMPAR-dependent synaptic plasticity may occur under physiological conditions. Moreover, I also presented the first ever demonstration of the ability of CP-AMPARs to induce and sustain a longer-lasting protein synthesis dependent form of L-LTP in the CA1 region of the hippocampus, previously thought to be a hallmark of NMDARs alone.

In collaboration with my fellow researcher Zikai Zhou, we demonstrated that CP-AMPAR-dependent LTP was completely blocked by postsynaptic inclusion of 30 mM BAPTA (Asrar et al., 2009b), indicating that calcium ions in the postsynaptic neurons are necessary. Consistent with this, past studies have revealed that GluR2-lacking AMPARs
exhibit significant Ca\textsuperscript{2+} permeability (Geiger et al., 1995; Jia et al., 1996) that is similar to that of NMDARs; hence, the Ca\textsuperscript{2+} influx from these receptors is likely to be sufficient for triggering Ca\textsuperscript{2+}-dependent events in the postsynaptic neurons. Ca\textsuperscript{2+} transients as a consequence of synaptically activated CP-AMPARs have also been demonstrated in the cortex and shown to amplify the transient amplitude when co-expressed with NMDARs, suggesting the existence of mechanisms that allow the proportional scaling of each receptor type independent of presynaptic miniature-release factors (Wang et al., 2002).

Since CP-AMPAR-dependent LTP was also inducible in the presence of high voltage activated calcium channel blockers (Jia et al., 1996), the most probable calcium source in this form of plasticity is the Ca\textsuperscript{2+} influx from CP-AMPARs. This was confirmed by my results showing that CP-AMPAR-dependent plasticity induced by either 2 trains or 4 trains of 100 Hz was completely blocked by the selective CP-AMPAR inhibitor IEM-1460 in GluR2-/− and GluR2+/− mutants respectively. In contrast to GluR2-/− animals, the same concentration of IEM-1460 (100 µM) had a minor but non-significant effect on the basal synaptic response in GluR2+/− slices, suggesting that CP-AMPARs may not play an important role in basal transmission at CA1 synapses, reinforcing the idea that Ca\textsuperscript{2+} influx from newly recruited forms of these receptors may aid the consolidation of previously induced plasticity in the region (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008; but see Gray et al., 2007; Adesnik and Nicoll, 2007). The presence of IEM-1460 significantly reduced but did not completely inhibit the synaptic response in GluR2-/− mutant slices, suggesting the possibility that basal transmission may be mediated by AMPAR functions that are not blocked by this drug. The activity-dependent properties of IEM-1460 at the concentration used may have also been insufficient to reach and
effectively block all the CP-AMPARs present in knockout animals. Moreover, the lower amounts of hippocampal CP-AMPARs in contrast to other AMPAR subtypes in wild-type animals may reflect their role in LTP-specific related tasks rather than in the maintenance of continual basal synaptic transmission.

**F.3 Signalling mechanisms**

**F.3.1. Lack of CaMKII involvement in CP-AMPAR-dependent plasticity**

Considering the critical role for Ca\(^{2+}\) in CP-AMPAR-dependent LTP, it is surprising to discover that CaMKII is not required for this form of E-LTP as the CaMKII-specific inhibitor KN-62 had no effect (Figure 9). Furthermore, the general kinase inhibitor staurosporine, which is also known to block the actions of PKC, PKA, and protein tyrosine kinases in addition to CaMKII (Rüegg and Burgess, 1989; Sanchez-Martinez et al., 2003), also had no inhibitory effects on CP-AMPAR-dependent E-LTP (Figure 7). The absence of effect by these two inhibitors is not due to the ineffectiveness of the drugs because they strongly inhibited NMDAR-dependent E-LTP in slices prepared from wild-type littermate animals (Figure 6,8). Moreover, the enhanced E-LTP (consisting of both NMDAR- and CP-AMPAR-dependent components in the absence of D,L-AP5, Figure 10) seen in GluR2-/- mice (Jia et al., 1996) was also substantially, but not completely, attenuated in the presence of KN-62. This would suggest that the CaMKII inhibitor acted only towards blocking the NMDAR-dependent component of the enhanced E-LTP induced in GluR2-/- mice when D,L-AP5 was not present, while not altering the CP-AMPAR-dependent component, corresponding with my pharmacological inhibitor results in wild-type and mutant animals respectively. These results indicate that Ca\(^{2+}\) influx from
CP-AMPARs instigates a unique synaptic signalling process that is different from the activation of NMDARs. Additionally, calmodulin and CaMKII are associated with the NMDAR but not AMPAR complex (Strack and Colbran, 1998; Gardoni et al., 1998; Leonard et al., 1999), suggesting that the spatial distribution of calmodulin/CaMKII may play a role in activation.

