Regulation of GluA1 phosphorylation by the neuropeptide PACAP38

By

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Abstract

Dynamic changes in synaptic strength are thought to be critical for higher brain function such as learning and memory. Alterations in synaptic strength result from modulation of AMPA receptor function and trafficking to synaptic sites. The phosphorylation state of AMPA receptor subunits is one mechanism by which cells regulate receptor function and membrane trafficking. Receptor phosphorylation is in turn regulated by extracellular signals; these include neuronal activity and signaling though neuromodulators such as dopamine, norepinephrine as well as neuropeptides. Although numerous studies have reported that the neuropeptide PACAP38 alters CA1 synaptic strength and GluA1 synaptic localization, its effect on AMPA receptor phosphorylation state has not been explored. We determined that PACAP38-mediated stimulation of hippocampal cultures increased phosphorylation of S845, and decreased phosphorylation of T840 on the GluA1 AMPA receptor subunit. Increases in GluA1 S845 phosphorylation primarily occurred via PAC1 and VPAC2 receptor activation, whereas a reduction in GluA1 T840 phosphorylation was largely driven by PAC1 receptor activation and to a lesser extent by VPAC1 and VPAC2 receptor activation. GluA1 S845 phosphorylation could be blocked by a PKA inhibitor, and GluA1 T840 dephosphorylation could be blocked by a PP1/PP2A inhibitor and was partly blocked by a NMDA receptor antagonist. These results demonstrate that the neuropeptide PACAP38 inversely regulates the phosphorylation of two distinct sites on GluA1 and may play an important role modulating AMPA receptor function and synaptic plasticity in the brain.

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List of abbreviations

5-HT  serotonin
ACh  acetylcholine
ACSF  Artificial Cerebrospinal Fluid
ADAM10  a disintegrin and metalloproteinase domain containing protein 10
AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazolepropionionic acid
APV  (2R)-amino-5-phosphonovaleric acid
CAMKII  Ca2+/calmodulin-dependent protein kinases II
cAMP  cyclic adenosine monophosphate
CHO  Chinese hamster ovary
CNS  central nervous system
C-terminus  carboxyl-terminus
DIV  days in vitro
DOC  sodium deoxycholate
EPSC  excitatory postsynaptic potential
EPSP  excitatory postsynaptic potential
HEK293  human embryonic kidney 293 cells
KI  knock-in
KO  knockout
LTD  long term depression
LTP  long term potentiation
MAPK  mitogen-activated protein kinase
NE  norepinephrine
<table>
<thead>
<tr>
<th>Short Form</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino-terminus</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP protein kinase/protein kinase G</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PSD</td>
<td>post-synaptic density</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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Chapter 1. Introduction
The synapse

The brain consists of an intricate network of neurons and non-neuronal cells which come together to perform numerous tasks including processing incoming sensory information, sending out action commands, storing information, and learning. Within the brain, the neuron is the primary unit used to collect, process, and transmit information. These roles are reflected in the typical structure of a neuron which consists of a cell body, the dendrites, a region primarily responsible for receiving information from other neurons, and the axon, a region responsible for sending out information. The site of communication between the presynaptic and postsynaptic neuron is the synapse, and this is composed of a presynaptic axon terminals that lies in close opposition to the dendrite or soma of the postsynaptic cell. During synaptic transmission, an action potential is generated in the presynaptic neuron, travels down the axon, and causes neurotransmitter containing synaptic vesicles to fuse with the presynaptic membrane and release their contents into the synaptic cleft. These neurotransmitters can then bind to and activate their corresponding receptors on the postsynaptic cell.

Within the CNS, GABA and the ionotropic GABA_A receptor are the major mediators of fast, inhibitory neurotransmission, and inhibitory synapses are often formed on the cell body and dendrite (Sheng and Kim 2011). Due to the fact that the GABA_A receptor is permeable to Cl^- ions and its reversal potential is more negative than the action potential threshold, GABA_A receptor activation generally produces a hyperpolarizing current that opposes action potential generation. On the other hand, glutamate is the major excitatory neurotransmitter, and the AMPA receptor conducts the
majority of fast, excitatory synaptic transmission. Another important glutamate receptor is the NMDA receptor. Unlike the AMPA receptor which is activated solely by glutamate, NMDA receptor activation requires both glutamate binding and postsynaptic depolarization. Upon opening, \( \text{Na}^+ \), \( \text{K}^+ \), and \( \text{Ca}^{2+} \) ions can pass through the channel. In contrast, the AMPA receptor is just permeable to \( \text{Na}^+ \) and \( \text{K}^+ \) ions.

Excitatory synapses most often occur at a dendritic structure called the dendritic spine which is an actin rich dendritic protrusion consisting of a spine head and spine neck. As a result of this configuration, the dendritic spine acts to compartmentalize neuronal signals (Hayashi and Majewska 2005). A prominent subdomain of the dendritic spine is the postsynaptic density (PSD), a protein rich structure that is attached to the postsynaptic membrane and opposes the presynaptic site. The PSD is composed of receptors including AMPA receptors, NMDA receptors, and metabotropic glutamate receptors. It also contains cytoskeletal components, adhesions molecules, kinases, phosphatases, and other signaling molecules. PSD scaffold proteins include the MAGUK family of proteins, Shank, and Homer, and function to link together different PSD components (Sheng and Kim 2011). Altogether this diverse group of proteins come together to establish a postsynaptic signal transduction apparatus.

PSD and spine size can reflect receptor content such that there is a positive correlation between AMPA receptor immunoreactivity and PSD size (Takumi et al 1999, Nusser et al 1998) as well as number of functional AMPA receptors and spine volume (Matsuzaki et al 2001). Furthermore, activity dependent increases in spine size are associated with an increase in AMPA receptor mediated currents (Harvey and Svoboda
2007, Matsuzaki et al 2004). Spines can also vary in the type of receptors that they contain. For example, the silent synapse contains NMDA receptors but not AMPA receptors (Takumi et al 1999, Petralia et al 1999, Isaac et al 1995, Liao et al 1995). During processes such as long term potentiation silent synapses can be unsilenced through the recruitment of AMPA receptors (Isaac et al 1995, Liao et al 1995, Gomperts et al 1998, Liao et al 1999, Liao et al 2001). Altogether these examples demonstrate that dendritic spine are heterogeneous and can be dynamically regulated.

**The AMPA receptor**

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropimate (AMPA) receptors are tetrameric receptors composed of the GluA1, 2, 3, or 4 subunits. All subunits consist of an extracellular N-terminus, followed by four membrane domains, and finally an intracellular C-terminus. While the N-terminal and membrane domains are highly homologous across subunits, the C-terminal domain is variable and consequently contains a number of subunit specific posttranslational modifications and interaction sites. Phosphorylation sites found on the GluA1 C-terminus include S816, S818, S831, T840, and S845 and on the GluA2 C-terminus they include S880 and Y876 (Lu and Roche 2012). Additionally, the GluA1 C-terminus mediates binding to SAP97 and 4.1N, and the GluA2 C-terminus mediates binding to PICK1, GRIP1/2, NSF, AP-2, BRAG2 (Anggono and Huganir 2012).

Within the adult hippocampus, AMPA receptors primarily consist of GluA1/2 and GluA2/3 complexes (Wenthold et al 1996, Lu et al 2009). As a result of subunit specific

AMPA receptor recruitment and removal from synaptic sites is a complex process involving multiple trafficking steps. First, newly synthesized or recycling AMPA receptors are delivered to the cell surface via exocytosis. While AMPA receptor exocytosis at extrasynaptic sites has been observed, there is much debate as to whether or not AMPA receptors exocytosis also occurs at synaptic sites (Lin et al 2009, Makino and Malinow 2009, Yudowski et al 2007, Wang et al 2008, Kennedy et al 2010, Patterson et al 2010). From extrasynaptic sites, AMPA receptors can diffuse into and back out of synaptic sites (Bats et al 2007, Petrini et al 2009, Tardin et al 2003). Studies have shown that AMPA receptor mobility as well as synaptic retention is regulated by activity, calcium signaling, proteins interactions, and CaMKII (Bats et al 2007, Borgdorff et al 2002, Tardin et al 2003, Opazo et al 2010, Petrini et al 2009). Furthermore, during long
term potentiation, the majority of AMPA receptors are delivered to synaptic sites by lateral diffusion (Makino and Malinow 2009). Finally, surface AMPA receptors are removed from the cell surface by clathrin-mediated endocytosis and traffic to either the lysosome for degradation or the recycling endosome for redelivery to the cell surface (Huganir and Nicoll 2013, Shepard and Huganir 2007).

A number of proteins have been shown to regulate AMPA receptor trafficking and localization. PSD95 binding to the TARP Stargazin has been shown to promote AMPA receptor synaptic retention (Bats et al 2007). Conversely, mGluR and NMDA receptor dependent long term depression is dependent on the GluA2 binding partner BRAG2 (Scholz et al 2010). During LTD, GluA2 Y876 dephosphorylation stimulates BRAG2 activation of Arf6 (Scholz et al 2010). It has been proposed that Arf6 then recruits components of the clathrin coat ultimately bringing AP2 and GluA2 close and enabling the formation of AMPA receptor containing clathrin-coated pits (Scholz et al 2010). Altogether, these are just a few examples of how AMPA receptor trafficking is regulated. Regulation at any one of the AMPA receptor trafficking steps could alter surface and synaptic AMPA receptor levels.

