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The Role of EPAC Signaling in Memory Consolidation and Sleep Deprivation

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The Role of EPAC Signaling in Memory Consolidation and Sleep Deprivation

**Abstract**
It is well established that cAMP signaling within neurons plays a major role in the formation of long-term memories. cAMP has three targets, protein kinase A (PKA), hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels, and exchange protein activated by cAMP (Epac). Studies have revealed that both PKA and HCN channels are important for long-term memory formation. However, little is known about the role of Epac in this process. Epac is a cAMP-dependent guanine nucleotide exchange factor for the small G proteins including Rap1. The Epac2 isoform is highly expressed in the forebrain. This dissertation examines the role of Epac in memory formation in several aspects. First, I show that activation of Epac within the hippocampus via intrahippocampal injection of Epac specific agonist 8-pCPT-2'-O-Me-cAMP was able to enhance long-term memory formation in a PKA independent fashion. Next, I show that the levels of Rap1 activity, the direct target of Epac, increased during the memory formation. Furthermore, mice injected with Epac2shRNA adeno-associated virus in the hippocampus showed decreased Epac2 protein levels and impaired memory consolidation. These results demonstrate that cAMP-Epac-Rap1 signaling plays an important role for memory consolidation, which alters prevailing assumptions that cAMP signaling modulates memory formation solely through PKA and HCN. Additionally, sleep deprivation appears to cause memory deficits by impairing cAMP signaling. Here, I show that the activity of Rap1 decreases after sleep deprivation, suggesting the memory deficits caused by sleep deprivation may be related to reductions in Epac signaling. These findings broaden our understanding of cAMP signaling in memory formation and sleep deprivation by adding Epac/Rap1 to the signaling pathways modulating memory formation.

**Degree Type**
Dissertation

**Degree Name**
Doctor of Philosophy (PhD)

**Graduate Group**
Biology

**First Advisor**
Edwin (Ted) G. Abel

**Second Advisor**
Nancy M. Bonini

**Keywords**
cAMP, Epac, memory, sleep deprivation

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THE ROLE OF EPAC SIGNALING IN MEMORY CONSOLIDATION AND SLEEP DEPRIVATION

Nan Ma

A DISSERTATION

in

Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2011

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Acknowledgements

This thesis would not have been possible without the help and support of my colleagues, family and friends. First, I would like thank Dr Ted Abel for being a great teacher and mentor. Ted is extremely dedicated to his students with his passion for science. He guided and supported me through the whole process while never showing any signs of impatience or disappointment. I would also like to thank my colleague Dr Pepe Hernandez for helping me with the mice behavioral experiments and paper-writing. I would also like to thank all the members of the Abel lab. It’s been a great five years working with you guys.

Thanks to my thesis committee for the valuable advice and intellectual guidance: Nancy Bonini, Wade H. Berrettini, Dejian Ren, Marc F. Schmidt and Steven A. Thomas. Thanks to the Lynch lab animal facility staff, especially J.B., for taking good care of the experimental mice. Also thanks to the Biology Graduate Group, and coordinator Colleen Gasiorowski.

Last but not least, I would like to thank my parents for their unconditional love and support. I would also like to thank my husband, Shuo Zhang, for always being so supportive and considerate.
ABSTRACT

THE ROLE OF EPAC SIGNALING IN MEMORY CONSOLIDATION AND SLEEP DEPRIVATION

Nan Ma
Ted Abel

It is well established that cAMP signaling within neurons plays a major role in the formation of long-term memories. cAMP has three targets, protein kinase A (PKA), hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels, and exchange protein activated by cAMP (Epac). Studies have revealed that both PKA and HCN channels are important for long-term memory formation. However, little is known about the role of Epac in this process. Epac is a cAMP- dependent guanine nucleotide exchange factor for the small G proteins including Rap1. The Epac2 isoform is highly expressed in the forebrain. This dissertation examines the role of Epac in memory formation in several aspects. First, I show that activation of Epac within the hippocampus via intrahippocampal injection of Epac specific agonist 8-pCPT-2’-O-Me-cAMP was able to enhance long-term memory formation in a PKA independent fashion. Next, I show that the levels of Rap1 activity, the direct target of Epac, increased during the memory formation. Furthermore, mice injected with Epac2shRNA adeno-associated virus in the hippocampus showed decreased Epac2 protein levels and impaired memory consolidation. These results demonstrate that cAMP-Epac-Rap1 signaling plays an important role for
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Chapter 1 Introduction

Abstract

cAMP signaling plays a critical role in the molecular process underlying memory formation. cAMP and two of its targets, PKA and HCN, have been extensively studied, and the role of these molecules in synaptic plasticity and memory formation are well described. Nevertheless, the function of a third target of cAMP, Epac, is less well understood. This introduction is focused on recent studies of Epac signaling and neuronal function, where evidence suggests that Epac may be involved in memory formation process. Additionally, I discuss how sleep may affect memory formation via cAMP signaling and the potential role that Epac may play in this process.

Memory is defined as the retention of changes in behavior that result from experience (Abel and Nguyen 2008). Once acquired, memory is gradually converted from a temporally form (short-form memory) into a stable form (long-term memory) that can last for days, months and years. The formation of long-term memory is called memory consolidation (Figure 1.1A), which requires gene expression and protein synthesis. The hippocampus is crucial for memory consolidation in rodents and humans (Becker et al.)
1981; Zola-Morgan et al. 1986). In addition, hippocampus-dependent memory is mediated via hippocampal synaptic plasticity, the persistent alterations in synaptic strength in specific neuronal pathways (Figure 1.1 B) (Bliss and Collingridge 1993; Moser et al. 1998; Lynch 2004). Cyclic-adenosine 3’, 5’-monophosphate (cAMP) signaling is critical for different forms of synaptic plasticity and memory in invertebrates and vertebrates and is an important molecular mechanism underlying memory consolidation. Protein kinase A (PKA) and hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels were thought to be the only two direct effectors of cAMP, until a novel category of cAMP mediators called Epac (exchange proteins directly activated by cAMP) were reported in 1998. Epacs function in a PKA-independent manner and therefore represent a novel mechanism for modulating signaling transduction within the cAMP cascade (de Rooij et al. 1998; Kawasaki et al. 1998). In this Introduction, I first summarize the pharmacological evidence and transgenic studies that demonstrate the role of cAMP signaling in memory consolidation. Then, the studies defining the role of the two other targets of cAMP, PKA and HCN, in synaptic plasticity and memory are discussed. Next, I focus on the newly found cAMP target Epac. The current work on Epac signaling and evidence of its involvement in cognitive function and memory formation are discussed. Lastly, I introduce the interaction between cAMP signaling and sleep deprivation. cAMP signaling is impaired by sleep deprivation and this appears to be the molecular mechanisms by which sleep deprivation impacts memory consolidation.
(Vecsey et al., 2009). The introduction gives an overall picture of cAMP signaling modulating memory consolidation and the evidence that Epac is involved in this process.

1.1 Overview of cAMP signaling and memory

1.1.1 cAMP is crucial for synaptic plasticity and memory consolidation.

The first evidence indicating the involvement of cAMP in memory dates back to 1976. cAMP was reported to facilitate synaptic transmission in *Aplysia*. Such facilitation was associated with *Aplysia* learning reflex (Brunelli et al. 1976; Castellucci and Kandel 1976). In mammals, cAMP was also found to be involved in long-term synaptic potentiation (LTP) in the hippocampus. LTP is extensively studied because it mimics the synaptic changes during memory formation. In the rat, L-LTP activates adenylyl cyclase and leads to increased cAMP levels in the CA1 region (Chetkovich et al. 1991; Chetkovich and Sweatt 1993). Applying the cAMP analog Sp-cAMPS (Nguyen et al. 1994) or the cAMP activator forskolin with phosphodiesterase inhibitor induces LTP (Chavez-Noriega and Stevens 1992). In behavioral studies, hippocampal cAMP levels were found to increase after training in inhibitory avoidance tasks (Bernabeu et al. 1996). Intra-hippocampal injection of cAMP analog Sp-cAMPS (Vianna et al. 2000; Ma et al. 2009) enhances long-term memory. These experiments clearly demonstrate that cAMP activates synaptic plasticity and memory consolidation.
More evidence from transgenic studies further demonstrates that cAMP signaling is required for memory consolidation. These studies were first started in *Drosophila*. By using an odor-electric shock associative learning paradigm, researchers found several *Drosophila* mutants that have memory deficits. Some of these mutants are linked to cAMP signaling cascade. For instance, the mutant *dunce* has a deficiency in the gene encoding phosphodiesterase (Byers, et al. 1981), the mutant *rutabaga* has a deficiency in adenylyl cyclase, the enzyme that synthesizes cAMP from ATP (Levin et al. 1992), the mutant *amnesiac* has a mutation in a gene for a neuropeptide that binds to a G protein–coupled receptor that stimulates adenylyl cyclase (Feany and Quinn 1995). Later, researchers started to generate similar transgenic models in mice to see if the similar behavioral phenotypes apply. One class of models was targeted at adenylyl cyclase (AC) knock-outs and the other was targeted at G-protein coupled receptors.