F.3.2 Importance of ERK/MAPK signalling in CP-AMPAR-induced forms of plasticity

In contrast to CaMKII, my results provide evidence for the involvement of the ERK/MAPK signalling cascade (Figures 12, 13) in CP-AMPAR-dependent plasticity. This was uncovered through the utilization of two separate pairs of structurally unrelated inhibitors geared towards this kinase system (PD98059 and U0126 respectively). The varying degrees of attenuation of CP-AMPAR-dependent E-LTP by the above-mentioned drugs was likely due to differences in concentration and the modalities of action of these particular inhibitors. Interestingly, CP-AMPAR dependent L-LTP was not completely inhibited by the same concentration of PD98059 (Figure 23) that abolished CP-AMPAR dependent E-LTP in knockout slices (Figure 12). This may be explained by the possibility that the stronger stimulation protocols utilized for L-LTP need a longer period of time for plasticity to completely decay, thus necessitating an extended period of post-inductive recording. Furthermore, mechanistic differences between E-LTP and L-LTP have been previously described (Lynch, 2004), suggesting that the induction of the latter form of plasticity may enlist additional biochemical pathways that are unique and insensitive to ERK/MAPK inhibitors. These notions are supported by the fact that L-LTP
induced in wild-type slices was also not completely blocked by the same concentration of PD98059 in a similar time frame (Figure 21), in comparison to the total abolishment of E-LTP induced by 2 trains of 100 Hz in the same animals (Figure 11).

The association of the ERK/MAPK pathway with this form of plasticity is of particular interest as it has also been shown to be important for NMDAR-dependent LTP (English and Sweatt, 1997). I would therefore hypothesize that this signalling pathway may serve as a common target for both NMDAR- and CP-AMPAR-dependent forms of LTP. Given that CP-AMPAR-dependent LTP is independent of CaMKII, my thesis work would suggest a model where CP-AMPARs may act downstream of NMDARs and CaMKII (see Diagram 6). While my results do not specifically address the role of CP-AMPARs in NMDAR-dependent forms of plasticity, they are consistent with the idea that the CP-AMPAR-activated signalling process may serve as a mechanism for the consolidation of NMDAR-dependent LTP (Plant et al., 2006). In support of this theory, a recent study implicated an alternate member of the CaMK family (CaMKI) in the specific synaptic recruitment of CP-AMPARs during TBS-LTP in the CA1 region (Guire et al., 2008). Moreover, the mutual dependence of both NMDAR- and CP-AMPAR-induced types of L-LTP on new protein synthesis may suggest that $Ca^{2+}$ influx through newly recruited GluR2-lacking receptors could also be an important facilitator of traditional longer-lasting forms of plasticity in the hippocampus.
Diagram 6. Postulated role of CaMKII in the transient insertion of CP-AMPARs. CaMKII is well-known as a crucial factor in the induction of NMDAR-dependent plasticity. In light of recent studies which suggest a role for CP-AMPARs in NMDAR-dependent forms of LTP, my electrophysiological results suggest that CaMKII may act upstream of CP-AMPARs, leading to the rapid insertion of these receptors at the level of the postsynaptic membrane following titanic stimulation of NMDARs. The newly inserted CP-AMPARs may then aid in the consolidation of plasticity through activation of the ERK/MPK signalling cascade.
An alternate interpretation of my results concerning the role of CaMKII in the above scenario is that CP-AMPARs may be transiently inserted at the level of the synapse through CaMKII-independent mechanisms, and CaMKII may actually play a role in the subsequent removal of CP-AMPARs from the synapse and/or insertion of calcium impermeable (GluR2-containing) AMPARs in the facilitation of NMDAR-induced plasticity. These questions can be specifically addressed through the use of electrophysiological studies investigating the trafficking of GluR2-lacking and GluR2-containing receptors prior to and following the induction of plasticity (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008; Gray et al., 2007; Adesnik and Nicoll, 2007) in the presence of CaMKII inhibitors.