**LTP and LTD**

Two well-studied processes during which synaptic connections are strengthened or weakened are NMDA receptor dependent long term potentiation (LTP) and long term depression (LTD) respectively. Long term potentiation was first described by Bliss, Lomo, and Gardner-Medwin who demonstrated that repetitive stimulation of dentate
granule cell excitatory synapses produced a long lasting potentiation of responses (Lomo
subsequent studies LTD was also shown to occur in the hippocampus (Dudek and Baer
1992, Mulkey and Malenka 1992). While LTP and LTD have been shown to occur in a
number of other brain regions, the hippocampus’s ordered circuitry, robustness of LTP
and LTD, and importance for learning on memory makes it a model structure to work on.
In particular, many studies have focused on hippocampal Schaffer collateral-CA1
synapses.

Within the hippocampus, LTP and LTD induction require NMDA receptor
activity, an increase in postsynaptic Ca\(^{2+}\), and postsynaptic depolarization (Collingridge
Bear 1992, Mulkey and Malenka 1992). Based on these finding and an understanding of
NMDA receptor activation, it believed that LTP and LTD occur as a result of
simultaneous glutamate release and postsynaptic depolarization which leads to NMDA
receptor activation and Ca\(^{2+}\) influx into the postsynaptic cell. This Ca\(^{2+}\) then goes on to
activate different signaling cascades required for LTP and LTD induction. Despite the
fact that both LTP and LTD are associated with an influx of Ca\(^{2+}\) ions, it thought that
differences in Ca\(^{2+}\) concentrations and temporal profile determine whether LTP or LTD
occur (Lee 2006).

During LTP, changes in synaptic strength could occur as a result of changes in
presynaptic glutamate release, postsynaptic AMPA receptor function, or postsynaptic
AMPA receptor levels. The most direct evidence for a postsynaptic component of
expression comes from glutamate uncaging experiments. In these experiments it was shown that repetitive glutamate uncaging results in spine enlargement and increased AMPA receptor mediated currents (Harvey and Svoboda 2007, Matsuzaki et al 2004). While this result shows that LTP stimulation alters AMPA receptor transduction at the postsynaptic site, it does not determine whether synaptic potentiation is due to changes in AMPA receptor trafficking or conductance. Both an increase in AMPA receptor unitary conductance (Benke et al 1998, Luthi et al 2004, Palmer et al 2004) and GluA1 delivery to spines (Shi et al 1999, Hayashi et al 2000, Kakegawa et al 2004, Makino and Malinow 2009, Shi et al 2001) are observed during LTP. However, the relative contribution of AMPA receptor conductance and trafficking to LTP is currently unclear. Just as LTP increases synaptic GluA1 levels, LTD also alters AMPA receptor trafficking. LTD stimulation is accompanied by increased AMPA receptor endocytosis (Ehlers et al 2000, Beattie et al 2000, Lee et al 2004), a decrease in surface, synaptic GluA1 (Carrol et al 1999), and decreased GluA1 in synaptoneurosomes (Heynen et al 2001). Altogether these studies demonstrate that LTP and LTD regulate AMPA receptor trafficking and function.

**AMPA receptor phosphorylation**

The postsynaptic cell can regulate synaptic strength through changes in AMPA receptor conductance, trafficking, and tethering at synaptic sites. Such changes can be achieved through alterations in AMPA receptor expression, binding partners, and posttranslational modifications (Lu and Roche 2012). A number of GluA1 and GluA2
phosphorylation sites have been shown to play a role in AMPAR trafficking and synaptic plasticity. On the GluA1 subunit, GluA1 S831 and S845 are two well studies sites which have been shown to be phosphorylated by distinct kinases and to differentially regulate AMPA receptor function and trafficking. While GluA1 S831 is phosphorylated by CaMKII and PKC (Roche et al 1996, Barria et al 1997, Mammen et al 1997), GluA1 S845 is phosphorylated by PKA and cGMP dependent protein kinase II (Roche et al 1996, Mammen et al 1997, Serulle et al 2007). In regards to channel function, GluA1 S831 phosphorylation increases single channel conductance (Derkach et al 1999, Kristensen et al 2011), whereas GluA1 S845 phosphorylation increases open channel probability (Banke et al 2000). Lastly, PKA activation has been shown to upregulate surface GluA1 levels in a GluA1 pS845 dependent mechanism, and NMDA induced GluA1 internalization is mediated by GluA1 S845 dephosphorylation (Man et al 2007, Lee et al 2003). Based on the finding that GluA1 S845 phosphorylation increases surface GluA1 levels but has no effect on synaptic transmission, it has been proposed that GluA1 S845 phosphorylation alter extrasynaptic but not synaptic GluA1 levels (Oh et al 2006, Gao et al 2006, Serulle et al 2007).

In addition to regulating channel function and trafficking, these phosphorylation sites have been implicated in synaptic plasticity. During LTP, LTD, and homeostatic scaling, GluA1 S831 and S845 phosphorylation changes are observed (Lee et al 2000, Barria et al 1997, Lee et al 1998, Lee et al 2000, Goel et al 2006, Deiring et al 2014). Given that LTP induces GluA1 S831 phosphorylation (Barria et al 1997, Lee et al 2000), CaMKII activity is necessary for LTP (Silva et al 1992, Malinow et al 1989), GluA1
S831 phosphorylation increases channel conductance (Derkach et al 1999, Kristensen et al 2011), and LTP is associated with an increase in AMPA receptor conductance (Benke et al 1998, Luthi et al 2004, Palmer et al 2004), it was proposed that LTP induced conductance changes were the result of GluA1 S831 phosphorylation. However LTP is intact in GluA1 S831A knock-in mouse (Lee et al 2010) indicating that it is not required for the expression of LTP. Interestingly, LTP is impaired in the GluA1 S845A, S831A double knock-in mouse (Lee et al 2003) but is intact in the GluA1 S845A knock-in mouse (Lee et al 2010). One possible explanation for this observation is that GluA1 S831, S845, and S818 phosphorylation compensate for one another. Alternatively, it is possible that GluA1 S831 and S845 phosphorylation alter the threshold for LTP induction. In support of this hypothesis, the threshold for LTP induction is lower in the GluA1 S831D, S845D knock-in mouse (Makino et al 2011), and norepinephrine facilitated LTP is impaired in the GluA1 S845A, S845A knock-in mouse (Hu et al 2007). This regulation of LTP threshold may be due to altered levels of extrasynaptic AMPA receptors which would be consistent with GluA1 S845 phosphorylation trafficking data. In summary, knock-in studies suggest that GluA1 S831 and S845 phosphorylation are not necessary for LTP, but may modulate LTP.

During LTD, GluA1 S831 phosphorylation is unchanged. In contrast, GluA1 S845 is dephosphorylated and this process is dependent on PP1/PP2A activity (Lee et al 2000, Lee et al 1998). Consistent with these findings, LTD is impaired in the GluA1 S845A knock-in mouse but intact in the GluA1 S831A knock-in animal (Lee et al 2010). Unfortunately, LTD is intact in the GluA1 knockout (Selcher et al 2012). Similar to the
LTP data, this result may be due to compensation, or it is possible that while GluA1 S845 is not necessary for LTP but modulates it.

Another GluA1 phosphorylation site is the GluA1 T840 site. In vitro and in vivo experiments have shown that GluA1 T840 phosphorylation is regulated by PKC and PP1/PP2A (Lee et al 2007, Gray et al 2014, Delgado et al 2007). While Gray et al (2014) demonstrated that CaMKII and p70S6 kinase could phosphorylation GluA1 T840 in vitro, another study did not observe CaMKII dependent GluA1 T840 phosphorylation (Lee et al 2007). The only evidence for a physiological regulation of GluA1 T840 phosphorylation comes from the finding that NMDA stimulation induces GluA1 T840 dephosphorylation which is dependent on PP1/PP2A activity (Gray et al 2014, Delgado et al 2007). In terms of functional importance, it is unknown how GluA1 T840 phosphorylation affects AMPA receptor trafficking. However, GluA1 T840 phosphorylation has been shown to enhance channel conductance (Jenkins et al 2014). Another role of GluA1 S840 phosphorylation may be to regulate GluA1 S845 phosphorylation. Due to the close proximity of the GluA1 T840 and the S845 it is possible that phosphorylation status of at one site regulates phosphorylation at the other. Indeed, in HEK-293 cells expressing a full length WT, GluA1 T840A or GluA1 T840D mutants, PKA induced GluA1 S845 phosphorylation was more dramatic with GluA1 T840A expression and reduced with GluA1 S845D expression (Gray et al 2014). Similarly, PKC dependent phosphorylation of GluA1 T840 site was impaired in GluA1 S845D C-tail mutants. Interestingly, CaMKII phosphorylation of GluA1 T840 was unaffected by the C-tail GluA1 S845D mutant. Further study is needed to determine if
and how crosstalk between GluA1 S831 and S845 phosphorylation regulate AMPA receptor phosphorylation, trafficking and synaptic plasticity.