There are nine AC isoforms expressed in the brain. The sequence of the *rutabaga* gene is most closely related to AC1 (Levin et al. 1992). AC1 is primarily expressed in the brain, particularly in the hippocampus and cortex (Xia et al. 1993). Hence, it became a likely candidate for learning and memory in mammalian systems. AC1 knock-out mice (AC1<sup>−/−</sup>) were generated in 1995 (Wu et al. 1995). These mice have normal growth, motor coordination, and lifespan (Abdel-Majid et al. 1998) but decreased Ca2+-stimulated cAMP, which is correlated with a decrease in CA1/CA3 hippocampal and cerebellar LTP. AC1<sup>−/−</sup> mice also show a partial reduction in mossy fiber LTP. Consistent with the mild
synaptic plasticity deficits, the AC1\(^{-/-}\) mice exhibit a mild memory deficit. AC1\(^{-/-}\) mice are unable to exhibit quadrant preference in probe trials in water maze, but the learning curves of the water maze are normal. These mild LTP and memory deficits indicate that the function of AC1 is compensated by other isoforms of AC that have overlapping functions with AC1. One possible candidate is AC8, which is also highly expressed in numerous brain regions, including the hippocampus (Muglia et al. 1999). Therefore AC8 knock-out mice were generated (AC8\(^{+/+}\)) and crossed with AC1\(^{-/-}\) to create a double knock out (DKO). Although individual AC1 and AC8 knock-outs exhibit normal L-LTP and fear-associated memory, DKO mice exhibit a nearly complete loss of mossy fiber LTP, loss of L-LTP and long-term memory deficits in passive avoidance task and contextual fear conditioning task (Wang et al. 2003). Mice with over-expression of AC1 in the forebrain, on the other hand, showed elevated LTP, increased memory for object recognition and slower rates of extinction for contextual memory (Wang et al. 2004).

Another transgenic mouse model of cAMP signaling is targeted at G protein coupled receptor, which is the direct activator of adenylyl cyclase. Researchers overexpressed a constitutively active form of Gs\(\alpha\) in the forebrain (Gs\(\alpha^*\)) in the mice, trying to mimic the behavioral deficits in Drosophila Gs mutant (Bourtchouladze et al. 2006). Interestingly, Gs\(\alpha^*\) mice show increased adenylyl cyclase activity but decreased cAMP levels in the hippocampus, which is probably due to a compensatory up-regulation in PDE activity (Bourtchouladze et al. 2006) (Kelly et al. 2007). Because of reduced hippocampal cAMP
level, Gsα* mice exhibit learning and memory deficits in the Morris water maze task as well as short-term and long-term memory deficits in contextual fear conditioning task. Surprisingly, the mice exhibit enhanced basal synaptic transmission and L-LTP. Such difference between behavioral phenotype (learning and memory impairments) and electrophysiological phenotype (enhanced LTP) is also observed in transgenic mice with Giα1 gene disruption (Pineda et al. 2004). Giα1–/– mice show increased adenylyl cyclase activity, persistent E-LTP but deficits in long-term contextual fear memory and object recognition memory. The phenotype of these two lines indicates that cAMP is required for memory formation and alternation of G protein coupled receptor may affect unspecific synaptic plasticity related signaling. To minimize the unspecific effects of modulating G protein coupled receptor, another transgenic line was generated, where an octopamine-activated invertebrate G protein couple receptor was expressed in the mouse forebrain (Apoa1). There is no endogenous octopamine synthesized in mouse, so the transgenic receptor can only be activated by octopamine injection. Therefore, the cAMP level in these mice can be activated in a more controlled and timely fashion (Isiegas et al. 2008). After applying octopamine, Apoa1 mice showed a rapid hippocampal cAMP elevation, enhanced hippocampus E-LTP, and enhanced short term and long-term contextual fear memory. All these transgenic studies, together with pharmacological studies demonstrate the crucial role of cAMP in synaptic plasticity and memory.

1.1.2 The role of PKA, a major target of cAMP, in memory consolidation
The role of PKA in learning and memory is well reviewed in Brandon et al. 1997 and Abel and Nguyen 2008. The mammalian PKA family includes four regulatory subunits (RIα, RIβ, RIIα, RIIβ) and three catalytic subunits (Ca, Cβ, Cγ). Each subunit is encoded by a unique gene (McKnight et al. 1988). Each PKA is composed of two dimerized regulatory subunits binding with two catalytic subunits. Each regulatory subunit contains two cAMP binding sites. The catalytic subunits are inactive in the absence of cAMP. When cAMP binds to the regulatory subunits, the catalytic subunits disassociate from the regulatory subunits and are able to phosphorylate targets. There are two major types of PKA: type I (with RIα and RIβ dimers) and type II (with RIIα and RIIβ dimers), (Tasken et al. 1993; Francis and Corbin 1999; Skalhegg and Tasken 2000). Among these regulatory subunits, RIα serves as a buffering system to maintain the overall PKA activity levels. When the expression levels of other regulatory subunits or catalytic units are dramatically altered (over expressed or knocked out), the expression levels of RIα change in the direction against the perturbations (Uhler and McKnight, 1987; Otten and McKnight, 1989; Cummings et al. 1996).

Hippocampal PKA activity increases after induction of L-LTP (Roberson and Sweatt, 1996) or training with a hippocampus-dependent task (Izquierdo et al. 2000). Pharmacological studies provide additional evidence of PKA modulating synaptic plasticity and memory consolidation. The most commonly used reagents for PKA studies are PKA activator Sp-cAMPS, PKA inhibitors Rp-cAMP, H-89, KT5720, and PKI. Sp-cAMPS facilitates LTP and enhances long-term memory (Nguyen et al. 1994; Vianna et
PKA inhibitors Rp-cAMP and KT5720, on the other hand, block L-LTP induced by of three high-frequency trains of stimuli (Frey, et al.1993; Huang and Kandel 1994). Rodents treated with Rp-cAMP or H-89 exhibited memory deficits (Mohammad et al. 2004) (Ma et al. 2009). However, those pharmacological tools are limited because they lack specificity. For instance, Sp-cAMPS and Rp-cAMP affect HCN ion channels (Kramer and Tibbs, 1996) and Epac (Christensen et al. 2003) in addition to their effects on PKA. KT5720 blocks JNK1 and p38MAPK, and H-89 inhibits almost all the members of MAPKs (Davies et al. 2000). Among them, PKI has the best specificity. Therefore, the requirement for PKA activity in synaptic plasticity is best demonstrated by the experiment where post-synaptic injection of PKI impairs the expression of L-LTP (Duffy and Nguyen, 2003).

Because of the limitations of pharmacological reagents, genetically-modified animal models are needed to better understand the role of PKA in synaptic plasticity and memory. Because of the “buffering” effect of RIα described before, mice lacking RIβ, or Cβ 1 have no changes in the level of total hippocampal PKA activities, and therefore no memory deficits are detected (Huang et al., 1995) (Qi et al., 1996). Mice lacking RIIβ have approximately 50% hippocampal PKA activity reduction but normal LTP and no deficits in hippocampus-dependent learning (Brandon et al.1997), Mice lacking RIα are lethal at early embryonic stage (Amieux et al. 1997) and cannot be used for behavioral analysis.
Instead of knocking out PKA subunits, Ted Abel and his colleague used a different strategy by creating a transgenic mouse line expressing a dominant negative form of the regulatory subunit of PKA, R(AB), in neurons within the forebrain (Abel et al. 1997). This transgene interferes with endogenous type I PKA and therefore reduces the potential compensatory effects of RIα. The R(AB) mice showed a 50% reduction in basal hippocampal PKA activity and a 25% reduction in total hippocampal PKA activity. R(AB) mice showed normal E-LTP but impaired L-LTP. Furthermore, in the hidden platform version of the Morris water maze, these mice exhibit normal learning, but memory deficits in retrieval tests. R(AB) mice also show selective deficits in long-term memory for contextual fear conditioning, where short-term memory for contextual fear as well as cued fear conditioning is intact. More recently, another R(AB) transgenic line was generated using the tetracycline system (teto-R(AB)). In this transgenic model, the expression of R(AB) can be temporally regulated and therefore prevent the effect of R(AB) expression during forebrain development. Studies on teto-R(AB) mice have confirmed that genetic inhibition of neuronal PKA in adulthood selectively impairs contextual long-term memory (Isiegas et al., 2006). Together, these findings clearly demonstrate that PKA is essential for synaptic plasticity and memory consolidation.

1.1.3 HCN channels and memory consolidation

9
Hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels are cation channels that are activated by membrane hyperpolarization and are modulated by the binding of cyclic nucleotides. There are four known HCN subunit isoforms, HCN1–HCN4. In mammals, all four isoforms are expressed in the central nervous system at various levels (HCN1 expression level is the highest) and combine in diverse ways to form hetero-tetrameric channels (Craven and Zagotta, 2006). With hyperpolarization, HCN channels activate and generate an inward current termed the Ih current. The binding of cAMP or cGMP facilitates the activation of Ih by accelerating the opening kinetics and shifting the voltage dependence of activation to more depolarized voltages (Ludwig, et al. 1998).

HCN channels are involved in many functions that are important for learning and memory: stabilizing membrane potential and membrane resistance, regulating synaptic transmission, dendritic integration, and oscillations (Morris et al. 2004; Herrmann et al. 2007). HCN1 knock-out mice (HCN1−/−) showed severe deficits on rotarod and visible platform water maze task. These motor learning deficits prevented further behavioral analysis. Therefore, another transgenic line of HCN1 was generated using the loxp/cre recombination system to constrain the deletion of HCN1 in the forebrain region (HCN1fl/fl,cre) (Nolan et al. 2004). These mice showed normal motor learning and activity. Surprisingly, HCN1fl/fl,cre mice exhibits enhanced spatial learning and memory but unaffected long-term contextual fear memory. The enhanced spatial learning and memory
is also associated with enhanced hippocampal theta rhythms activity and enhanced LTP at the perforant-CA1 pathway. Interestingly, Schaffer collateral LTP is not altered in these mice. Their findings demonstrate that HCN1 function as a negative regulator to constrain the synaptic plasticity that is involved in specific forms of learning and memory and synaptic plasticity in the hippocampus.