To further emphasize the association of CP-AMPARs with the ERK/MAPK signalling cascade, I performed acute hippocampal slice treatment studies which are commonly implemented for obtaining a chemical form of LTP as well as other forms of plasticity. To rule out any possible involvement of other sources of calcium other than CP-AMPARs, all treatment experiments were performed in the presence of the NMDA antagonist D,L-AP5 and the L-type calcium channel antagonist Nifedipine. Following treatment with AMPA, protein homogenate samples from both wild-type and GluR2 mutant mice underwent western blotting for the analysis of activated (or phosphorylated) ERK/MAPK protein levels. Slice samples obtained from wild-type mice revealed no significant increase in the level of p-ERK/MAPK, suggesting that chemical stimulation by AMPA alone does not activate the signalling pathway in these animals. This corresponds with my electrophysiological data, where, despite having a greater number of CP-AMPARs than wild-type mice, GluR2 heterozygous (GluR2+/-) mutants did not
reveal a significant alteration of the basal synaptic response in the presence of the antagonist IEM-1460. These results provide additional evidence that CP-AMPARs are not present significantly at the level of the synapse or do not contribute to synaptic transmission under basal conditions, and that their recruitment by the stimulation of NMDARs is a critical factor in the implementation of their activity in NMDAR-induced forms of plasticity seen in pyramidal neurons that dominate the CA1 region of the hippocampus. In contrast, in GluR2 knockout mice (GluR2-/-), where all the AMPARs (including those at the synapse) present are calcium permeable, we see a significant increase in the level of p-ERK/MAPK. These results complement my electrophysiological studies by revealing that chemical stimulation of synaptic CP-AMPARs leads to the activation of ERK/MAPK signalling, a well-known and critical element to numerous forms of synaptic plasticity. To confirm this increase was induced solely through CP-AMPARs, the administration of IEM-1460 completely inhibited ERK/MAPK activity in response to AMPA during slice treatment studies, similar to the inhibition of plasticity in the presence of this drug in GluR2 knockout mice. Taken together, these results reveal the critical dependence of the activity of CP-AMPARs on the ERK/MAPK signalling cascade, a factor which is probably essential for their role in synaptic plasticity at cells where they already predominate at the level of the synapse (such as interneurons), or are later recruited to due to the action of NMDARs or other receptors (such as pyramidal neurons).
F.3.3 The involvement of PAK1 cascade in CP-AMPAR-dependent plasticity

My results revealing the importance of ERK/MAPK signalling for the fulfillment of CP-AMPAR activity led to additional questions, the most pertinent of which was what factors may play a role in the activation of this cascade. One factor that has been implicated in facilitating ERK/MAPK activity during NMDAR-dependent plasticity is CaMKII. Since my electrophysiological results suggest that CaMKII and other members of the CaMK family may play a role in the synaptic recruitment of CP-AMPARs rather than a direct involvement in signalling activation, I ruled out this possibility. As mentioned previously, one crucial component of the ERK/MAPK signalling cascade is Ras. Utilizing an inhibitor of Ras/Rho (FTase 1), I confirmed previous studies that implicated this factor in NMDAR-dependent forms of plasticity ($P < 0.05$; see appendix Figure 44). I extended these studies to reveal that Ras was also involved in CP-AMPAR-dependent L-LTP, where FTase 1 reduced plasticity to a statistically similar level to the MEK inhibitor PD98059 (see appendix Figure 44), providing further verification of the involvement of the ERK/MAPK signalling in this unique form of plasticity. Since there was direct evidence to implicate the Ras-Raf-MEK-ERK/MAPK cascade in this form of plasticity, I decided to look at other known signalling factors that are associated with this kinase network. One such factor is the Rho-GTPase associated protein kinase PAK1. This serine/threonine kinase family is a key effector of the small Rho-GTPases of the Rac1 and Cdc42 family. PAK1 has been shown to provide the means by which Rho GTPases can activate the ERK/MAPK cascade in non-neuronal systems. PAK1 can “prime”
MEK1 by phosphorylating it at Ser298, preparing it for its interaction with Raf-1 (Frost et al., 1997).