**PACAP38 and the PACAP38 receptors**

In addition to glutamate and GABA, many other transmitters can be found within the brain. Transmitters that exert neuromodulatory effects on the neurons are termed neuromodulators and can regulate a wide range of neuronal properties including intrinsic membrane excitability, presynaptic vesicle release, the expression and function of postsynaptic receptors, and synaptic plasticity (Nadim and Butcher 2014, Sjostrom et al 2008). Additionally, depending on the identity of the neuromodulator, a particular neuromodulator may act on target neurons that are located near or far from their site of release, and may drive changes over short and long periods of time. The diverse actions of neuromodulators is reflected by this somewhat vague definition given by Katz (1999): neuromodulation is “any communication between neurons, caused by release of a chemical, that is either not fast, or not point-to-point, or not simply excitation or inhibition.” Transmitters that can exert neuromodulatory effects include small molecules such as acetylcholine, dopamine, norepinephrine (NE), serotonin (5-HT), and glutamate, or peptides such as opioid peptides, neuropeptide Y, and tachykinins. Synthesis, storage, and release mechanisms differ between small molecule transmitters and peptides. While the majority of small molecule transmitters are synthesized in nerve terminals, stored in synaptic vesicles, released at specialized nerve terminal structures called active zones, and act at nearby sites (Kandel et al 2000), neuropeptides are synthesized in the cell
body, stored in large dense-core vesicles, can be released from dendritic and axonal sites, and can act at targets that are nearby, intermediate distances, or far away (van den Pol 2012).

The diverse actions of neuromodulators is partly due to the fact that many neuromodulators act through G protein-coupled receptors. Unlike ionotropic receptors that simply allow ions to pass through them upon receptor activation, G protein-coupled receptor activation can turn on a number of different signaling cascades to generate diverse changes throughout the cell. Furthermore, depending on the receptor subtype, cellular localization, cell type, and brain region, the same neuromodulator can regulate a range of neuronal functions. For example, acetylcholine and dopamine have been shown to regulate voltage-gated Na⁺ channels in hippocampal pyramidal neurons (Cantrell et al 2001). In prefrontal cortex pyramidal neurons, serotonin has been reported to act through the 5-HT₂ receptor to regulate GABAₐ currents (Feng et al 2001) and to act through the 5-HT₁₅ receptor to regulate AMPA receptor currents (Cai et al 2002). Serotonin has also been shown to regulate intrinsic membrane properties of pyramidal and stellate cells in the entorhinal cortex (Ma et al 2007). Altogether neuromodulators can targets a range of cellular processes including Na⁺, K⁺, and Ca²⁺ currents, and receptor trafficking to regulate neuronal function at presynaptic and postsynaptic sites (Nadim and Bucher 2014, Sjostrom et al 2008, Lee and Kirkwood 2011). Just as LTP and LTD can regulate AMPA receptor phosphorylation and trafficking, so too can neuromodulators. For example, in nucleus accumbens cultures and neostriatal slices a D₁ dopamine receptor agonist was shown to increase GluA1 S845 phosphorylation (Snyder et al 2000, Chao et al 2002a),
and in nucleus accumbens, hippocampal, and prefrontal cortical cultures a D1 receptor agonist increased extrasynaptic surface GluA1 levels (Chao et al 2002b, Sun et al 2005, Gao et al 2006). Similarly NE stimulation of hippocampal slices has been shown to upregulate GluA1 S831 and S845 phosphorylation (Hu et al 2007) and extrasynaptic surface GluA1 levels (He et al 2011), and to lower the threshold for long term potentiation (Hu et al 2007). Likewise, in the visual cortex cholinergic and adrenergic neuromodulation has been shown to regulate plasticity in AMPA receptor phosphorylation dependent manner (Seol et al 2007).

While neuropeptides have been shown to regulate learning and memory (Borbely et al 2013), few studies have looked at how neuropeptides affect AMPA receptor trafficking and phosphorylation. One such neuropeptide is PACAP38. PACAP38 is a neuropeptide that has been shown to regulate hippocampal synaptic strength (Kondo et al 1997, Cirrana et al 2003, Roberto et al 2000, Roberto et al 2001), AMPA receptor mediated synaptic transmission (Costa et al 2009), and GluA1 trafficking (Gardoni et al 2012). To generate the mature form of PACAP38, the PACAP gene is transcribed and translated to generate a 176 amino acid precursor call prepro-PACAP (Hosoya et al 1992). Prepro-PACAP is then cleaved and modified to generate 3 different peptides: PACAP related protein, PACAP 27, and PACAP38 (Ozaki et al 1992). Within the CNS, PACAP38 is the predominant form (Dickson and Finlayson 2009) and its expression is highest in the hypothalamus (Arimura et al 1991, Ghatei et al 1993). Some other brain regions where PACAP38 expression is observed include the nucleus accumbens,

PACAP38 can bind to and activate 3 different G-protein coupled receptors, the PAC1, VPAC1, and VPAC2 receptors. In addition to recognizing PACAP38, some of these receptors are also activated by the neuropeptide VIP. While the PAC1 receptor shows a much greater affinity for PACAP27 and PACAP38 in comparison to VIP, the VPAC1 and VPAC2 receptor bind equally well to PACAP27, PACAP38, and VIP (Dickson and Finlayson 2009). Another layer of complexity arises from the existence of different PAC1, VPAC1, VPAC2 receptor splice variants which in some cases exhibit altered ligand affinity and downstream signaling. Whereas the VPAC1 and VPAC2 receptor variants are uncommon, there are numerous PAC1 receptor variants and some of these are highly expressed (Dickson and Finlayson 2009). The two major PAC1 receptor variants found within the cortex and hippocampus are Pac1 short and Pac1 hop variant (Zhou et al 2000, Spengler et al 1993).

Similar to the widespread distribution of PACAP38 expression, the PAC1, VPAC1, and VPAC2 receptors are found in many different brain regions. High levels of the PAC1 receptor are found in the olfactory bulb, cerebellum, hypothalamus, brainstem, and thalamus, hippocampus (Joo et al 2004, Shioda et al 1997, Hashimoto et al 1996). VPAC1 receptor expression was found to be high in the cerebral cortex, hippocampus, thalamus, deep cerebellar nuclei, hypothalamus, brainstem (Joo et al 2004, Usdin et al 1994). Lastly the VPAC2 receptor is abundant in the cerebral cortex, hippocampus, amygdalar region, cerebellum, hypothalamus, thalamus, brainstem (Joo et al 2004, Usdin
et al 1994, Sheward et al 1995, Lutz et al 1993). With regard to hippocampal distribution, many of these studies report conflicting results. While high levels of the PAC1 receptor (Shioda et al 1997) and VPAC2 receptor (Joo et al 2004) were seen in dentate gyrus granule cells and CA1-3 pyramidal cells, other studies saw low levels of the PAC1 and VPAC2 receptor in the hippocampus and expression in different cell types (Joo et al 2004, Hashimoto et al 1996, Usdin et al 1994). Data is similarly conflicting on VPAC1 receptor hippocampal expression (Usdin et al 1994, Joo et al 2004). Differences across experiments may be due to dissimilarities in experimental approach and protocols.

A variety of signaling cascades lie downstream of PACAP receptor activation. In general, the VPAC1, VPAC2, and PAC1 receptors are potent activators of adenylyl cyclase. Additionally, various PACAP receptors have been shown activate phospholipase D, phospholipase C, PKC, MAPK, RhoA GTPases, and tyrosine kinases, and to elevate intracellular Ca^{2+} levels (Dickson and Finlayson 2009, Vaudry et al 2009). The ability of the PACAP to stimulate different signaling cascades can vary depending on the cell type and the PACAP receptor being activated (Dickson and Finlayson 2009, Vaudry et al 2009). For example, in a CHO overexpression system, PLD activation by VPAC1, VPAC2 and PAC1 “hop” receptor was dependent on ARF activity whereas PLD activation by the PAC1 “null” receptor was independent of ARF activity (McCulloch et al 2000). In another study using CHO cells transfected with VPAC1 or VPAC2 receptor, VPAC1 receptor activation produced a more robust increase in intracellular calcium levels (Langer et al 2001). As already mentioned, the PACAP38 splice variants can show altered ligand affinity and downstream signaling. In the case of the PAC1-hip variant,
adenylyl cyclase activation is reduced and there is no PLC activation (Sprengler et al 1993). The Pac1 short and PAC1-hop form can both stimulate cAMP and inositol phosphate production (Spengler et al 1993). While the above studies demonstrate that the PACAP receptors can regulate many different signaling cascades, they have not systematically studied which cascades are activated by the PAC1, VPAC1, VPAC2 receptor and how this varies with cell type.