1.2 Epac is a newly found target of cAMP

1.2.1 Introduction of Epac family

Epac are cAMP–dependent guanine nucleotide exchange factors (GEFs) for the small G proteins. There are two isoforms of Epac, Epac1 and Epac2 (Figure 1.2A), which are coded by two distinct genes that share extensive sequence homology. Epac1 is expressed ubiquitously in all tissues, whereas Epac2 is predominantly expressed in the brain (Figure 1.2B), pituitary, adrenal gland, and pancreas (de Rooij et al. 1998; Kawasaki et al. 1998). Recently, a shorter N-terminal splice variant of Epac2 named Epac2B has been identified in the adrenal gland (Niimura, et al., 2009). Both Epac1 and Epac2 are multi-domain proteins containing an N-terminal regulatory region and a C-terminal catalytic region. The regulatory region is composed of one (Epac1) or two (Epac2) cyclic nucleotide binding (CNB) domains and a DEP (Dishevelled, Egl-10, and Pleckstrin) domain, which determines the subcellular localization of Epac (de Rooij et al. 2000). The catalytic region consists of a RAS exchange motif (REM) domain that stabilizes the G-protein
exchange activity, a RAS association (RA) domain that may be involved in protein translocation (Li, et al. 2006), and a CDC25- homology domain where the exchange of GDP for GTP on Rap takes place.

The crystal structure analysis of Epac2 (Rehmann, et al 2006) and NMR spectroscopy of Epac1 (Das, et al 2008) revealed the detailed conformation changes that occur during Epac activation. In the absence of cAMP, the regulatory region containing the cAMP-binding domain directly interacts with the catalytic region and blocks the small G protein binding site. Binding of cAMP at the CNB domain allows the regulatory region to move away from the catalytic region. The exposing of the catalytic site therefore leads to Rap activation (Figure 1.2C). Although Epac2 has two CNB domains, only one of them (the conserved one between Epac1 and Epac2) is involved in the conformation change during activation. The other CNB (called CNB-A) binds cAMP with a 20-fold lower affinity and seems unnecessary for cAMP-dependent Epac activation. Recently, evidence suggests that CNB-A may be involved Epac2 subcellular localization (Niimura, et al., 2009).

Epac1 and Epac2 have distinctive subcellular distribution patterns. Epac1 is localized at the peri-nuclear mitochondria through a specific N-terminal sequence that is not presented in Epac2 (Li, et al. 2006; Qiao et al. 2002). Instead, the N terminus of Epac2 (CNB-A) is required for Epac2 localization. When activated with cAMP, recombinant
full length Epac2 / GFP fusion protein localized near the plasma membrane, while the shorter N-terminal splice variant Epac2B (with no CNB-A) was found primarily in the cytoplasm but not near the membrane (Niimura, et al., 2009). Besides CNB-A, the RA domain of Epac2 is also involved in the Epac2 localization to the plasma membrane. This domain specifically binds to Ras, which has lipid modification at the c-terminus that binds to the lipid membrane. Therefore, when RA domain is exposed by Epac2 activation, the interaction between Ras and Epac2 via RA domain clusters Epac to plasma membrane (Li, et al. 2006) (Liu et al., 2008). Other domains may also be involved in Epac compartmentalization. In COS cells, the peri-nuclear targeting of Epac1 is due to the interaction between a portion of the GEF domain and with light chain (LC) 2 of microtubule- associated protein (MAP) 1B (Borland, et al.,2006). In cultured rat neonatal ventriculocytes and mouse primary cortical neurons, Epac1 or Epac2 is found to anchor with different AKAP proteins at different subcellular location as well (Dodge-Kafka et al., 2005) (Nijholt et al. 2008). The subcellular distribution differences between Epac1 and Epac2 indicate that they may serve different physiology functions.

1.2.2 Epac specific agonist 8-(4-chloro-phenylthio)-2’-O-methyladenosine-3’,5’ - cAMP
As described before, most cAMP analogs lack specificity and are able to activate all cAMP targets. Fortunately, it was noticed that Epac CNB domain lacks a highly
conserved glutamate residue that is presented in all other known CNB domains, including PKA and HCNs (Figure 1.3A) (Enserink et al. 2002). This residue difference makes it possible to design a cAMP analog specific for Epac. The cAMP analog with 2’-hydroxyl group replaced by 2’-O- methyladenosine (2’-O-Me) binds specifically to Epac. The binding affinity is further improved by the adding of a parachlorophenylthio at position 8 on the adenine moiety of 2’-O-Me-cAMP. This new cAMP analog, 8-((4-chloro-phenylthio)-2’-O-methyladenosine- 3’,5’-cAMP (8-pCPT), has high affinity for Epac and greater potency and efficacy than cAMP (Figure 1.3B,3C). 8-pCPT activates both Epac1 and Epac2 and is widely used to explore Epac signaling in cells and tissue. However, it has been recently reported that, depending upon the model and the doses used, 8-pCPT may indirectly activate cAMP/PKA pathways through the inhibition of PDE (Poppe, et al., 2008). In addition, metabolites of 8-pCPT may regulate gene expression through an unknown signaling pathway (Enyeart and Enyeart 2009). Therefore, validation of 8-pCPT specificity in specific cell type and animal model is still necessary. So far, there is no specific antagonist for Epac. So temporal (siRNA or antisense) or permanent genetic modification technique are required to demonstrate the necessity for Epac signaling in memory and synaptic plasticity.

1.2.3 The effectors of Epac
With the tools described above, molecular targets and effectors of Epac have been discovered. Here, several Epac targets that are also involved in the regulation of synaptic plasticity and memory are discussed. Rap1, the first identified target of Epac, is an important regulator of synaptic plasticity and memory. Transgenic mice with less Rap1 activity in the forebrain exhibit impaired hippocampal Schaffer collateral LTP induced by either forskolin or theta frequency stimulation, impaired spatial memory and context discrimination (Morozov et al. 2003).

Another important type of modulator of synaptic plasticity, MAPKs, are also effectors of Epac. Epac activates ERK1/2 (Wang et al. 2006; Lin et al.,2003), JNK (Eid et al. 2008; Monaghan et al. 2008) and p38MAPK (Hochbaum et al. 2003) in different cell types. All three types of MAPKs are important for memory formation. ERKs play a crucial part in synaptic plasticity by regulation of translation. Dysregulation of ERK is directly linked with a learning disorder in humans (Sweatt, 2004). JNK is involved in different forms of synaptic plasticity and several aspects of memory consolidation in the hippocampus (Sherrin et al. 2011). p38MAPK activity is required for both short-term memory and long term memory formation (Alonso et al. 2003).

In addition, insulin secretion studies demonstrated that Epac2 modulates exocytosis by binding Rim2 or Piccolo (Kashima et al. 2001) (Fujimoto et al., 2002). While in neurons,
Rim family and Piccolo are located at pre-synaptic sites and thought to play a role in neurotransmitter release (Ohtsuka, 2011). In addition, the highly homologous isoform of Rim2, Rim1, is crucial for the pre-synaptic component of cAMP-dependent mossy fiber LTP, Schaffer collateral late-phase LTP and learning and memory (Castillo et al. 2002; Powell et al. 2004).

The effectors of Epac also include transcription and translation regulators. Epac activation increased C/EBP DNA binding activity and induced activation of C/EBP reporter in human umbilical vein endothelial cells and mouse embryonic fibroblast cells. Deletion of C/EBP isoforms blocked the cAMP-dependent expression of C/EBP target gene (Yarwood et al. 2008). Those C/EBP isoforms are again involved in molecular mechanism underlying memory (Taubenfeld et al. 2001) (Taubenfeld et al. 2001). A study in human prostate cancer cells reported that Epac-mediated cellular effects require activation of mTOR signaling cascades, suggesting that mTOR is an effector of Epac as well (Misra and Pizzo, 2009). mTOR is a key regulator of translational initiation. Pharmacological studies demonstrate that mTOR signaling is required for L-LTP (Gelinas et al. 2007) and various forms of memory (Gafford et al. 2011).

1.3 Epac signaling and neuronal functions.
1.3.1 Epac signaling and neurotransmitter release

Because neurotransmitter release at the synapse is thought to regulate by cAMP signaling, it is natural to consider the possibility that Epac signaling is, at least partially, involved in this process. Gamma-aminobutyric acid (GABA) inhibits synaptic transmission by down regulation of cAMP levels. Application of the Epac activator 8-pCPT is able to counteract the inhibitory effect of GABA on synaptic transmission, which indicates the Epac signaling enhances this type of neurotransmitter release (Sakaba and Neher, 2003). More studies in different types of neurons confirmed that Epac activation enhances neurotransmitter release and evokes excitatory postsynaptic current (Kaneko and Takahashi, 2004; Zhong and Zucher, 2005; Huang and Hsu, 2006; Gekel and Neher, 2008). Epac signaling is also found to modulate neuronal excitability in cultured cerebellar granule cells (Ster et al., 2007).

1.3.2 Epac signaling and synaptic plasticity

As cAMP signaling is critical for many forms of synaptic plasticity, the role of Epac signaling in synaptic plasticity is also been explored (Figure 1.4). In mouse hippocampal slices, activation of Epac by 8-pCPT enhances maintenance of Schaffer collateral LTP without affecting basal synaptic transmission or initial LTP induction. The persistence of this form of LTP requires ERK and protein synthesis, but not transcription. Epac activation can also facilitate maintenance of chemically induced LTP (Gelinas et al.)
2008). These finding suggest that Epac is an important modulator for some forms of protein synthesis-dependent LTP.

Under certain conditions, Epac activation also induces another form of synaptic plasticity, long-term depression (LTD), in the Schaffer collateral of hippocampus. The induction of this form of LTD requires translation but not transcription. Further study reveals that this Epac-LTD depends on postsynaptic mechanisms including Rap-1 and p38 MAPK and might be induced by stimulation of PACAP receptors (Ster et al., 2009).