Additionally, it can directly phosphorylate Raf-1 (Coles and Shaw, 2002), providing a supplementary augmentation of the ERK/MAPK pathway. Despite the strong signalling evidence linking PAK1 with the ERK/MAPK cascade, very little is known in regards to the participation of PAK1 in neuronal synaptic plasticity. In this regard, I performed L-LTP electrophysiological studies in PAK1-/- knockout mice. These mutants revealed a largely diminished form of plasticity in comparison to wild-type controls, providing the first evidence associating PAK1 with NMDAR-dependent forms of L-LTP. I demonstrated that this diminution was specifically related to plasticity, as both basal synaptic strength and presynaptic function were unchanged in these mutant animals. It is also unlikely that this reduction in L-LTP was due to developmental defects in the mutants, as both synapses and dendritic spines were normal in the knockout mice (Asrar et al., 2009a). This form of plasticity was also not further susceptible to the presence of the MEK inhibitor PD98059, indicating that the deficient L-LTP seen in PAK1 mice might be due to the lack of PAK1-dependent activation of the ERK/MAPK signalling pathway. One interpretation of the above results in PD98059 L-LTP treatment studies was that this inhibitor was ineffective in knockout animals in general. To rule out this possibility, I tested this inhibitor in ROCK2 mutant mice. ROCK2 is another type of Rho GTPase-associated protein kinase whose mutants also display a reduced level of NMDAR-dependent L-LTP in comparison to wild-type animals. However, unlike PAK1, ROCK2 has no known association with the ERK/MAPK signalling cascade. In the presence of PD98059, the already deficient L-LTP seen in ROCK2 mutants was further attenuated,
strongly indicating that the lack of effectiveness on L-LTP seen in PAK1 knockouts was not due to the inability of the drug in blocking the ERK/MAPK pathway (see Figure 40). To test whether the PAK1-ERK/MAPK interaction extended to CP-AMPAR-dependent forms of plasticity, I performed additional experiments with GluR2PAK1 double knockout mice. In a similar manner to NMDAR-dependent forms of plasticity, both GluR2-/PAK1-/- and GluR2+/PAK1-/- mice displayed significantly attenuated CP-AMPAR-dependent L-LTP in comparison to GluR2-/ and GluR2+/ mutants respectively. The diminished L-LTP seen in GluR2+/PAK1 mutants was statistically similar to the amount of plasticity seen in GluR2+/ mice treated with PD98059. These results provided further evidence of the importance of PAK1 in the possible activation of ERK/MAPK during multiple forms of potentiation.

Figure 40. Comparison of PD98059 treatment during L-LTP recordings in wild-type, PAK1-/- and ROCK2-/- mutant mice (A) The administration of the MEK inhibitor PD98059 significantly lowered L-LTP in wild-type and ROCK2-/- slices, but did not have any effect on plasticity induced in PAK1-/- mice. Error bars represent SEM.
Figure 40.
To further emphasize the possible interaction of PAK1 with the ERK/MAPK cascade through CP-AMPARs, I performed additional slice treatment studies. Experiments in PAK1-/- mice revealed no significant increase in the level of p-ERK/MAPK in response to AMPA treatment in the presence of both D,L-AP5 and Nifedipine, in a similar manner to wild-type animals. This would correspond with previous electrophysiological and treatment studies that indicate that the basal level of synaptic CP-AMPARs is not sufficient to activate signalling. In contrast to GluR2/- animals, GluR2PAK1 double knockout mice also revealed no significant changes in p-ERK/MAPK levels following administration of AMPA. Therefore, despite the presence of CP-AMPARs at the synapse in these mutants, the ERK/MAPK signalling pathway was not sufficiently activated when PAK1 was also absent in these knockout mice. In conjunction with electrophysiological studies, these results strongly suggest that PAK1 has an important role in the activation of the ERK/MAPK signalling cascade through CP-AMPARs.

**F.3.4 Role of receptor trafficking in CP-AMPAR-induced forms of plasticity**

My results are consistent with the hypothesis that the expression mechanisms of CP-AMPAR-dependent LTP are postsynaptic. This is based on the findings that PPF does not change after both E-LTP or L-LTP induction (Figure 3,18). Additionally, studies performed by my collaborator Zikai Zhou demonstrated that postsynaptic injection of exocytosis-inhibiting tetanus toxin (75 nM) largely blocks this form of LTP (Asrar et al., 2009b). The requirement of AMPAR insertion for CP-AMPAR-dependent LTP is also
consistent with the postulation that CP-AMPAR-induced LTP is downstream of NMDAR-dependent LTP. This mechanism has been suggested to help consolidate synaptic enhancement, as newly inserted GluR2-lacking CP-AMPARs are gradually replaced by GluR2-containing receptors (Plant et al., 2006; Lu et al., 2007; Guire et al., 2008), a process that is likely dependent on exocytosis.