**PACAP38 regulates synaptic strength**

In the hippocampus, PACAP38 stimulation has been shown to alter synaptic strength (Kondo et al 1997, Cirrana et al 2003, Roberto et al 2000, Roberto et al 2001) and AMPA receptor mediated currents (Costa et al 2009). The effect of PACAP38 on synaptic transmission is dependent on the region of study and concentration of PACAP38. At the perforant path-dentate granule cell synapse in the dentate gyrus, PACAP38 (1 μM) enhanced synaptic transmission (Kondo et al 1997). In contrast, PACAP38 (1 μM) was shown to depressed synaptic transmission at the Schaffer collateral-CA1 synapse (Kondo et al 1997, Roberto et al 2000). Furthermore, at the Schaffer collateral-CA1 synapse, PACAP38 was shown to have a dose dependent effect on synaptic transmission such that low doses (0.05 nM) of PACAP38 enhanced synaptic transmission, high doses (1μM) depressed it, and intermediate doses (0.1-0.5 μM) first depressed then enhanced synaptic transmission (Roberto et al 2001). Similar results were reported by Costa et al (2009) who demonstrated that PACAP38 also had a dose dependent effect on AMPA receptor mediated currents.
Using different inhibitors, studies have investigated the involvement of the PACAP38 receptors and downstream signaling molecules in this process. One study found that the PAC1/VPAC2 receptor antagonist, PACAP 6-38, blocked the enhancement of AMPA receptor mediated currents but had no effect on PACAP38 mediated depression (Costa et al 2009). This same study also found that PACAP6-38 alone decreased synaptic strength suggesting that endogenous PACAP38 regulates synaptic transmission (Costa et al 2009). While this and other studies refer to PACAP6-38 as a PAC1 receptor antagonist, PACAP6-38 shows similar affinity for the PAC1 and VPAC2 receptor and similar inhibition of PAC1 of VPAC2 receptor mediated cAMP production (Gourlet et al 1995, Robberecht et al 1992, Moro et al 1999, Dickinson et al 1997). Since PACAP38 has been shown to enhance acetylcholine release and enhance NMDA receptor currents (Masuo et al 1993, Mcdonald et al 2005, Yaka et al 2003), studies have tested the importance of muscarinic acetylcholine (ACh) receptors and NMDA receptors for PACAP38 dependent changes in synaptic strength. While the PACAP38 dependent enhancement of CA1 synaptic transmission was blocked by a muscarinic ACh receptor antagonist and was partially blocked by the NMDA receptor antagonist (Roberto et al 2001, Roberto et al 2000), inhibition of these receptors had no effect on PACAP38 dependent depression (Roberto et al 2001, Kondo et al 1997). Other studies found that a PKA inhibitor blocked the PACAP38 dependent depression of synaptic transmission, the enhancement of AMPA receptor mediated currents, and the depression of AMPA receptor mediated currents (Ciranna et al 20003, Costa et al 2009). As this results conflicts with another study showing that PKA inhibitors did not affect
PACAP38 depression of synaptic transmission (Kondo et al 1997), further investigation is required. Lastly, PACAP38 dependent depression of synaptic transmission was unimpaired by a PKC and CaMKII inhibitors and inclusion of a Ca\(^{2+}\) chelator in the intracellular solution (Kondo et al 1997, Roberto et al 2001).

Similar to experiments conducted in the hippocampus, PACAP38 has been shown to regulated synaptic transmission in the amygdala (Cho et al 2012). At the basolateral nucleus-central nucleus (BLA-CeL) synapse, PACAP38 stimulation potentiated synaptic transmission, enhanced AMPA receptor EPSCs and the \(\text{EPSC}_{\text{AMPAR}}/\text{EPSC}_{\text{NMDAR}}\) ratio, but had no effect on NMDA receptor EPSCs. PACAP38 mediated potentiation was blocked by a VPAC1 receptor antagonist, a GluA1 C-terminal peptide that has previously been shown to block LTP, a PKA inhibitor, and a CamKII inhibitor. In future studies it would be interesting to establish the similarities and differences between PACAP38 regulation of synaptic transmission at the Schaffer collateral-CA1 synapse and the BLA-CeL synapse.

In addition to regulating synaptic transmission and AMPA receptor mediated currents, PACAP38 has been shown to regulate GluA1 synaptic localization. Gardoni et al (2012) found that stimulation of primary, hippocampal cultures (DIV10) with PACAP38 (300nM, 30 minutes) led to reduced GluA1/Shank co-localization, a decrease in spine width and percentage of mushroom spines, and an increase in the percentage of stubby and thin spines. Changes in GluA1 distribution was partially blocked by a PKA inhibitor and a VPAC1/VPAC2 antagonist, VIP(6-38) (Gardoni et al 2012). Given that PACAP38 induced changes in GluA1 localization and spine morphology were inhibited by a
disintegrin and metalloproteinase domain containing protein 10 (ADAM10) inhibitor, they proposed that ADAM10 played a role in this process (Gardoni et al 2012). Furthermore they found that morphology changes could be blocked by a N-Cadherin mutant which could not be cleaved by ADAM10. Since this study demonstrates that PACAP38 regulates AMPA receptor trafficking, it would be interesting to see if PACAP38 dependent changes in synaptic strength is the result of altered AMPA receptor trafficking.

Role of AMPA receptor phosphorylation and PACAP in learning

It has long been hypothesized that synaptic plasticity is a mechanism underlying learning and memory. Consistent with this theory, LTP and LTD occur in brain regions important for learning, and learning has been shown to alter synaptic strength and AMPA receptor trafficking (Huganir and Nicoll 2013, Kessels and Malinow 2009). In a study by Whitlock et al (2006), one-trial inhibitory avoidance training led to an enhancement of GluA1 and GluA2 in synaptoneurosomes and induced LTP in the hippocampus (Whitlock et al 2006). Similarly, recruitment of GFP-GluA1 to CA1 mushroom spines is observed after contextual fear conditioning (Matsuo et al 2008). Since AMPA receptor phosphorylation has been shown to regulate synaptic plasticity and AMPA receptor trafficking, studies have investigated the role of AMPA receptor phosphorylation in learning and memory. The GluA1 S831, S845A double knock-in mouse show impaired retention in the Morris Water Maze (Lee et al 2003). In another study, it was shown that mice exposed to a fearful stimuli displayed elevated GluA1 S831 and S845
phosphorylation in the hippocampus. Furthermore, this phosphorylation increase was blocked by an antagonist targeting the β-adrenergic receptor, the receptor which mediates norepinephrine (NE) signaling. To investigate previous reports that NE enhanced learning (Cahill et al 1994, McGaugh and Roozendaal 2002, Frankland et al 2004), mice were injected with epinephrine so as to elevate NE levels and then trained and tested with a hippocampal dependent learning assay, contextual fear conditioning. Consistent with a previous report, epinephrine enhanced contextual fear learning (Frankland et al 2004). However the NE mediated enhancement of contextual fear learning was absent in the GluA1 S831A, S845A knock-in mouse. Based on this finding, the authors concluded that GluA1 S831 and S845 phosphorylation play a role in the emotional regulation of learning. Although I have just focused on hippocampal dependent learning assays, GluA1, GluA1 interactions, and GluA1 phosphorylation has also been shown to be important for behaviors and other forms of learning that are independent of hippocampal function (Kessels and Malinow 2009, Clem et al 2010).

Few studies have looked at the ability of PACAP38 to regulate synaptic plasticity and learning. In the PAC1 receptor KO mouse, LTP at the perforant path-dentate granule cell synapse was reduced when induced with a threshold stimulus but unimpaired when induced with a suprathreshold stimulus (Matsuyama et al 2003). In passive avoidance learning, a form of learning dependent on hippocampal function, intracerebroventricular administration of PACAP38 after the acquisition trial improved memory retention (Sacchetti et al 2000, Telegdy and Kokavsky 2000). This improvement was dose dependent such that intermediate doses enhanced learning while high doses had no effect
(Sacchetti et al 2000). Similarly, PACAP knockout mice are impaired in contextual fear conditioning and novel object recognition (Takuma et al 2014), and PAC1 receptor knockouts exhibit impaired contextual fear conditioning (Otto et al 2001). Seeing as the PAC1 receptor KO exhibits normal learning in the Morris Water Maze, not all hippocampal learning is impaired in the PAC1 receptor knockout (Otto et al 2001, Sauvage et al 2000). In summary, learning and memory studies in knockout and knock-in mice demonstrate that AMPA receptor phosphorylation, PACAP38, and the PACAP receptors regulate some forms of learning and memory.
Chapters 2. Materials and Methods
Reagents and antibodies

Maxadillan, (Lys15, Arg16,Leu27)-VIP(1-7)-GRF(8-27) which is abbreviated as K,R,L-VIP-GRF, PACAP6-38, and PG97-269 were purchased from Bachem. PACAP38, Bay 55-9837, Go6983, d-APV, KT5823, H89, were purchased from Tocris. Okadaic acid was purchased from LC Laboratories and cyclosporine A was purchased from Sigma-Aldrich. Commercial antibodies GluA1 pT840 (Abcam, AB12108), GluA1 pS845 (Millipore, AB5849), GluA1 pS831 (Millipore, AB5847), GFP (Abcam, AB13970), were used. Antibodies against the GluA1 N terminus (JH4296, 4.9D), GluA2 N terminus (monoclonal, ascites), and GFP (JH4030) were generated in house.