1.3.3 Epac signaling and memory
The last eight years of research of the function of Epac in the nervous system already reveals a wide range of important roles in cAMP-regulated cell signaling. All these findings indicate that Epac may play a role in the learning and memory. In 2008, two different groups reported that under different experiment settings, Epac activation via 8-pCPT alone, or together with PKA activation enhances hippocampus-dependent memory retrieval (Kelly et al. 2008; Ouyang et al. 2008). Later, the role of Epac in memory retrieval was confirmed (Figure 1.5). Mice exhibit memory retrieval deficits when the hippocampal Epac2 expression was temporarily knocking down via siRNA injection (Ostroveanu et al. 2010). However, little is known about the role of Epac in memory consolidation. This problem is addressed in this thesis by investigating the memory
performance in mice with enhanced Epac activity and reduced Epac activity. The detailed
descriptions are in Chapter 2 and Chapter 3.

1.3.4 Epac and cognitive deficits
Pathology studies have found that alteration in Epac activity levels are related to several
cognitive diseases. For example, increased Epac2 levels are found in the prefrontal cortex
and hippocampus of depression-related suicides (Dwivedi, et al. 2006). In addition, a
genome screen identified Epac2 as a candidate gene for autism (Bacchelli et al. 2003).
The mRNA level of Epac1 is elevated whereas the level of Epac2 is reduced in the frontal
cortex and hippocampus of the brain in Alzheimer’s patients (McPhee et al. 2005).
Recently, Epac-Rap1 signaling is also found to regulate a key protein in the development
of Alzheimer’s disease (Robert, et al. 2005; Zaldua et al., 2007). Because learning and
memory deficits are often symptoms of these diseases, the study of Epac function in
memory consolidation is important to understand the mechanism and to find the
treatment of these deficits.

1.4 Sleep, memory and cAMP signaling.
It has been proposed that one important function of sleep is to facilitate memory
consolidation. In human studies, participants with a period of post-learning sleep perform
better in word recognition and word pair association tasks (Benson and Feinberg, 1975; Schoen and Badia, 1984; Drosopoulos et al. 2006; Gais et al., 2006), indicating improved memory consolidation. In rodent studies, sleep deprivation impairs LTP (Vecsey et al. 2009), memory retention performance in Morris water maze (Guan et al., 2004) and different forms of associative learning tasks (Graves et al. 2003).

Researchers have focused on electrophysiological properties of the sleeping brain and proposed different mechanisms through which sleep facilitates memory consolidation (Tononi and Cirelli, 2006; Marshall and Born, 2007). However, little is known about the molecular mechanism underlying the interaction between sleep and memory consolidation. Our lab demonstrated that sleep deprivation reduces cAMP signaling and impairs cAMP-and PKA-dependent forms of LTP and hippocampus dependent memory. Furthermore, treatment of mice with phosphodiesterase inhibitors rescued those sleep-deprivation-induced deficits (Figure 4.1) (Vecsey et al. 2009). Therefore, it is possible that sleep influences memory consolidation by modulating cAMP signaling cascades. Because Epac-Rap1 signaling is involved in memory consolidation, I explore the impact of sleep deprivation on Rap1 activity.
Figure 1.1 Memory consolidation and synaptic plasticity

A). Using a classical conditioning scheme to demonstrate memory consolidation process.

B). The hippocampal synaptic plasticity is believed to underlie the memory consolidation. The *in vitro* hippocampal long-term potentiation (LTP) at Schaffer collateral fiber is usually triggered to mimic the synaptic plasticity happened during memory consolidation.
Figure 1.1

Modified from Cardin and Abel, 1999
Figure 1.2 Epac is a novel target for cAMP

A). Domain architecture of the Epac proteins. The regulatory region contains one or two CNB (cyclic nucleotide – binding) domains and a DEP (Disheveled, Egl-10, and Pleckstrin) domain. The catalytical region harbors the enzymatically active CDC25-homology domain (CDC25-HD) that is stabilized by the Ras exchange motif (REM) domain, with a Ras-association (RA) domain between the two.

B). The expression pattern for Epac1(a) and Epac2 (b) in Rat brain. The mRNA in situ shows that Epac1 expressed at low level in the brain while Epac2 is highly expressed in the Cortex(Ctx), Hippocampus (H), Cerebellum (Cb) and Olfactory bolb (Ob). Other abbreviations: Caudoputamen (CP), Thalamus (Th) Pons (P) and S substantia nigra (SN).

C). The crystal structure of inactive Epac2 and active Epac2 in complex with Rap1B and the cyclic nucleotide. For simplicity, only the catalytic region and the CNB-B domain (indicated with a dotted line in panel A) are shown. In the inactive conformation, the CNB-B domain sterically hinders binding of Rap to the CDC25-HD, which is relieved by a conformational change induced by binding of cAMP.
Figure 1.2

Modified from Gloerich and Bos, 2010
& Kawasaki et al., 1998
Figure 1.3 8-pCPT-2’-O-Me-cAMP is an Epac specific agonist

A) The cAMP binding (CNB) site of Epac proteins lacks a highly conserved glutamate (E) residue that is presented in all other known CNB domains, which make it possible to design an Epac binding specific cAMP analog.

B) The chemical structure of the 8-pCPT-2’-O-Me-cAMP. The proton of the 2-OH group of the ribose of cAMP has been replaced with a methyl (-CH3) group, and the proton at the 8- position of the base has been replaced with a 4-chlorophenylthio (pCPT) group. The 2’O-Me group increases the specificity for Epac proteins and the 8-pCPT group increases binding affinity for Epac.

C) The binding affinities for Epac and PKA of cAMP analogs. Indeed, 8-pCPT has the highest binding affinity (Ki) for Epac and relative low binding affinities for PKA.
Figure 1.3

A

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<tr>
<td>Epac2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>CAP</td>
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</tr>
</tbody>
</table>

B

![Chemical Structure]

8-pCPT group

2'-O-Me group

C

<table>
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<th>Compound</th>
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<th>K_i PKA R1 α, Bl</th>
<th>K_i PKA RII α, All</th>
<th>K_i PKA RII αBI</th>
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<td>0.0016</td>
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</table>

Modified from Enserink et al. 2002
& Christensen et al. 2003
Figure 1.4 Activation of Epac enhances synaptic plasticity in the hippocampus.

A). The Epac agonist 8-pCPT has no effect on basal synaptic transmission.

B). Pairing 8-pCPT with 1 x 100 Hz stimulation enhances maintenance, but not induction, of LTP relative to controls.

C). The enhancement of LTP described above requires protein synthesis, but not transcription or PKA. The LTP histogram of pairing 1 x100 Hz stimulation with 8-pCPT and several drug treatments, levels of potentiation were compared 120 min after HFS. Emetine, a translation inhibitor, blocked the enhancement of LTP by 8-pCPT. Act D, a transcription inhibitor, or KT5720, a PKA inhibitor, had no significant effect on LTP enhanced by 8-pCPT.
Figure 1.4

Modified from Gelinas et al. 2008
Figure 1.5 Epac is essential for memory retrieval.

After contextual fear conditioning, mice were received 3 daily injections of Epac2 siRNA. On day 4, mice were injected with Epac agonist 8-pCPT and then tested for memory retrieval. Compared with untreated mice, injection of 8-pCPT along enhanced memory retrieval. Injection of Epac2 siRNA, on the other hand, impaired memory retrieval and blocked the enhancement effect caused by 8-pCPT.
Figure 1.5

Modified from Ostroveanu et al. 2010
Chapter 2 Epac enhances long-term memory formation independent of protein kinase A

Abstract

It is well-established that cAMP signaling within neurons plays a major role in the formation of long-term memories - signaling thought to proceed through protein kinase A (PKA). However, here we show that exchange protein activated by cAMP (Epac) is able to enhance the formation of long-term memory in the hippocampus and appears to do so independently of PKA, thus demonstrating the importance of Epac-mediated signaling in memory consolidation.

2.1 Introduction

The molecular mechanisms underlying long-term memory consolidation mediated by cyclic adenosine monophosphate (cAMP) signaling have been extensively studied. It is thought that cAMP regulates memory formation mainly by activating the cAMP sensitive PKA. Once activated, PKA can then phosphorylate various downstream kinases and
transcription factors required for memory formation (Abel and Nguyen, 2008). However, with the discovery of another major target of cAMP known as Epac (de Rooij et al., 1998; Kawasaki et al., 1998), there may be reason to reconsider the precise nature of cAMP in memory formation. Although Epac-mediated signaling has been previously studied in various in vitro preparations, hippocampal long-term potentiation studies (Gelinas et al., 2008; Holz et al., 2008) and now in memory retrieval paradigms (Kelly et al., 2008; Ouyang et al., 2008), its role in long-term memory formation or consolidation has yet to be addressed. In the present study, we examine the effects of Epac activation within the hippocampus on memory consolidation for contextual fear.

2.2 Materials and Methods

2.2.1 Animals

The following experiments were conducted with singly housed 2-4 month old male C57BL/6J mice (Jackson Laboratories). Animals were provided with water and rodent chow ad libitum and maintained on a 12 h light/dark cycle. All experiments conducted in accordance with the policies of the Institutional Animal Care and Use Committee of the University of Pennsylvania and the National Institutes of Health.
2.2.2 Cannulation and Hippocampal infusion

First, mice were anesthetized with isoflurane, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and implanted with a 22 gauge guide cannulae (Plastics One, Roanoke, VA) (stereotaxic coordinates: 1.7 mm posterior to bregma, 1.5 mm from midline and -1.5 mm from the skull surface). Animals were allowed to recover for one week before being trained in the contextual fear conditioning paradigm. Right after the conditioning, drugs were injected bilaterally (0.25 μl/min for 2 min) through injection cannulae that were connected to Hamilton microsyringes via polyethylene tubes using a microinfusion pump (Harvard Apparatus, Holliston, MA). Another minute was allowed for diffusion before the injection cannulae were removed.

2.2.3 Fear conditioning

Fear conditioning experiments were performed as previously described (Graves, et al., 2003). Mice were handled for 1-2 min on 3 consecutive days before fear conditioning.

For contextual fear conditioning, mice were placed in a standard conditioning chamber (Med Associates) for 2 min 28 s after which a single 2 s footshock was given. Mice were
left in the chamber for an additional 30 s before being returned to their home cage. Long-term memory was assessed by measuring freezing behavior (defined as complete immobility except for breathing) during a 5 min test session in the same chamber 24 h after training.