In summary, I have identified a distinct signalling pathway underlying the long-lasting synaptic enhancement initiated by Ca\(^{2+}\) influx through CP-AMPARs at synapses in the hippocampal CA1 region. My findings are consistent with the recent notions that CP-AMPARs are essential to classical NMDAR-dependent forms of LTP by demonstrating for the first time that plasticity induced through the former receptors can last for extended periods and depend on new protein synthesis in a manner similar to the latter receptors. My results also demonstrate that these two forms of LTP share the downstream ERK/MAPK cascade in their signalling, suggesting that CP-AMPARs may play an important role in the post-inductory phase of NMDAR-dependent forms of potentiation. This CP-AMPAR-dependent synaptic process may also represent a vital and general mechanism that underlies signalling at synapses in circumstances where the expression of GluR2 is dynamically regulated, including during development, and pathological conditions.
G. Future Directions

G.1 Further research on CP-AMPARs

My present study has revealed the involvement of the ERK/MAPK signalling cascade in CP-AMPAR-dependent forms of both E-LTP and L-LTP. Since the activity of CaMKII has been ruled out in this form of plasticity, an intriguing question still remains on what alternate factors may activate the ERK/MAPK pathway in response to calcium influx under these circumstances. In this regard, I have conducted some preliminary studies looking at the ERK-/MAPK cascade-related factors Ras and PI3K (Phosphatidylinositol-3-kinase).

G.1.1 Ras

The most commonly studied GTPases are those related to the Ras family. Ras is a small guanine nucleotide-binding protein that has been implicated in calcium signalling cascades for the activation of downstream effectors such as PI3K and ERK. Several mechanisms of Ras activation (Lynch, 2004) by increased calcium levels have been demonstrated:

a) RasGRF activation by calmodulin (Farnsworth et al., 1995).
b) Activation of adenylyl cyclase by calmodulin followed by cAMP activation of CNrasGEF (Pham et al., 2000).
c) Transactivation of EGF receptor-like molecule by calcium (Zwick et al., 1997).
d) Direct activation of Ras by nitric oxide produced by calmodulin-activated NO synthase (Yun et al., 1998)
Utilizing an inhibitor of Ras (FTase inhibitor 1, 200 nM), my preliminary studies found that both NMDAR-dependent and CP-AMPAR-induced L-LTP was significantly attenuated (see appendix Figures 44, 45; p-value < 0.05) in wild-type and GluR2 mutant slices respectively. Therefore, considering the importance of calcium influx and the ERK/MAPK signalling cascade respectively in CP-AMPAR-induced LTP, and the well-documented relation of each of these factors with Ras (Lynch, 2004), the investigation of the latter factor would be of significant interest in this form of plasticity.

**G.1.2 PI3K (Phosphatidylinositol-3-kinase)**

Another downstream factor that has figured prominently in recent years with regard to studies in synaptic plasticity is PI3K (Phosphatidylinositol-3-kinase). The PI3K inhibitor wortmannin successfully abolished LTP induced in the CA1 region (Sanna et al., 2002) and dentate gyrus (Kelly and Lynch, 2000) respectively. Further studies conducted in rats established the importance of PI3K in the consolidation and acquisition of memory (Barros et al., 2001). The activation of the ERK/MAPK cascade was also shown to be mediated by PI3K through calcium influx from both NMDARs (Perkinton et al., 2002) and AMPARs (Perkinton et al., 1999) respectively. The use of the PI3K inhibitor LY294002 was also shown to inhibit the activation of PAK by Ras and PI3K (Tang et al., 1999). PI3K was also implicated in the induction of LTP in the hippocampal CA1 region through mechanisms independent of the ERK pathway (Opazo et al., 2003). My preliminary electrophysiological studies involving this factor revealed that the presence of the PI3K inhibitor LY294002 (20 μM) completely blocked both NMDAR-dependent
LTP (see Appendix Figure 41) in the wild-type animals (vehicle treated = 163 ± 7%; LY294002 = 110 ± 5.2%; $P < 0.001$) and CP-AMPAR-dependent LTP (see Appendix Figure 42) in GluR2 -/- mice (D,L-AP5 + vehicle = 150 ± 4.4; D,L-AP5 + LY294002 = 107 ± 4.6; $P < 0.001$). Accordingly, administration of wortmannin (1 µM), another PI3K inhibitor that is structurally unrelated to LY294002, also attenuated CP-AMPAR dependent LTP in knockout slices (D,L-AP5 + vehicle = 150 ± 4.4; D,L-AP5 + wortmannin = 117 ± 3.5; $P < 0.001$; see Appendix Figure 43). Hence, further research on the involvement of PI3K in CP-AMPAR-induced plasticity through either ERK-dependent or ERK-independent pathways may be of considerable interest.