Cell culture

Hippocampal cultures were prepared from E18 rat pups. Neurons were plated onto poly-L-lysine coated plates containing NM5: Neurobasal growth medium (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone), 2% B27 (Invitrogen), 50U/ml PenStrep (GIBCO), and 2mM Glutamax (GIBCO). Unless otherwise noted, one day after plating, this media was completely replaced with NM0: Neurobasal growth medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 50U/ml PenStrep (GIBCO), and 2mM Glutamax. Every 3-4 days thereafter half of the media was replaced with fresh NM0. In some instances, hippocampal cultures were maintained in glia conditioned Neurobasal medium. At DIV3-4 these cultures were treated with NM1 (Neurobasal growth medium supplemented with 1% fetal bovine serum, 2% B27, 50U/ml PenStrep, and 2mM Glutamax) containing 5µM uridine and 5µM (+)-5-flour-2’-deoxyuridine.
Thereafter, every 3-4 days half of the culture media was replaced with new glia conditioned NM1. Mouse cortical cultures were similarly maintained in NM1 and the glia conditioned NM1.

**Preparation of whole brain lysate**

Whole brains from WT or “penta” knock-in mice were lysed with NL buffer (1% SDS, 150 mM NaCl, 50 mM Tris pH 7.4, 2mM EGTA, 50 mM NaF, 10 mM NaPPi, PICA+B, 1µM okadaic acid). Samples were sonicated and incubated at 95°C for 5 minutes. The protein concentration of each sample was measured using the BCA Protein Assay (Thermo Scientific) and samples were diluted to equivalent concentrations. Cell lysate was diluted 10 fold with dilution buffer (Final concentration: 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.4, 2mM EGTA, 50 mM NaF, 10 mM NaPPi) and GluA1 was immunoprecipitated. Input and IP samples were visualized by western blot.

**PACAP38 stimulation and immunoprecipitation in hippocampal neurons**

At DIV14 hippocampal cells were stimulated with NM0 containing 1nM PACAP38 for 10 minutes, unless otherwise noted. Cells were then rinsed with ACSF and lysed with NL buffer (1% SDS, 150 mM NaCl, 50 mM Tris pH 7.4, 2mM EGTA, 50 mM NaF, 10 mM NaPPi, PICA+B, 1µM okadaic acid), incubated at 95°C for 5 minutes, sonicated, and spun down at 16,000 g for 10 minutes. Cell lysate was diluted 10 fold with dilution buffer (Final concentration: 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.4, 2mM EGTA, 50 mM NaF, 10 mM NaPPi) and incubated with a GluA1 antibody (JH4296) and
protein A sepharose overnight. The following day, beads were washed three times with
Wash buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.4, 2 mM
EGTA, 50 mM NaF, 10 mM NaPPi) and protein eluted with 2xSDS sample buffer.
Samples were separated on a 5% SDS-PAGE gel, transferred to a nitrocellulose
membrane, blocked with Odyssey Blocking Buffer, and incubated with antibody. Blots
were then washed, incubated with Alexa Fluor 680 and 800 secondary antibodies,
washed, and imaged using an Odyssey Imaging System. Data was analyzed using
Odyssey software.

Biotinylation
Hippocampal neurons were maintained in GCM. At DIV 17, cultures were stimulated
with 1 nM PACAP38 for 10 or 30 minutes. After stimulation, cells were rinsed with
chilled ACSF and incubated with 1 mg/ml Sulfo-NHS-biotin (Thermo Scientific) on ice
for 25 minutes. To quench the reaction, cells were washed two times with 50 mM
glycine/ACSF. Cells were then lysed with RIPA buffer (1% Triton X-100, 0.4% DOC,
0.1% SDS, 100 mM NaCl, 25 mM Tris pH 7.4, 50 mM NaF, 10 mM NaPPi, 2 mM
EDTA), solubilized for 1 hour, and spun down at 13,000 rpm for 10 minutes. Lysate was
removed and incubated with streptavidin agarose beads (Thermo Scientific) at 4°C
overnight. Beads were washed 3x with RIPA buffer and proteins eluted using 2x Load
dye. Samples were visualized by western blot.

DNA Constructs, plasmid transfection, and immunocytochemistry
Mutations were cloned into the GluA1 T840 site using SOE PCR. Mutations were introduced into a N-terminal, GFP tagged GluA1 construct. For immunocytochemistry experiments, hippocampal neurons were maintained in glia conditioned growth medium (GCM) and fed two times a week until the time of transfection. At DIV 15 cells were placed in Neurobasal medium supplemented with 0.5 mM glutamine and were transfected using Lipofectamine 2000. Following transfection, cells were then returned to GCM. On DIV 17 neurons were stimulated with 1 nM PACAP38 for 10 minutes. Neurons were then rinsed with chilled ACSF and live stained with a rabbit GFP antibody (JH4030) for 25 minutes at 10°C. Subsequently, excess antibody was rinsed off with ACSF, neurons were fixed with 4% folmaldehyde/4% sucrose/PBS, and permeabilized with 0.25% Triton-X 100/PBS. Cells were blocked with 10% BSA/PBS and then incubated with a chicken GFP antibody (Abcam, ab13970) overnight at 4°C. Following primary antibody incubation, cells were rinsed with PBS, incubated with Alexa conjugated secondary antibody, rinsed with PBS, and finally coverslips were mounted. Coverslips were imaged using a 63x oil-immersion objective on a Zeiss LSM510 confocal microscope. Images were analyzed using Metamorph (MDS Analytical Technologies, Inc.). For each experimental set, a threshold for GFP surface and total images was set based on the unstimulated WT GluA1 condition. The threshold was set such that diffuse staining was excluded and GFP punctae were visualized. This threshold was applied to all images within that experimental set. When quantifying an image, several dendritic segments per cell were selected. For each region, we then measured the surface integrated intensity and divided it by total integrated intensity.
Chapter 3. Results
3.1 Effect of PACAP38 on AMPA receptor phosphorylation

In the hippocampus, PACAP38 has been shown to regulate synaptic strength (Kondo et al. 1997, Roberto et al. 2000, Roberto et al. 2001, Ciranna et al. 2003, Costa et al. 2009), AMPA receptor mediated currents (Costa et al. 2009), and GluA1 synaptic localization (Gardoni et al. 2012). Additionally, AMPA receptor phosphorylation regulates receptor trafficking and function (Huganir and Nicoll 2013). Given the ability of PACAP38 to regulate AMPA receptors, we hypothesized that PACAP38 may also regulates AMPA receptor phosphorylation. To study the effect of PACAP38 on AMPAR phosphorylation, we stimulated mature (DIV 14), dissociated hippocampal cultures with a low and high dose of PACAP38. Following stimulation, cells were lysed and AMPA receptor phosphorylation was examined by western blot. PACAP38 stimulation resulted in elevated GluA1 S845 phosphorylation, reduced GluA1 T840 phosphorylation, and had no effect on GluA1 S831 phosphorylation (Figure 3.1-1).

Previous reports have validated the specificity of the GluA1 pS831, pS845, and pT840 antibodies (Diering et al. 2014, Delgado et al. 2007, Gray et al. 2014). Since the GluA1 pT840 antibody detected several bands, we confirmed the specificity of our antibody using the GluA1 “penta” knock-in mouse which harbors mutations at GluA1 S831A, T838A, S839A, T840A, and S845A (Lee et al. 2007). When WT whole brain lysate was probed with the GluA1 pT840 antibody, we observed a prominent band co-migrating with GluA1 (Figure 3.1-1). This band diminished to negligible levels in “penta” samples. Similarly, in GluA1 immunoprecipitation experiments, the GluA1 pT840 band was present in WT but not “penta” samples. Although several non-specific
bands were observed in the input, these bands were absent following GluA1 immunoprecipitation. Thus, subsequent experiments involving the GluA1 pT840 antibody were performed exclusively upon GluA1 immunoprecipitated complexes.

Due to the close proximity of the GluA1 T840 and S845 phosphorylation sites, the PACAP38 dependent increase in S845 phosphorylation may impair binding of the GluA1 pT840 antibody. Consequently, the decrease in GluA1 pT840 signal could be an artifact. To investigate this possibility, we preformed PACAP38 stimulation experiments in cortical cultures prepared from WT and GluA1 S845A knock-in mice. A PACAP38 dependent decrease in GluA1 T840 phosphorylation was observed in both WT and GluA1 S845A knock-in cultures (Figure 3.1-2) demonstrating that PACAP38 stimulation regulates both GluA1 S845 and T840 phosphorylation. Furthermore, this along with control experiments in the “penta” mouse demonstrates that PACAP38 stimulation induces an increase in GluA1 S845 phosphorylation and a decrease in GluA1 T840 phosphorylation.
Figure 3.1-1. Regulation of GluA1 phosphorylation by the neuropeptide PACAP38

(A) Hippocampal cultures (DIV 14) were stimulated with different concentrations (nM) of PACAP38 for 10 minutes. Stimulation was followed by cell lysis and western blot analysis. (B) GluA1 was immunoprecipitated from whole brain lysate prepared from WT and “penta” knock-in mice. Input and GluA1 IP samples were visualized by western blot.
Figure 3.1-2. PACAP38 induced GluA1 phosphorylation in cortical cultures generated from WT, GluA1 S845A, and penta knock-in mice.