For cued fear conditioning, mice were placed into a context (trained context) for 3 min. At 2 min, a tone (CS) was played in the chamber for 30 s and at 2 min 28 s, a single 0.75 mA footshock (US) was given for 2 s. At 2 min 30 s, both the tone and the footshock stopped and the mice were allowed to explore the chamber A for additional 30 s and then were placed back to their home cages. The mice then received a bilateral hippocampal infusion of the 8-pCPT. Twenty-four hours later, the mice were placed into a different context for 5 min. At 2 min, the same tone (CS) they heard during the training session was played in the chamber B for 3 min. The freezing behavior of the mice were scored and analyzed as pre-CS and post-CS.

2.2.4 Biochemistry
For Rap1 activity assay, hippocampus extracts containing 1500 μg of protein were assayed with a Rap1 activation kit (Millipore). Briefly, lysates were incubated with glutathione-agarose beads precoupled with glutathione-S-transferase (GST) fusion protein, which contains a Rap1 binding domain from the human guanine nucleotide exchange factor specific for the Ras protein Ral. The GST fusion protein precipitates active Rap1 (GTP-Rap1) from the extracts. Samples were then analyzed by western blotting using a Rap1 antibody (Millipore).

For Western blots, the tissue were homogenized in RIPA buffer (Sigma) in the presence of 1% proteinase inhibitor cocktail and photestease inhibitor cocktail (Sigma). The homogenates were centrifuged at 10, 000 g at 4°C for 20 min. The supernatants were applied to 4-12% SDS–PAGE gel, and the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The blot was blocked at room temperature for 1 h in standard TBST Blocking buffer (Thermo). The primary antibodies used were anti-phoesphor MAPK44/42 (1:2000 Cell signaling), pSer845 at GluR1 (1:2000, Millipore), β-tubulin (1:20000, Sigma) and total GluR1 (1:2000, Millipore). The secondary antibodies were horseradish peroxidase (HRP) -conjugated sheep anti-rabbit IgG 1:5000
and HRP-conjugated rabbit anti-mouse IgG 1:20 000. The protein amount was quantified using ImageJ and normalized to β-tubulin.

2.3 Results

2.3.1 Activation of cAMP signaling enhances memory consolidation.

To begin our examination of Epac in memory formation, we tested whether a single intrahippocampal infusion of the PDE resistant cAMP analog Sp-cAMPS (Yusta et al., 1988) would enhance the consolidation of contextual fear memory. Although Sp-cAMPS is commonly used as an activator of PKA, it is also capable of activating Epac (Christensen et al., 2003). In this experiment, mice were fear conditioned with a single low intensity footshock (0.75 mA) to better detect drug-related increases in freezing. This procedure is often used to avoid ceiling effects in freezing levels that might be observed if higher footshock intensities are used (Tronson et al., 2006). Immediately after training, mice received 0, 4.5 or 9 μg Sp-cAMPS (Sigma-Aldrich) bilaterally into the dorsal hippocampus. Long-term memory was assessed 24 h later by measuring freezing behavior in the training context. Mice that received 4.5 μg of Sp-cAMPS showed significantly enhanced long-term memory relative to both the vehicle and high dose groups (F2,25 = 4.11, P = 0.03, ANOVA) resulting in an inverted U-shaped dose-
response curve (Figure 2.1). Hormetic or biphasic responses of this type are characteristic of a broad range of physiological and behavioral responses and may represent an over-compensation in response to disruptions in homeostasis (Calabrese and Baldwin, 2001). Post-hoc analysis revealed that the low dose froze significantly more than either the vehicle or high dose groups (Ps < 0.05, Student-Newman-Keuls). These data and others (Souza et al., 2002) implicate, but cannot distinguish between, Epac and PKA signaling in memory consolidation. Indeed, since Epac and PKA recruit, at least to some extent, different downstream effectors (Holz et al., 2008), such a distinction is critical to further understand the nature of memory consolidation.

2.3.2 Activation of Epac signaling enhances memory consolidation.

To distinguish the Epac signaling and PKA signaling, we repeated the above experiment using the Epac-specific agonist 8-(4-chlorophenylthio)-2'-O–methyladenosine-3', 5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP) (Enserink et al., 2002). In designing this cAMP analog, specific chemical group substitutions were made at positions critical for high affinity binding of cAMP to PKA thereby generating a potent and highly selective agonist for Epac (Enserink et al., 2002; Christensen et al., 2003; Bos, 2006; Ster et al., 2007). Mice were infused with 0, 0.5 or 5 μg 8-pCPT-2'-O-Me-cAMP (Axxora),
and conditioned as described above. ANOVA revealed a significant effect of drug treatment on memory formation (F2, 26 = 10.68, P = 0.0004) again resulting in an inverted U-shaped dose-response curve (Figure 2.2). Post-hoc analysis showed that this difference was due to enhanced freezing in the low dose group relative to both the vehicle and high dose groups (Ps < 0.01, Student-Newman-Keuls). These data suggest that activation of Epac may be sufficient to enhance memory formation and suggest the existence of an alternative pathway through which cAMP regulates consolidation.

To further characterize the effect of 8-pCPT on memory consolidation, an altered-context experiment was performed. Two different batches of BL6 mice were cannulated and trained in context A and received intrahippocampal infusion of 0.5 ug of 8-pCPT right afterwards. For one batch of mice, they were placed in context B (altered context) 24 hour later where their freezing behavior was assessed. Freezing were measure again in the context A (conditioning context) 24 hour later. For the other batch of mice, they were placed in context A first and context B afterwards. The 8-pCPT infused group showed significantly enhanced freezing (p=0.03) in the training context without affect freezing in the altered context (Figure 2.3), suggesting that Epac activation enhances memory consolidation but not generalized freezing. Next, we conducted cued fear conditioning on
a separate batch of mice to rule out the possibility that hippocampal activation of Epac may also enhance hippocampus-independent memory. The freezing levels of 8-pCPT infused mice were no different from that of the saline group, either before and after the tone was played (Figure 2.4). These results indicate that the 8-pCPT infusion specifically enhances hippocampus-dependent memory consolidation without generalizing non-specific fear.

2.3.3 Activation of Epac activates Rap1 activity but not PKA activity.

To support the notion that Epac signaling is an alternative pathway through which cAMP regulates memory consolidation, a number of additional experiments were conducted to examine the function and specificity of the Epac agonist in vivo. Because Epac has previously been reported to activate the neuroplasticity-related G protein Rap1 in vitro (de Rooij et al., 1998), we tested whether 8-pCPT-2′-O-Me-cAMP would also activate Rap1 in the hippocampus. Using a within subject design, separate groups of mice were infused with 8-pCPT-2′-O-Me-cAMP (0.5 μg) in one hippocampus and saline in the other. The hemisphere that received drug was counterbalanced. Hippocampi were then removed and assayed for Rap1 activity. As expected, agonist-infused hippocampi had significantly higher levels of active Rap1 relative to saline-infused hippocampi (P = 0.05, paired t-test,
Figure 2.5). Together with previous findings linking Rap1 to hippocampus-based memory formation (Morozov et al., 2003), these results indicate that Epac-mediated memory enhancements may act through an upregulation in Rap1 signaling.

Due to the possibility that 8-pCPT-2′-O-Me-cAMP might also activate PKA, we determined the specificity of the Epac agonist on a molecular level. To do so, we took advantage of the fact that PKA is specifically required to phosphorylate the GluR1 subunit of AMPA-type glutamate receptors at Ser845 (Lee et al., 2000; Whitlock et al., 2006). Thus, pSer845 levels can be assayed to measure PKA activity and the specificity of the Epac agonist. Here, a within subjects design was used to measure pSer845 levels after intrahippocampal infusion of either vehicle, Sp-cAMPS (4.5 μg), 8-pCPT-2′-O-Me-cAMP (0.5 μg) or 8-pCPT-2′-O-Me-cAMP (0.5 μg) + PKA inhibitor PKI-(14-22)-amide (PKI, Assay Designs) (4 μg). Mice were sacrificed 15 or 45 min after drug infusion after which the hippocampi were removed and assayed by western blotting for phospho- and total GluR1 levels. These time points were chosen to evaluate whether the Epac agonist might activate PKA directly (i.e., with kinetics similar to cAMP) or indirectly, requiring a longer period of time to alter pSer845 levels. Figure 2.6A shows that mice sacrificed 15 min after Sp-cAMPS infusions had significantly greater pSer845 levels (P = 0.04, paired
t-test) in the drug-infused side relative to the saline-treated side. Similarly, Sp-cAMPS-treated mice sacrificed 45 min later showed a strong trend toward a significant increase in pSer845 levels (P = 0.06, paired t-test), possibly due to the long half life of Sp-cAMPS. Interestingly, pSer845 levels appear to be increased in the vehicle-treated hippocampi of mice that also received Sp-cAMPS relative to other vehicle groups. This could be due to the propagation of activity induced in the drug-treated hemisphere to the contralateral side via commissural connectivity. Nevertheless, the Epac agonist alone had no effect on pSer845 levels nor did the agonist in combination with PKI at either time point (P’s > 0.05, paired t-tests). Finally, total GluR1 levels were not significantly altered in any of the treatment groups or time points (P’s > 0.05, paired t-tests, Figure 2.6B).