**G.2 The Big Picture**

Research in the field of synaptic plasticity in the CA1 region of the hippocampus has played a vital role in our understanding of the fundamental mechanisms involved in learning and memory. A large majority of these studies have concentrated on plasticity occurring as a result of calcium influx through NMDARs. In addition to this, studies have also revealed the presence of calcium permeable (or GluR2-lacking) AMPARs in both pyramidal neurons and interneurons in the hippocampus. Investigations have demonstrated them to play a critical role in both physiological and pathophysiological processes in the central nervous system (CNS). Adding to the possibility that CP-AMPARs may induce a unique form of plasticity in their own regard, recent studies also implicated these receptors in playing a critical role in the classical NMDAR-dependent form of LTP. The development of GluR2 (the AMPAR subunit that prevents calcium influx through these receptors) knockout mice has allowed for a viable model from which
an in-depth study of CP-AMPARs can be conducted. My studies have revealed that these receptors have the ability to induce various forms of plasticity through unique signalling pathways. However, a major question that still remains is whether plasticity induced through CP-AMPARs has an increased relevance in relation to particular types of behavior and memory tasks in comparison to other forms of potentiation. Considering the growth and behavioral deficits seen in GluR2-/- mice, the GluR2+/-(heterozygous) mice could provide a more appropriate environment for these types of investigations. Conditional and hippocampal-specific knockouts could also be created to completely rule out the involvement of developmental compensation interfering with results. An experimental protocol would be needed where specific drugs/toxins that inhibit CP-AMPARs would be administered to animals prior to undergoing a particular task or test. The results of these experiments should prove very interesting, considering the dual nature of the involvement of CP-AMPARs in both NMDAR-dependent and NMDAR-independent forms of plasticity at pyramidal neurons and interneurons respectively. The role of CP-AMPARs in regulating LTP at interneurons in the basolateral amygdala suggests a probable role for these receptors in fear memory (Mahanty and Sah, 1998). CP-AMPARs have also been implicated in a number of pathological conditions, including ischemia, epileptic seizures and drug addiction (Pellegrini-Giampietro et al., 1997; Bellone and Lüscher, 2006; Liu and Zukin, 2007; Mameli et al., 2007; Conrad et al., 2008). Therefore, the therapeutic interest in understanding the properties and mechanisms underlying these receptors is vast. Additionally, the notion that CP-AMPARs are crucial for the expression and regulation of NMDAR-dependent tasks greatly amplifies their importance as a therapeutical target. These functions may be
exemplified by the actions of the NMDAR-dependent antagonist Ketamine, which has a number of effects in humans, including analgesic, anesthetic, hallucinatory, increasing blood pressure, and bronchodilation. Despite their relatively low numbers in the CA1 region of the hippocampus, studies in LTP have revealed that the inhibition of CP-AMPARs completely blocked NMDAR-dependent plasticity. Therefore, CP-AMPARs may represent a viable alternative target in the suppression of NMDAR-induced processes.
H. Significance of the present study