Cultured cortical neurons were prepared from WT, GluA1 S845A, and penta knock-in animals. At DIV 17 cultures were stimulated with PACAP38 (1nM) for 10 minutes. Stimulation was followed by GluA1 immunoprecipitation and western blot.
3.2 Dose sensitivity and time course of PACAP38 dependent phosphorylation changes

To better understand how PACAP38 regulates AMPA receptor phosphorylation, we examined the dose sensitivity and time course of PACAP38 effects on GluA1 phosphorylation. For dose response experiments, hippocampal cultures were stimulated for 10 minutes with doses of PACAP 38 ranging from 0.005 nM to 100 nM (Figure 3.2-1). A 0.05 nM dose of PACAP38 significantly decreased GluA1 T840 phosphorylation, and this was maximally decreased upon 1 nM dose PACAP38 application. Similarly, a significant increase in GluA1 S845 phosphorylation was observed with a 0.05 nM dose of PACAP38, reaching a maximum at 0.5 nM. We next investigated the time course of PACAP38 dependent phosphorylation changes by stimulating hippocampal cultures with PACAP38 (1 nM) for different durations of time (Figure 3.2-2). Two minute stimulation with PACAP38 produced a significant reduction in GluA1 T840 phosphorylation and this was maximally reduced following 10 minute stimulation. At the S845 site, a significant increase was observed at the 2 minute time point, and a maximal increase was seen at the 30 minute time point. Taking into account the dose response and time course data, we thereafter performed PACAP38 stimulation experiments using a 1 nM dose of PACAP38 for 10 minutes.
Figure 3.2-1. Dose response of PACAP38 dependent changes.

(A) Hippocampal neurons (DIV 14) were stimulated with different concentrations (nM) of PACAP38 for 10 minutes. Stimulation was followed by GluA1 immunoprecipitation and western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, ANOVA, Tukey posttest. n≥6.
Figure 3.2-2. Time course of PACAP38 dependent phosphorylation changes.

(A) Hippocampal neurons (DIV 14) were stimulated for different durations of time with 1 nM PACAP38. Stimulation was followed by GluA1 immunoprecipitation and western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, ANOVA, Tukey posttest. n≥6.
3.3 Investigation of the PACAP38 receptors responsible for GluA1 phosphorylation changes

PACAP38 can bind to and activate three different GPCRs, the VPAC1, VPAC2, and PAC1 receptor. In order to test the ability of these receptors to induce AMPAR phosphorylation changes, we stimulated cultures with a VPAC1, VPAC2, or PAC1 receptor agonist (Figure 3.3-1). When cultures were stimulated with the VPAC1 receptor agonist, K,R,L-VIP-GRF, we observed a minor decrease in GluA1 T840 phosphorylation and a minor increase in GluA1 pS845 phosphorylation. Stimulation with the VPAC2 receptor agonist, Bay 55-9837, resulted in a moderate decrease in GluA1 T840 phosphorylation and a robust increase in GluA1 S845 phosphorylation (Figure 3.3-1). Since Bay 55-9837 can weakly activate the VPAC1 receptor (Tsutsumi et al 2002), we cannot rule out the possibility that some of this phosphorylation change is due to VPAC1 receptor activation. Lastly, application of the PAC1 receptor agonist, Maxadillan, most closely reproduced changes observed with PACAP38 stimulation, namely a strong decrease in GluA1 T840 phosphorylation and a strong increase in S845 phosphorylation (Figure 3.3-1).

We next wanted to see if PAC1, VPAC1, and VPAC2 receptor antagonists blocked PACAP38 stimulated AMPA receptor phosphorylation in a similar manner. In preliminary experiments, we found that a VPAC1 receptor antagonist, PG 97-269, had no noticeable effect on the PACAP38 dependent decrease in GluA1 T840 phosphorylation and increase in S845 phosphorylation (Figure 3.3-2). On the other hand, the PACAP38 dependent increase in S845 phosphorylation and decrease in T840 phosphorylation was
impaired by the PAC1/VPAC2 receptor antagonist, PACAP6-38 (Figure 3.3-2). PACAP6-38 does not completely block GluA1 phosphorylation changes, so we preformed stimulation experiments in the presence of both PACAP6-38 and PG 97-269. The PACAP38 induced increase in GluA1 S845 phosphorylation was not significantly different between the PACAP6-38 and the PACAP6-38+PG 96-269 condition. At the T840 site, this inhibitor cocktail had no significant effect on the PACAP38 dependent phosphorylation decrease. Since our sample size is small, we expect to see significance with more experiments. A drawback of the PACAP6-38 inhibitor is that we cannot determine the relative contribution of the PAC1 and VPAC2 receptor. Further investigation is complicated by the fact that there is no specific VPAC2 receptor antagonist. While M65 is a PAC1 receptor specific antagonist, the inability of M65 to block Maxadillan induced phosphorylation changes in control experiments currently prevents us from using M65. Nonetheless, our PACAP receptor agonist and antagonist experiments demonstrate that PACAP38 primarily acts through the PAC1 and VPAC2 receptors to stimulate an increase in GluA1 S845 phosphorylation and a decrease in GluA1 T840.
Figure 3.3-1. Regulation of GluA1 phosphorylation by PACAP receptor agonists.

(A) Hippocampal neurons (DIV14) were stimulated with the PAC1 receptor agonist (Maxadillan, 100 nM), the VPAC2 receptor agonist (Bay 55-987, 100 nM), or the VPAC1 receptor agonist (K,R,L-VIP-GRF, 1 μM) for 10 minutes. Stimulation was followed by GluA1 immunoprecipitation and western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluR1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, ANOVA, Tukey posttest. n≥6
Figure 3.3-2. Effect of PACAP receptor antagonists on GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated with combination of P6-38 (10µM) or PG97-269 (4µM) for 15 minutes, and then stimulated with PACAP38 (1 nM, 10 minutes). Cells were lysed, GluA1 immunoprecipitated, and samples visualized by western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluR1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, ANOVA, Tukey post test. n≥6
3.4 Identification of kinases or phosphatases responsible for PACAP38 induced GluA1 phosphorylation changes

PACAP38 could modulate phosphorylation at the GluA1 T840 or the S845 sites through the regulation of kinase or phosphatase activity. Since PACAP38 has been shown to increase PKA activity (Dickson and Finlayson 2009) and PKA can phosphorylate GluA1 at S845 (Roche et al 1996), we investigated the role of PKA in PACAP38 dependent phosphorylation changes. For these experiments we used the PKA inhibitor H89. In the presence of H89 alone, there was a trend towards reduced GluA1 S845 phosphorylation. Furthermore, H89 blocked the PACAP38 dependent increase in GluA1 S845 phosphorylation but had no effect on the PACAP38 dependent reduction in GluA1 T840 phosphorylation (Figure 3.4-1). Seeing as H89 has a moderate inhibitory effect on cGMP-dependent protein G (PKG) (Chijiwa et al 1990) and PKG can phosphorylate GluA1 S845 (Serulle et al 2007), we investigated ability of a PKG inhibitor, KT5823, to inhibit PACAP38 induced phosphorylation changes. In preliminary experiments, KT5823 did not impair the PACAP38 mediated increase in GluA1 S845 phosphorylation and decrease in GluA1 T840 phosphorylation (Figure 3.4-2). Activation of PKC has also been shown to regulate GluA1 T840 phosphorylation (Lee et al 2007, Gray et al 2014). It is possible the reduction in GluA1 T840 phosphorylation is caused by a down-regulation of PKC activity. Application of the PKC inhibitor, Go6983, resulted in a significant decrease in GluA1 T840 phosphorylation which is consistent with the finding that that GluA1 T840 is phosphorylated by PKC (Lee...
et al 2007, Gray et al 2014). Despite this basal effect, Go6983 did not inhibit the ability of PACAP38 to stimulate phosphorylation changes at the T840 or S845 site (Figure 3.4-3). These data suggest that while PACAP38 can modulate PKA to effect changes specific to S845 phosphorylation state, the PACAP38 induced increase in S845 phosphorylation and decrease in T840 phosphorylation is not due to changes in PKC activity.