2.3.4 Activation of Epac signaling ameliorates memory deficits caused by PKA signaling inhibition.

Next, we examined the effect of the Epac agonist when coinfused with specific inhibitors of PKA. This approach has also been used to validate the action of this agonist in hippocampal long-term potentiation studies (Gelinas et al., 2008) and in various in vitro studies (Holz et al., 2008). First, we examined the effects of the Epac agonist when coinfused with the PKI. This form of PKI is myristoylated on the N-terminus to allow
cell membrane permeability and selectively inhibits the free catalytic subunit of PKA (Glass et al., 1989). In this experiment, a single 1.5 mA footshock was used to detect memory deficits caused by PKA inhibition while avoiding potential floor effects related to the expression of non hippocampus-mediated freezing. Figure 2.7A shows that the infusion of PKI (4 μg) alone significantly impaired the consolidation of contextual fear memory. However, these deficits were overcome when 8-pCPT-2'-O-Me-cAMP (0.5 μg) was coinfused with PKI (4 μg). ANOVA revealed an overall effect of treatment group on freezing (F2, 26 = 4.71, P = 0.02) where post-hoc analysis demonstrated significant differences in freezing between the PKI group relative to both the vehicle and the Epac agonist + PKI groups (Ps < 0.05, Student-Newman-Keuls). We also examined whether memory deficits induced by the well-characterized PKA inhibitor Rp-cAMPS (Dostmann, 1995) could be rescued with the Epac agonist. As expected, mice receiving intrahippocampal infusions of Rp-cAMPS (9 μg) after a 1.5 mA footshock showed impaired contextual fear memory (Sananbenesi et al., 2002) but displayed normal memory when the Epac agonist (0.5 μg) and PKA inhibitor were coinfused. ANOVA revealed an overall effect of treatment group on freezing (F2, 25 = 4.04, P = 0.03) where post-hoc analysis demonstrated significant differences in freezing between the Rp-cAMPS group relative to both the vehicle and the Epac agonist + Rp-cAMPS groups (Ps < 0.05, Student-Newman-Keuls). Thus, the data provide strong behavioral evidence that
Epac can modulate memory independent of PKA and can rescue deficits caused by reduced PKA signaling.

2.4 Discussion

The purpose of the present study was to determine whether the cAMP-sensitive molecule Epac can influence memory formation previously thought to occur mainly through the activity of PKA. We demonstrated that treatment of specific Epac agonist is able to enhance memory consolidation and to rescue PKA-related memory deficits. Also, we showed at the molecular level that those behavioral effects were not due to increases in PKA activity. Our findings demonstrate that Epac is able to modulate memory consolidation thereby broadening our understanding of cAMP signaling in memory formation. While there may be some interaction between the Epac and PKA signaling pathways (Nijholt et al., 2008), the nature of this interaction and its effect on memory consolidation remains to be determined. It is worth noting that cAMP-dependent PKA signaling does not have uniform effects on memory formation within all brain regions (Arnsten et al., 2005). Likewise, the effects of Epac activation in different regions will require further investigation. Importantly, these results provide the basis for the development of novel cognition-enhancing drugs that target specific components of the
cAMP-Epac signaling pathway, rather than broad activators of cAMP (e.g. rolipram (Bach et al., 1999)). Indeed, such an approach might be warranted in that mutations in the Epac gene and altered levels of expression have been linked to Autism (Bacchelli et al., 2003) and Alzheimer’s disease (McPhee et al., 2005), respectively.

2.5 Acknowledgements

This work was supported by NMH P50-MH064045 to TA (R. Gur, PI). PJH was supported by a NIH T32NS00743-11 grant in developmental disabilities (M. Robinson, PI) and by NSF Minority Postdoctoral Fellowship 0706858. We thank Dr. Steven Thomas for his suggestions in experiment design and Christopher G. Vecsey for comments on the manuscript.

2.6 Contribution

This chapter is published in Learning and memory as Brief Communication. All experiments are conducted by Nan Ma and Pepe Hernandez.
Figure 2.1 cAMP activation in the hippocampus enhances long-term contextual fear memory.

Effect of a single post-training intrahippocampal infusion of the PKA/Epac activator Sp-cAMPS (0 μg, n = 10; 4.5 μg, n = 8; 9 μg, n = 10). An inverted U-shaped dose response curves were obtained where the low doses significantly enhanced memory measured 24 h after fear conditioning (F2,25 = 4.11, P = 0.03, ANOVA). Post-hoc analysis revealed that the low dose froze significantly more than either the vehicle or high dose groups (Ps < 0.05, Student-Newman-Keuls), Data are expressed as mean ± s.e.m. *P < 0.05, **P < 0.01.
Figure 2.2 Epac activation in the hippocampus enhances long-term contextual fear memory.

Effect of a single post-training intrahippocampal infusion of the Epac-specific activator 8-pCPT-2'-O-Me-cAMP (0 μg, n = 10; 0.5 μg, n = 10; 5 μg, n = 9). Again, an inverted U-shaped dose response curves were obtained where the low doses significantly enhanced memory measured 24 h after fear conditioning (F2, 26 = 10.68, P = 0.0004). Post-hoc analysis showed that this difference was due to enhanced freezing in the low dose group relative to both the vehicle and high dose groups (Ps < 0.01, Student-Newman-Keuls). Data are expressed as mean ± s.e.m. **P < 0.01.
Figure 2.3 Post-training infusion of the Epac agonist does not enhance the freezing level in the altered context.

Mice received 0.5 ug 8-pCPT intrahippocampal infusion showed significantly higher freezing levels in the long-term memory test compared with saline injected mice (p=0.034). However, the freezing levels in the altered context were the same as saline infused mice. Data are expressed as mean ± s.e.m. *P < 0.05.
Figure 2.3

- Saline n=15
- 8-pCPT 0.5 ug n=14

% freezing

Altered context | Trained context

* Statistical significance
Figure 2.4 Infusion of the Epac-specific agonist does not affect cued fear memory consolidation

8-pCPT intrahippocampal infused mice showed similar freezing levels as the saline treated mice in the 24 h memory test for cued fear conditioning (hippocampus-independent) task. Data are expressed as mean ± s.e.m.
Figure 2.4

- Saline n=10
- 8-pCPT 0.5 μg n=10

% freezing

pre-CS
CS
Figure 2.5 Infusion of 8-pCPT increases hippocampal Rap1 activity.

Quantitative analysis of active Rap1 immunoreactivity 5 min after infusion of 0.5 μg 8-pCPT revealed significantly higher hippocampal GTP-Rap1 levels in the hemisphere that received 8-pCPT infusions (p=0.05, paired t-test). A representative immunoblot of active Rap1 in the hippocampus. Data are expressed as mean ± s.e.m. *P < 0.05,
Figure 2.5

![Graph showing relative Rap1 activity](image)

- **Saline n=6**
- **8-pCPT n=6**

![Western blot images](image)

- **GAPDH**
- **Total Rap1**
- **Active Rap1**

Values:
- **37**
- **20**
Figure 2.6 Infusion of the Epac-specific agonist does not change PKA-dependent phosphorylation of GluR1 receptor subunits in the hippocampus.

(A) phospho-GluR1 (pSer845) and (B) total GluR1 levels in the hippocampus 15 or 45 min post-drug infusion (open and shaded bars, respectively). (A) pSer845 levels were significantly increased 15 min after unilateral infusion of Sp-cAMPS (4.5 μg) relative to the saline-treated side (*P = 0.04). Infusions of the Epac agonist or the combination of the agonist and PKI had no effect at either time point relative to their respective vehicle-treated hippocampi (8-pCPT-2’-O-Me-cAMP, 0.5 μg; PKI (4 μg) + 8-pCPT-2’-O-Me-cAMP (0.5 μg)). A strong trend (P = 0.06) toward an increase in pSer845 levels was observed for the Sp-cAMPS-infused hippocampi at 45 min. n = 5 for all treatment groups and time points (B) Total GluR1 levels were unaffected by drug treatments at either time point. (C,D) Representative immunoblots of pSer845, β-tubulin (and total GluR1 levels 15 min (C) or 45 min (D) after infusion. All values were normalized to β-tubulin levels within individual samples to control for differences in total protein loaded and then to mice that received mock injections (black bars in A and B, n = 4). Data are expressed as mean ± s.e.m.
Figure 2.6

A  
**phospho-GluR1 (Ser845) Levels**

- Fold change relative to mock infusion
- 15 min post-infusion
- 45 min post-infusion

B  
**Total GluR1 Levels**

- Fold change relative to mock infusion
- 15 min post-infusion
- 45 min post-infusion

C  
15 min post-infusion

D  
45 min post-infusion

- pSer845
- β-tubulin
- Total GluR1
Figure 2.7 Coinfusion of the Epac-specific activator with a PKA inhibitor leaves fear memory intact.

(A) Inhibition of PKA with PKI significantly impaired contextual fear memory relative to saline-treated animals, whereas the combination of PKI and 8-pCPT-2′-O-Me-cAMP blocked this effect (saline, n = 10; PKI, 4 μg, n = 10; PKI (4 μg) + 8-pCPT-2′-O-Me-cAMP (0.5 μg), n = 9). (B) The coinfusion of 8-pCPT-2′-O-Me-cAMP with Rp-cAMPS enhanced long-term memory relative to Rp-cAMPS infusion alone (saline, n = 9; Rp-cAMPS, 9 μg, n = 9; Rp-cAMPS (9 μg)+ 8-pCPT-2′-O-Me-cAMP (0.5 μg), n = 10). Data are expressed as mean ± s.e.m. *P < 0.05.
Figure 2.7

A

- Saline n=10
- 4 μg PKI n=10
- 4 μg PKI + 0.5 μg 8-pCPT n=10

B

- Saline n=10
- 9 μg Rp-cAMPs n=8
- 9 μg Rp-cAMPs + 0.5 μg 8-pCPT n=8
Chapter 3 Reduction of hippocampal Epac2 levels

impairs memory consolidation

Abstract

The Activation of Epac-Rap1 signaling enhances long-term contextual fear memory. Here, I show that hippocampal Rap1 activity increases after training in a contextual fear task. Furthermore, mice injected intrahippocampally with Epac2shRNA AAV exhibited low hippocampal Epac2 expression and exhibited impaired hippocampus-dependent memory, demonstrating that Epac-Rap1 signaling is involved in memory consolidation.