Calcium signalling has been demonstrated to be a crucial factor in the occurrence of numerous physiological and pathophysiological functions. In relation to synaptic plasticity, it is critical for both the presynaptic release of the neurotransmitter glutamate as well as the instigation of postsynaptic kinase signalling that ultimately leads to the delivery of AMPARs to the synapse and LTP. CP-AMPARs are presently widely in the brain, and have been suggested to represent a vital source of Ca\(^{2+}\) influx during numerous fundamental processes in the CNS. Despite their relative scarcity in the hippocampal CA1 region, the inhibition of the activity of these receptors led to the complete blockage of classical NMDAR-induced forms of LTP. Since the depolarization of NMDARs already results in a large calcium influx in the postsynaptic region, the additional enlistment of CP-AMPARs to facilitate LTP would suggest the recruitment of additional signalling pathways by the latter receptors. My studies revealed that, unlike NMDARs, CP-AMPARs act through a distinct signalling mechanism that is devoid of the activity of CaMKII. This would suggest that calcium influx from different types of calcium permeable receptors does not necessarily act through similar dynamics, and may result in the fulfillment of different functions in working towards the same goal. Furthermore, I demonstrated the ability of synaptic CP-AMPARs to be self-sufficient (NMDAR-independent) in the induction of various forms of E-LTP. Therefore, the presence of these receptors in regions where the incidence of plasticity is largely unknown (such as CaMKII-lacking interneurons) may indicate the existence of unique forms of synaptic enhancements employed in distinctive memory formations specific to these areas. The dependence of CP-AMPAR-dependent plasticity on the ERK/MAPK
cascade greatly emphasizes the importance of this pathway in numerous forms of potentiation in the CNS.

Additionally, I also confirmed the capacity of these receptors to be involved in a rarely seen form of NMDAR-independent L-LTP. This would suggest that CP-AMPARs could potentially act towards the consummation of NMDAR-dependent forms of long-lasting potentiation in addition to their own distinct type of long-term plasticity in the brain.

Lastly, the signalling mechanisms which I have divulged may also serve towards the better understanding of the role of CP-AMPARs in pathological activities. Therefore, further studies in this area may facilitate pharmacological research in respect to ischemia, epileptic seizures and substance abuse.
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APPENDIX
Figure 41. LY294002 inhibits the induction of E-LTP in wild-type mice (A) The administration of an induction protocol of 2 trains of 100 Hz in the Schaffer collateral pathway of the hippocampal CA1 region of wild-type (GluR2 +/+ ) mice displayed a significant enhancement of the synaptic response (vehicle treated = 163 ± 7%; n = 6) following a brief baseline period. This form of potentiation was completely attenuated in the presence of the PI3K inhibitor LY294002 (LY294002 treated = 110 ± 5.2%; n = 5; \( P < 0.0001 \)). This illustrated the dependence of the induction of synaptic plasticity in the wild-type CA1 region on the activity of the PI3K. (B) Comparison of the last 10 minutes of plasticity between LY294002 treated and vehicle treated slices in wild-type animals. 20 μM LY294002 was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 41.

A

- R2 +/- (vehicle)
- R2 +/- (LY294002)

Normalized field EPSP (%)

Time (min)

B

- R2 +/- (vehicle)
- R2 +/- (LY294002)

* $P < 0.001$

Normalized means of last 10 mins of LTP (%)

R2 +/-  

R2 +/-
**Figure 42.** LY294002 completely inhibits CP-AMPAR-dependent E-LTP in GluR2 knockout mice

(A) In contrast to wild-type animals, the use of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-lacking (GluR2 -/- ) mice demonstrated significant E-LTP (D,L-AP5 + vehicle = 150 ± 4.4%; n = 5) despite the administration of the NMDAR antagonist D,L-AP5. This form of plasticity was completely susceptible to the administration of the PI3K inhibitor LY294002 (D,L-AP5 + LY294002 treated = 107 ± 4.6%; n = 5; $P < 0.001$). This would suggest that the PI3K signalling cascade plays a crucial role in synaptic plasticity induced by CP-AMPARs.

(B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both LY294002 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 20 µM LY294002 was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 42.