Lastly we sought to determine whether phosphatases might play a role in PACAP38 regulation of GluA1 phosphorylation. We first investigated the ability of PP2B to regulate PACAP38 dependent phosphorylation changes. We found the PP2B inhibitor, cyclosporine A, led to a significant decrease in basal levels of GluA1 T840 phosphorylation. However, cyclosporine A was unable to block PACAP38 dependent phosphorylation changes at the GluA1 T840 and S845 sites (Figure 3.4-4). Consistent with published data (Lee et al 2007), the PP1/PP2A inhibitor, okadaic acid, led to a significant increase in basal GluA1 T840 phosphorylation (Figure 3.3-5). We also found that okadaic acid blocks PACAP38 dependent decrease in GluA1 T840 phosphorylation, but had no effect on the PACAP38 dependent GluA1 S845 phosphorylation. In summary our data shows that PKA is responsible for the PACAP38 induced increase in GluA1 S845 phosphorylation, and PP1/PP2A is responsible for PACAP38 induced decrease in GluA1 T840 phosphorylation.
Figure 3.4-1. PKA involvement in PACAP38 stimulated GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated with 10 µM H89 for 10 minutes and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples visualized by western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA, Bonferroni posttest. n≥6.
Figure 3.4-2. PKG involvement in GluA1 phosphorylation changes.

Hippocampal neurons (DIV14) were pre-incubated with KT5823 (1 or 4 µM) for 1 hour and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples examined by western blot.
Figure 3.4-3 PKC involvement in GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated 1 μM Go6983 for 10 minutes, and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples visualized by western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM. *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA, Bonferroni posttest. n≥6.
Figure 3.4-4. PP2B involvement in GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated with 2 µM cyclosporine A (CsA) for 15 minutes and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples visualized by western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA, Bonferroni posttest. n≥6.
Figure 3.4-5. PP1/PP2A involvement in GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated with 2 μM okadaic acid (OA) for 10 minutes and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples visualized by western blot. (B) Quantification of GluA1 T840 or S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA, Bonferroni posttest. n≥6.
3.5 Involvement of the NMDA receptor

PACAP38 has been shown to enhance NMDA receptor currents at Schaffer collateral-CA1 synapses (Mcdonald et al 2005, Yaka et al 2003). PACAP38 dependent changes in synaptic strength are also regulated by NMDA receptor activity such that the PACAP38 induced potentiation of synaptic transmission is partially be blocked by a NMDA receptor antagonist whereas depression is unaffected (Roberto et al 2001, Kondo et al 1997). Lastly, both PACAP38 and NMDA stimulate GluA1 T840 dephosphorylation which is dependent on PP1/PP2A activity (Delgado et al 2007, Gray et al 2014). Altogether this data suggests that NMDA receptor activity may be required for PACAP38 mediated changes in AMPA receptor phosphorylation. To test this possibility, we conducted PACAP38 stimulation experiments in the presence of the NMDA antagonist d-APV. We found that d-APV partially blocked the reduction in GluA1 pT840 phosphorylation but had no effect on the increase in GluA1 S845 phosphorylation (Figure 3.5-1). In control experiments, we confirmed that d-APV completely blocked NMDA dependent GluA1 T80 desphosphorylation (Figure3.5-2). In conclusion, NMDA receptor activity is partially responsible for the PACAP38 dependent decrease in GluA1 T840 phosphorylation.
Figure 3.5-1. NMDA receptor involvement in GluA1 phosphorylation changes.

(A) Hippocampal neurons (DIV14) were pre-incubated with D-APV (50 µM) for 45 minutes and then stimulated with PACAP38 (1 nM) for 10 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples examined by western blot. (B) Quantification of GluA1 T840 or GluA1 S845 phosphorylation normalized to GluA1. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA, Bonferroni posttest. n≥6.
Figure 3.5-2. NMDA receptor involvement in GluA1 phosphorylation changes.

Hippocampal neurons (DIV14) were pre-incubated with D-APV (50 µM) for 45 minutes and then stimulated with PACAP38 (1 nM) for 10 minutes or NMDA (50 µM) for 5 minutes. Cells were lysed, GluA1 immunoprecipitated, and samples examined by western blot.
3.6 Preliminary insights into the functional significance

So far we have established that PACAP38 can regulate GluA T840 and S845 phosphorylation, but the functional significance of these phosphorylation changes is unclear. One possibility is that PACAP38 dependent changes in GluA1 phosphorylation alter GluA1 function or synaptic trafficking. Such changes may then go on to alter synaptic strength and AMPA receptor mediated currents. We first tested ability of PACAP38 to regulate AMPA receptor localization. Using a biotinylation assay, we found that 10 or 30 minute PACAP38 stimulation (1 nM) had no effect on surface or total GluA1 and GluA2 levels (Figure 3.6-1). A drawback of the biotinylation assay is that synaptic proteins make up a very small percentage of the total surface population. Thus, changes occurring in the synaptic population may be obscured by a lack of change in the non-synaptic, surface population.

Therefore, we next used immunocytochemistry to study the effect of PACAP38 on GluA1 trafficking. In preliminary experiments, hippocampal cultures were transfected with wild type GFP GluA1, a GFP GluA1 T840A mutant, or a GFP GluA1 T840D mutant. The inclusion of GluA1 T840A and T840D mutants in these experiments allows us to determine if PACAP38 dependent changes in AMPA receptor localization are due to changes in GluA1 T840 phosphorylation. At DIV 17, hippocampal cultures were stimulated with PACAP38 (1 nM) for 10 minutes, and then stained for surface and total GFP. During image analysis, the threshold was set so as to exclude diffuse GFP staining and to visualize GFP puncta. This method allowed for more precise inspection of surface and total GluA1 puncta. We found that PACAP38 stimulation had no significant effect
on GluA1 surface/total levels (Figure 3.6-2). In cells transfected with the GluA1 T840A, there was a trend toward increased surface/total GluA1 levels after PACAP38 stimulation. Our sample size was small in this experiment, so we may see significance in some of these conditions with further experiments. Alternatively, increasing that duration of PACAP38 stimulation may produce more noticeable changes in GluA1 trafficking.
Figure 3.6-1. Effect of PACAP38 stimulation on surface and total AMPA receptor levels analyzed by a biotinylation assay.

Hippocampal neurons (DIV 17) were stimulated with PACAP38 (1 nM) for 10 or 30 minutes. Following stimulation, cultures were subjected to a surface biotinylation assay. Surface and total proteins were visualized by western blot.
Figure 3.7-2. Effect of PACAP38 stimulation on GFP GluA1 surface and total levels.

(A) At DIV15 Hippocampal cultures were transfected with GFP GluA1, GFP GluA1 T840A, GFP GluA1 T840D. Then at DIV17 cultures were stimulated with 1 nM PACAP38 for 10 minutes. Following stimulation, cells were stained for GFP surface and GFP total levels. (B) Quantification of surface GFP/total GFP levels. Error bars indicate ±SEM *P<0.05, **P<0.01, ***P<0.001, ANOVA, Tukey posttest.
Chapter 6. Discussion
PACAP38 has been shown to regulate synaptic transmission, AMPAR EPSCs, and GluA1 synaptic clustering (Kondo et al 1997, Cirrana et al 2003, Roberto et al 2000, Costa et al 2009, Roberto et al 2001, Gardoni et al 2012). Additionally, the PAC1R knockout exhibits impaired contextual fear conditioning (Otto et al 2001), and the PACAP38 knockout exhibit impaired contextual fear and novel object recognition (Takuma et al 2014). Despite the accumulating evidence that PACAP38 can regulate synaptic strength and learning, very little is known about how this regulation occurs.

Since AMPA receptor phosphorylation has been shown to regulate receptor recycling and to be important of learning (Lu and Roche 2012, Song and Huganir 2002), we hypothesized that PACAP38 may regulate AMPA receptor phosphorylation. In our study we demonstrated that PACAP38 stimulation of mature, hippocampal cultures results in an up-regulation of GluA1 S845 phosphorylation and a down-regulation of GluA1 T840 phosphorylation. We found that phosphorylation changes at the GluA1 T840 and S845 site result from a PACAP38 doses as low as 0.05 nM. A maximal decrease in GluA1 T840 phosphorylation was observed with a 1 nM dose, and a maximal increase in GluA1 S845 phosphorylation was observed with a 0.5 nM dose. Changes in GluA1 T840 and S845 phosphorylation were also found to occur quickly and could be observed with stimulations as short as 2 minutes. The PACAP38 induced decrease in GluA1 T840 phosphorylation was maximal with a 10 minute stimulation, whereas the PACAP38 induced increase in GluA1 S845 phosphorylation was maximal at 30 minutes.