3.1 Introduction

Both transgenic animal model studies and in vitro and in vivo pharmacological studies have demonstrated that cAMP signaling plays a role in memory consolidation (Bernabeu et al. 1996; Wang et al. 2003; Isiegas et al. 2008). Among the three known family of cAMP targets (protein kinase A (PKA), hyperpolarization-activated cyclic nucleotide–
modulated (HCN) channels and exchange protein activated by cAMP (Epac)), PKA is the most extensively studied. Once activated by cAMP, PKA phosphorylates various downstream kinases and transcription factors required for memory consolidation (Abel and Nguyen 2008). Among the HCNs, HCN1 is enriched in the hippocampus (Biel and Michalakis 2009) and is believed to contribute to the emergence of theta rhythm (Morris et al. 2004; Herrmann et al. 2007), which is important for learning and memory processes. In addition, the studies on HCN1 KO mice reveal that hippocampal HCN1 constrains spatial memory and synaptic plasticity (Nolan et al. 2004). As for the role of Epac, studies has shown that activation of hippocampal Epac signaling positively modulates synaptic plasticity (Gelinas et al. 2008; Ma et al. 2009) and memory retrieval (Ouyang et al. 2008; Ostroveanu et al. 2010). In Chapter 2, I demonstrated that the activation of Epac enhances memory consolidation.

In the previous chapter, I presented evidence that activation of Epac signaling increases the activity of Rap1, the major target for Epac. Mice with reduced Rap1 activity exhibited deficits in synaptic plasticity and several forms of memory (Morozov et al. 2003). Therefore, Epac signaling may modulate memory consolidation through Rap1. However, there is a missing link here: does Rap1 activity increase after a learning task, as both cAMP and PKA activity do (Bernabeu et al. 1996; Izquierdo et al. 2000)? In this chapter,
I first show that, similar to cAMP and PKA, Rap1 activity increases after training in a fear conditioning task.

To further study the role of Epac signaling in memory consolidation, I then determined whether Epac signaling is required for memory formation. The approaches to inhibit Epac activity are limited. The only Epac antagonist characterized, brefeldin A, has unspecific effects by blocking Arf-GEF, a small G-protein that is essential for vesicular trafficking, (Donaldson et al. 1992). As for transgenic mouse models, an Epac2 mutant mouse exhibited lethality at very young ages (Shibasaki et al. 2007) and therefore not suitable for behavioral analysis. To avoid the lethal effect of complete loss of Epac2 in development, I used a viral approach to stably express Epac2shRNA via adeno-associated virus (AAV). The AAV is widely used to stably express the carrier gene (or shRNA in this case) in cells or tissues. The Epac2 shRNA expressed by the virus is a small hairpin RNA, which specifically binds to and cleaves Epac2 mRNA and resulting in a reduction in Epac2 protein levels. Using intrahippocampal injections of Epac2shRNA AAV, I was able to assess the effect of reduced levels of Epac. Here I show results of my behavioral analyses of the virus-treated mice. These include analyses of exploratory behavior,
locomotor activity, anxiety levels, motor learning and memory, and hippocampus-dependent memory.

3.2 Materials and Methods

3.2.1 Intra-hippocampal viral injection

Mice were anesthetized with isoflurane, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and two holes were drilled at anteroposterior from bregma, −1.9 mm; lateral, ±1.5 mm after which a nano syringe needle was lowered to a depth of 1.5 mm from dura. One ul of virus (Epac2 shRNA or control virus, at viral titer of $10^9$) were delivered at the rate of 0.125 ul per m. After injection, the needle remained in place an additional 1 min to allow for diffusion. A heating blanket was used during the injection procedure to maintain a consistent body temperature. After injection, mice were singly housed and allowed to express the virus for 4 weeks to let the shRNA fully expressed. On the 5th week after surgery, mice were tested in open field, zero maze, and rotarod. On week 6, mice were fear conditioned. After the experiment, animals were sacrificed, and the hippocampi were collected for biochemical assays.
The Epac2shRNA AAV virus was provided by Dr. Simen from Yale University. The virus was created based on the methods described in (Wang et al. 2007). Briefly, Epac2shRNA was first ligated into a pAAV vector, which then co-transfected with pDG helper plasmid into HEK-293 cells to produce adeno-associated viral particles (AAV). The control shRNA virus was created containing a scrambled sequence (shRNA-scrEpac2) of the shRNA-Epac2shRNAa target sequence with no homology to any mammalian gene in the Genbank database.

3.2.2 Fear conditioning

Fear conditioning experiments were performed as described previously (Ma et al. 2009). Fear conditioning was performed in standard conditioning chambers (Med Associates Inc., Vermont, GA) and an automated video tracking system was used to measure freezing behavior (Clever Sys, Inc., Washington, DC). Mice were handled (in all experiments) for 1 min for 3 consecutive days before fear conditioning. For the contextual fear conditioning, mice were placed in the conditioning chamber and after 2 min 28 s received a mild 2 s, 1.5 mA footshock. Mice were left in the chamber for an additional 30 s before returning to the home cage. No tone was played at any time. Freezing behavior was scored during pre-shock and post-shock intervals during the
training session. Twenty-four hours after training, mice were placed back in the conditioning chamber for 5 min for long-term memory testing, and freezing behavior was scored.

Cued fear conditioning was performed as described previously (Graves et al. 2003), on the day of training, mice were placed in the conditioning chamber and after 2 min, a 2900 Hz 85 db tone was played in the chamber for 30 s. The tone co-terminated with a 2 s 1.5 mA footshock. Mice were left in the chamber for an additional 30 s before returning to the home cage. Freezing behavior was scored during pre-tone, post-tone, and post-shock sessions. Twenty-four hours after the training, mice were placed in a novel chamber with a different shape, size and odor (2% lemon Joy detergent) located in a different room from training chamber. After 2 min, the tone (2900 Hz, 85db) were played for 3 min and the freezing behavior were scored during 2 min pre-CS (pre-tone) session and 3 min post-CS (post-tone) session. Different batches of mice were used for contextual or cued fear conditioning.

3.2.3 Open field
The open field task was performed as described previously (Kelly et al. 2009). Mice were individually placed in a 41 × 41 cm San Diego Instruments Open Field box for 30 min. The box was equipped with 16 × 16 motion detector beams (every 2.54 cm). Ambulations, breaks of two contiguous beams, were calculated for the center part, periphery part, and total field.

3.2.4 Zero maze

The zero maze task was performed as described previously (Favilla et al. 2008). The zero maze apparatus consists of a raised circular track divided into two open and two closed quadrants. The track had an internal diameter of 40.5 cm and a width of 5.1 cm and was elevated off the floor at a height of 40 cm. The closed quadrants had walls 11 cm high. Mice were placed into the center of a closed quadrant and were allowed to explore the elevated zero maze for 5 min in a single session. Two anxiety-related variables were recorded including time spent in open quadrants and number of transitions into open quadrants, which was operationally defined as the time when all 4 paws first entered the open quadrant.
3.2.5 Rotarod test

The rotarod test was performed as previously described (Oliveira et al. 2006). The day before testing, mice were placed on the rotarod running at 4 rpm for 30 s. The next day, mice were placed on the same rotarod, which accelerated at a constant rate from 4 to 40 rpm in 5 min, for a maximum time of 5 min. Mice were allowed to perform the test until they fell from the rod and given three trials with 60 min inter-trial intervals. The test was repeated for 3 days and the duration of time each mouse stayed on the rod in each trial were recorded.

3.2.6 Order of testing

The mice were exposed to fear conditioning tasks only once, and the tasks were the last to be performed on any group of animals. Before contextual fear conditioning training, mice were tested in the open field, the zero maze and the rotarod in that sequence.

3.2.7 Rap1 activity assay

As described in Chapter 2. Briefly, hippocampal extracts containing 1500 μg of protein were then assayed with a Rap1 activation kit (Upstate). Briefly, lysates were incubated
with glutathione-agarose beads pre-coupled with glutathione-S-transferase (GST) fusion protein, which contains a Rap1 binding domain from the human guanine nucleotide exchange factor specific for the Ral. The GST fusion protein precipitates active Rap1 (GTP-Rap1) from the extracts. Samples were then analyzed by western blotting using a Rap1 antibody (Upstate).

3.2.8 Western blot analysis
The mouse hippocampi tissues were homogenized in Rap1 activation lysing buffer (Millipore) in the presence of 1% protease inhibitor and phosphatase inhibitor cocktail (Sigma). The homogenates were centrifuged at 10 000 g at 4 °C for 20 min. The supernatants were applied to 4-12% SDS–PAGE gel, and the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The blot was blocked at room temperature for 1 h in standard Tris-Buffered Saline and Tween 20 (TBST) Blocking buffer (Thermo). The primary antibodies used were anti-Epac2 antibody 1:500 (Santa Cruz, CA), anti-Rap1 1:1000 (millipore) and anti-beta tubulin 1:20 000(Sigma). The secondary antibodies were horseradish peroxidase (HRP)-conjugated donkey anti-sheep immunoglobulin (Ig)G 1:1000, HRP-conjugated sheep anti-rabbit IgG 1:2000 and HRP-conjugated rabbit anti-
mouse IgG 1:20 000. The protein amount was quantified using ImageJ and normalized to β-tubulin.

3.3 Results

3.3.1 Rap1 activity increases during memory consolidation.

Here, I further established the relationship between Rap1 activity and memory consolidation. C57BL/6J mice were contextual fear conditioned and hippocampal Rap1 activity was measured 30 min after conditioning. Hippocampal Rap1 activity was increased by 183.4±17.4% (t test, p<0.05) in fear conditioned mice compared to home cage control mice (Figure 3.1). Both fear conditioned mice and home cage control mice were sacrificed during a similar time of the day to control for circadian effects and effects of handling. This result is consistent with the previous observations that transgenic mice with decreased Rap1 activity showed impairments in synaptic plasticity and hippocampus-dependent memory tasks (Kawasaki et al. 1998; Morozov et al. 2003).