A

- □ R2 -/-(D,L-AP5 + vehicle)
- ○ R2 -/- (D,L-AP5 + LY294002)

B

- □ R2 -/- (D,L-AP5 + vehicle)
- ○ R2 -/- (D,L-AP5 + LY294002)

* $P < 0.001$
Figure 43. Wortmannin significantly inhibits CP-AMPAR-dependent E-LTP in GluR2 mutants (A) The usage of an induction protocol of 2 trains of 100 Hz in the CA1 region of the hippocampus of GluR2 knockout (GluR2 -/-) mice demonstrated a robust E-LTP (D,L-AP5 + vehicle = 150 ± 4.4%; n = 5) in spite of the administration of the NMDAR antagonist D,L-AP5. This type of potentiation was strongly attenuated in the presence of the PI3K inhibitor wortmannin (D,L-AP5 + wortmannin treated = 117 ± 3.5%; n = 5; $P < 0.001$). These results provide further evidence that the PI3K signalling cascade has an important role in the induction of synaptic plasticity through CP-AMPARs. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both wortmannin and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent E-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 1 µM wortmannin was added to the ACSF perfusate 15-20 minutes prior to E-LTP induction lasting until 5 minutes post-induction. Error bars represent SEM.
Figure 43.

A

- R2 -/- (D,L-AP5 + vehicle)
- R2 -/- (D,L-AP5 + Wortmannin)

B

- R2 -/- (D,L-AP5 + vehicle)
- R2 -/- (D,L-AP5 + Wortmannin)

* P < 0.001
Figure 44. The Ras/Rho inhibitor FTase 1 inhibits L-LTP in wild-type mice (A) The use of an induction protocol of 4 trains of 100 Hz in the Schaffer collateral pathway of the CA1 region of the hippocampus of wild-type (GluR2 +/+ ) mice revealed a long-lasting form of L-LTP (vehicle treated = 179 ± 6.3%; n = 5) following a brief baseline period. This form of potentiation was significantly inhibited by the administration of the Ras/Rho inhibitor FTase 1 (FTase 1 treated = 117 ± 9.7%; n = 5; P < 0.001). This demonstrated the importance of Ras/Rho signalling in hippocampal L-LTP induced in wild-type animals. (B) Comparison of the last 10 minutes of plasticity between FTase 1 treated and vehicle treated slices in wild-type animals. 200 nM FTase 1 was added to perfusate 15-20 minutes prior to L-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 44.

A

- • R2 +/+ (vehicle)
- ○ R2 +/+ (FTase 1)

Normalized field EPSP (%)

Time (min)

B

- Dark grey R2 +/+ (vehicle)
- Light grey R2 +/+ (FTase 1)

* $P < 0.001$

Normalized means of last 10 mins of LTP (%)
Figure 45. FTase 1 inhibits CP-AMPAR-dependent L-LTP induced in GluR2 mutant mice (A) Utilizing an induction protocol of 4 trains of 100 Hz in the CA1 region of the hippocampus of GluR2-less (GluR2 +/- ) mice demonstrated robust L-LTP (D,L-AP5 treated = 172 ± 11.5%; n = 4) despite the presence of the NMDAR antagonist D,L-AP5. This form of potentiation was strongly attenuated in the presence of the Ras/Rho inhibitor FTase 1 (D,L-AP5 + FTase 1 treated = 133 ± 8.2%; n = 6; P < 0.05). These results revealed the reliance of CP-AMPAR-dependent L-LTP on the Ras/Rho signalling cascade. (B) Comparison of the last 10 minutes of the length of the field EPSP slope of the synaptic response between both FTase 1 and D,L-AP5 treated slices versus D,L-AP5 alone treated slices. All field EPSP recordings of CP-AMPAR-dependent L-LTP in GluR2 mutants involved the addition of 100 µM D,L-AP5 to perfusate 15 minutes prior to induction, lasting until 5 minutes post-induction. 200 nM FTase 1 was added to perfusate 15-20 minutes prior to L-LTP induction and washed away 5 minutes post-induction. Error bars represent SEM.
Figure 45.

A

- R2 +/- (D,L-AP5 + vehicle)
- R2 +/- (D,L-AP5 + FTase 1)

Normalized field EPSP (%)

Time (min)

B

- R2 +/- (D,L-AP5 + vehicle)
- R2 +/- (D,L-AP5 + FTase 1)

Normalized means of last 10 mins of LTP (%)
LIST OF STUDENT PUBLICATIONS ASSOCIATED WITH THE PRESENT STUDY


Figures 1-24 and Figures 41-43 (as well as related text descriptions in the Introduction, Results and Discussion sections) are modified from results previously published in Asrar et al., 2009b in the journal PLoS ONE under the Creative Commons Attribution License (CCAL), which is accessible at http://creativecommons.org/licenses/by/2.5/