To investigate the role of the different PACAP38 receptors in this process, we stimulated hippocampal cultures with VPAC1, VPAC2 and PAC1 receptor agonists. A
phosphorylation increase at the S845 site was robustly driven by VPAC2 and PAC1 receptor activation, and a phosphorylation decrease at the T840 site was most robustly driven by PAC1 receptor activation. Similarly, we found that a VPAC2/PAC1 receptor antagonist impaired PACAP38 stimulated GluA1 T840 and S845 phosphorylation changes, while the VPAC1 antagonist had no effect. Overall the PACAP receptor agonist and antagonist data suggest that VPAC2 and PAC1 receptor activity are largely responsible for PACAP38 stimulated changes in AMPA receptor phosphorylation. However further investigation is needed to establish the relative importance of VPAC2 and PAC1 receptor activation. Troubleshooting use of M65, the PAC1 receptor antagonist, would offer one potential approach for answering this question. Alternatively, we could design shRNAs against the VPAC2 or PAC1 receptor, and see how VPAC2 and PAC1 receptor knockdown affects PACAP38 induced phosphorylation changes. Using a similar approach, the PACAP38 stimulation experiments could also be performed in cultures generated from PAC1 or VPAC2 receptor knockout animals.

Downstream of the PACAP38 receptors, we found that PP1/PP2A activity was necessary for the GluA1 T840 phosphorylation decrease. GluA1 T840 dephosphorylation was also partially blocked by a NMDA receptor antagonist. At the S845 site, the PACAP38 induced increase in GluA1 S845 phosphorylation was unaffected by a PP1/PP2A and PKC inhibitor but was blocked by a PKA inhibitor. These results demonstrate that PACAP38 regulates GluA1 T840 and S845 phosphorylation through independent signaling pathways. As demonstrated by Gray et al (2014), phosphorylation status at GluA1 T840 can regulate GluA1 S845 phosphorylation and vice versa.
However it seems unlikely that this interaction contributes to PACAP38 dependent changes in GluA1 T840 and S845 phosphorylation. If such an interaction were to occur, we would expect GluA1 T840 and GluA1 S845 phosphorylation changes to be regulated by overlapping signaling cascades. For example, PACAP38 stimulation activates PP1/PP2A which dephosphorylates GluA1 T840. Due to the interaction between the GluA1 T840 and S845 site, low levels of GluA1 T840 phosphorylation would promote PKA phosphorylation at the S845 site. Any inhibitor that blocks the PACAP38 induced decrease in GluA1 T840 phosphorylation should also impair the increase in GluA1 S845 phosphorylation. In the reverse situation, any inhibitors that block GluA1 S845 phosphorylation changes should also impair T840 phosphorylation changes. Instead we found that inhibitors which blocked the PACAP38 dependent increase in GluA1 S845 phosphorylation had no effect on GluA1 T840 dephosphorylation and vice versa.

Two ways in which PACAP38 mediated changes in AMPA receptor phosphorylation may alter synaptic transmission and AMPA currents is through changes in AMPA receptor function and trafficking. Since GluA1 T840 phosphorylation has been shown to increase AMPA receptor conductance (Jenkins et al 2014), the PACAP38 dependent decrease in GluA1 T840 phosphorylation may reduce AMPA receptor conductance and consequently reduce synaptic transmission. Similarly, GluA1 S845 phosphorylation has been shown to increase receptor open channel probability (Banks et al 2000), so the PACAP38 induced increased GluA1 S845 phosphorylation could alter AMPA receptor gating and enhance synaptic transmission. Conversely, a number of studies have shown that GluA1 845 phosphorylation regulates AMPA receptor
trafficking. GluA1 S845 phosphorylation has been shown to increase GluA1 membrane insertion (Man et al 2007) and there is a correlation between increased GluA1 S845 phosphorylation and elevated surface GluA1 levels (Oh et al 2006, Man et al 2007). The PACAP38 dependent increase in GluA1 S845 phosphorylation could increase GluA1 delivery to the cell surface. Even so, an increase in surface GluA1 levels may not alter synaptic transmission because GluA1 S845 phosphorylation has been proposed to regulate extrasynaptic but not synaptic GluA1 levels (Oh et al 2006). The ability of GluA1 T840 phosphorylation to regulate AMPA receptor trafficking is currently unknown, so this will be an important question to investigate.

There are still many remaining questions concerning PACAP38 regulation of synaptic transmission. It will be important to determine if PACAP38 dependent changes in synaptic strength are due to presynaptic or postsynaptic changes. The fact that PACAP stimulation alters AMPA receptor mediated currents and GluA1 trafficking suggests that there is a postsynaptic expression mechanism (Gardoni et al 2012, Costa et al 2009). Additionally, Roberto et al (2000) found that when PACAP38 enhanced synaptic transmission there was no effect on paired pulse facilitation. Since this study used just one inter-stimulus interval, it will be important to determine how PACAP38 affects the paired pulse ratio across a range of inter-stimulus intervals. A follow up question would be: if there is postsynaptic expression mechanism, are PACAP38 dependent changes in synaptic strength the result of altered AMPA receptor trafficking or function and is this mediated by changes in AMPA receptor phosphorylation?
Many of these questions can be addressed with immunocytochemistry and physiology experiments. In preliminary experiments looking at GluA1 localization, we found that PACAP38 had no significant effect on GFP GluA1 WT or mutant surface/total levels. However, we do see a trend suggesting altered trafficking of the GluA1 T840A mutant. More samples will be needed to determine how PACAP38 regulates GluA1 WT and phospho-mutant surface/total levels. In these experiments, we used a relatively short PACAP38 stimulus, so we will perform this experiment using a longer PACAP38 stimulus to see if a pronounced effect is observed. In order to establish the importance of GluA1 S845 phosphorylation changes, we will also perform immunocytochemistry experiments in cultures prepared from WT and GluA1 S845A knock-in mice. Another intriguing line of investigation is to look at the effect of PACAP38 stimulation on GluA1 synaptic targeting. Gardoni et al (2012) have shown that PACAP38 stimulation decreased GluA1/Shank co-localization and this was partially blocked by the PKA inhibitor, H89 (Gardoni et al 2012). The decrease in GluA1 synaptic targeting could be due to the PACAP38 dependent increase in GluA1 S845 phosphorylation or decrease in T840. In future experiments, it will be interesting to study WT or phosphor-mutant GluA1 co-localization with Shank before and after PACAP38 stimulation.

The above experiments investigate the ability of PACAP38 to regulate GluA1 localization, but they do not determine if PACAP38 dependent changes in synaptic transmission and AMPA receptor mediated currents occur as a result of GluA1 phosphorylation changes. This issue must be addressed using electrophysiology. Physiology can be performed in hippocampal slices prepared from GluA1 S845A knock-
in mice, penta knock-in mice, and WT mice. Using whole cell or field recording, we could measure the effect on PACAP38 stimulation on Schaffer collateral-CA1 synaptic transmission in slices prepared from WT and mutant animals. While our phosphorylation experiments were performed on hippocampal cultures, in physiology experiments the use of hippocampal slices is ideal because this preparation will preserve the hippocampal circuitry and allow for more thorough investigation of PACAP38 effects on CA1 and possibly dentate gyrus synaptic transmission. PACAP38 can regulate synaptic transmission in a dose dependent manner, so it will also be important to stimulate slices with a range of PACAP38 concentrations. It is possible that phosphorylation changes at the GluA1 T840 or S845 site does not regulate synaptic both strengthening and weakening of synaptic transmission but instead may be important for only depression or only potentiation. Due to the availability of a GluA1 S845 knock-in animal, we will be able to directly investigate the role of GluA1 S845 phosphorylation in the PACAP38 regulation of synaptic transmission. A drawback of the penta mouse is that we will not know if the phenotype is due to the GluA1 T840A or S845A mutation. A comparison of the results from GluA1 S845A knock-in and penta animal may give us some insight into the role of GluA1 T840 phosphorylation. In parallel with physiology experiments, we need to confirm that PACAP38 stimulation alters AMPA receptor phosphorylation in slices similar to the culture system.

It is also possible that the PACAP38 dependent increase in GluA1 S845 phosphorylation and decrease in T840 phosphorylation may not regulate synaptic transmission rather it may alter the threshold for synaptic plasticity. In support of this
hypothesis, LTP in the dentate gyrus of the PAC1 receptor knock-out is intact when induced with a suprathreshold tetanus, but it is impaired when induced with a subthreshold tetanus (Matsuyama et al 2003). It has also been suggested that GluA1 S845 phosphorylation regulates extrasynaptic GluA1 levels and primes synapses for LTP (Lu and Roche 2012, Lee and Kirkwood 2011, Oh et al 2006). Consistent with this theory, the neuromodulator norepinephrine (NE) has been shown to increase GluA1 S845 phosphorylation and to lower the threshold for long term potentiation (LTP) (Hu et al 2007). In the GluA1 S831, 845A knock-in mouse, NE-facilitated LTP is impaired (Hu et al 2007). Similarly, GluA1 S831/845D knock-in mice exhibit a lower threshold for LTP induction (Makino et al 2011). Considering the fact that the threshold for LTP induction is altered in the PAC1 receptor knockout and PACAP regulates S845 phosphorylation, it is possible that PACAP38-dependent changes in AMPA receptor phosphorylation alter the LTP threshold. Physiology experiments in the penta and GluA1 S845A knock-in animal can address this possibility. Altogether, these findings suggest that deficits in AMPAR phosphorylation may underlie the role of PACAP38 and the PAC1 in certain forms of learning (Otto et al 2001, Takuma et al 2014).
Chapter 7. Bibliography


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