3.3.2 Dorsal hippocampal injection of Epac2shRNA AAV virus decreases levels of Epac2 protein.
Next, I investigated whether reduction of Epac activity impairs memory consolidation. Epac2 is the predominant isoform expressed in the brain (Kawasaki et al. 1998) and was therefore chosen as the target of Epac2shRNA. AAV virus vector containing Epac2 shRNA was used to reduce the expression of Epac2 via bilateral intrahippocampal injection and/or infusion. In the hippocampus, the virus expressed a small hairpin RNA, which then bound to and cleaved Epac2 mRNA to reduce the protein expression levels of Epac2. Initially, two forms of Epac2shRNA AAV virus were tested for their abilities to reduce the Epac2 expression in vivo (Ep2aV and Ep2bV). Because the concentrations of the virus were relatively low (at viral titers of 10^7), the virus were infused for three consecutive days via hippocampal cannula (as described in Chapter 2), and the hippocampi were collected one week later to measure the Epac2 protein expression levels via western blot. Ep2bV injected mice showed lower reduction of Epac2 (54.4%±11.2%, *p=0.023, student’s t-test) and therefore this viral construct was used in the subsequent behavioral studies (Figure 3.2).

However, a different injection protocol was used for the following reason. We performed multiple behavioral tasks to assess the effect of Epac2 reduction on memory formation and other basal behaviors. This requires one week of recovery from the surgery and three weeks of behavioral testing. Including the 3 days of injection, the whole procedure took
4.5 weeks Because cannula do not remain attached for this length of time and the loss of cannula can cause damage to brain tissue and affect behavioral outcomes, we used direct injections instead (see Methods). Because mice were given a single injection, a higher concentration of virus (at viral titers of $10^9$) was used. The mice were also given a longer time before the behavioral tests to let the virus fully expressed. The mice were sacrificed after the completion of the behavioral evaluation process, which is 6 weeks after injection. As expected, the Epac2 protein level significantly decreased (Figure 3.3, t test, p<0.05) in the hippocampus of Epac2 shRNAb AAV virus (Ep2bV for short) injected mice compared to controls.

3.3.3 Mice with reduced hippocampal Epac2 expression showed impaired hippocampus dependent memory consolidation.

I hypothesized that hippocampus-dependent memory consolidation would be impaired in mice with reduced Epac activity. But first, I wanted to see if Ep2bV injected mice had any behavior alterations that might affect their performance in the hippocampus-dependent task. Open field was used to measure locomotor activity, zero maze was used to measure anxiety related behaviors, and rotarod was used to measure locomotor learning and memory. Both Epa2V and control virus injected mice spent similar time
exploring the central and peripheral parts of the arena in the open field test (Figure 3.4). In addition, the total exploring time is the same between the groups suggesting that they had similar locomotor activity. Both groups also spent similar time in open quadrants of the zero maze and had similar transition entries to open quadrants (Figure 3.5), indicating they had similar anxiety levels. The three-day learning curves on the rotarod of the Ep2bV injected mice were similar to that of the control group, suggesting they had normal motor learning and memory (Figure 3.6). In conclusion, no basal behavioral abnormalities in mice with reduced hippocampal Epac2 levels were found.

Next, hippocampus-dependent memory was evaluated using the contextual fear conditioning task. Six weeks after viral injection, the Ep2bV group showed significantly lower freezing levels compared to control group in the 24 h memory test (Figure 3.7), indicating impairment in memory formation. Different groups of mice were also tested in cued-fear condition task, a hippocampal-independent memory paradigm. Ep2bV injected mice showed similar freezing levels both in pre-CS session and in CS session of the 24 h memory tests (Figure 3.8), suggesting that hippocampus-independent memory is not affected by the viral injection.
3.4 Discussion

In this present study, I first showed that Rap1 activity increases after fear conditioning, indicating that Epac-Rap1 signaling may play a role in memory consolidation. I next found that Epac signaling is required for memory formation. Hippocampal injection of Epac2AAV shRNA reduced the expression of Epac2, a predominant member of Epac family. Although Epac signaling is reduced, the mice showed normal locomotor activity, anxiety levels and motor learning and memory. The mice showed long-term memory impairment in a hippocampus-dependent task, but hippocampus-independent memory formation is not affected.

According to cellular studies, The direct targets of Epac include Rap1, Rap2, Rims, and R-Ras (de Rooij et al. 1998; de Rooij et al. 2000; Lopez De Jesus et al. 2006). Among all these targets, Rap1 and Rims are mostly likely to be involved in the modulation of memory consolidation. Rap1 was reported to activate a distinct memory associate pool of MAPK42/44 and therefore affected synaptic plasticity (Morozov et al. 2003). MAPK42/44 activities increase 30 min after a learning task (Sindreu et al. 2007). Hippocampal slices treated with 15 min of Epac agonist 8-pCPT incubation also showed increase MAPK42/44 activities (Gelinas et al. 2008). I therefore tried to measure the
activity of MAPK42/44 15 min after the Epac agonist 8-pCPT injection (Figure 3.9) but was unable to detect any increases. Because Rap1 activity increased only 5 min after the 8-pCPT injection, it is possible that the MAPKs were activated at 5 min and return to normal levels by 15 min. A more comprehensive time course of MAPK activation after 8-pCPT injection would be helpful to confirm the link between Rap1 and MAPK.

Rim1 is another possible Epac target that could pre-synoptically regulate the memory consolidation. In vitro studies found that Epac2 directly binds to Rim1 and Rim2 and that activation of Epac leads to a Rim2-dependent exocytosis in PC12 cells (Ozaki et al. 2000; Kashima et al. 2001). Rim1, on the other hand, is enriched in the brain (Sun et al. 2003) and is crucial for the pre-synaptic component of cAMP-dependent mossy fiber LTP, Schaffer collateral late-phase LTP and learning and memory (Castillo et al. 2002; Powell et al. 2004). It is worth noting that PKA phosphorylation of Rim1 is not required for mossy fiber LTP or learning (Kaeser et al. 2008; Yang and Calakos 2010). Since Rim1 and Rim2 are highly homologous proteins (Mittelstaedt et al. 2010), it is possible that Epac2 acts via Rim2. To test this hypothesis, the role of Epac in mossy fiber LTP needs to be established and then the functional analysis of Rim1 and Epac2 is required. The Epac2KO line from Gabriel’s lab will be a good model for such tests.
The fact that Epac regulates memory consolidation suggests that transcription/translation processes are also regulated by Epac. Numerous cellular studies reported that Epac activation did not alter levels of phosphor-CREB (Delghandi et al. 2005; Chaudhuri et al. 2007), the most well-known transcription factor regulated memory consolidation. Rim mainly function at the pre-synaptic sites and has little interaction with transcription machinery. Then what may be the link between Epac and transcription/translation? There are two potential pathways. One is through the transcription factor CCAAT/enhancer binding protein (C/EBP), most likely isoforms C/EBPβ or C/EBPδ. The expression levels of these two isoforms significantly increases between 6 and 9 h after inhibitory avoidance training in rat (Taubenfeld et al. 2001). And reduction of hippocampal C/EBPβ impairs memory consolidation of inhibitory avoidance task (Taubenfeld et al. 2001). C/EBPδ knock-out mice, on the contrary, showed selective enhancement of contextual fear conditioning (Sterneck et al. 1998). Epac activation increased C/EBP DNA binding activity and induced activation of C/EBP reporter in human umbilical vein endothelial cells and mouse embryonic fibroblast cells. Deletion of C/EBP isoforms C/EBPβ or C/EBPδ blocked the cAMP-dependent expression of a C/EBP target gene (Yarwood et al.)
2008). Therefore, it is possible that Epac indirectly activates C/EBP and regulate memory consolidation-related gene expression.

Another potential pathway through which Epac regulates gene transcription and translation is mTOR signaling. mTOR is a key regulator of translational initiation. In rat hippocampus, mTOR is localized at the post-synaptic sites and applying rapamycin, an inhibitor of mTOR signaling, reduces the late phase of 4 train LTP (Gelinas et al. 2007). Pharmacological studies of rapamycin demonstrate that mTOR signaling is required for auditory cortex-dependent memory, amygdala-dependent fear memory and hippocampal-dependent fear memory (Gafford et al. 2011). A study in human prostate cancer cells reported that Epac-mediated cellular effects require activation of mTOR signaling cascades (Misra and Pizzo, 2009) Therefore, it is possible that Epac modulates translation via MTOR signaling.

Like Rap1 activity, studies in rats showed that the amount of cAMP and the activity of PKA in the hippocampus increases after leaning a step-down inhibitory avoidance task (Bernabeu et al. 1996; Izquierdo et al. 2000), cAMP increases 30 min and 180 min
after the training whereas PKA activity increases 5 min and 180 min, but not around 30 min (22 min) after the task. It is possible that cAMP levels keep elevated from 5 min to 30 min after training, and PKA responded quickly to the cAMP level changes, reached to the peak at 5 min and then quickly fell back to the base level. Since inhibitory avoidance and fear conditioning are tasks used for generating aversive hippocampus-dependent associative memory, the molecular activities in the hippocampus after inhibitory avoidance and fear conditioning tasks are comparable. My data showed that Epac activity elevated 30 min after training, at which time PKA activity supposes to be back to the baseline. This observation suggests that Epac appears to have a different response pattern to the cAMP changes, where Epac responds to cAMP either slower or persist longer than PKA. The two distinctive cAMP response patterns may shed some light on functional differentiation of these two cAMP targets. Another interesting observation is that there is another round of activation of cAMP and PKA 180 min after the learning. I also observed such trend of Epac activity enhancement (Figure 3.10, p=0.05) but at a later time point, 240 min after training. This is consistent with the conclusion that Epac may have a distinct response pattern to cAMP. In addition, I attempted to determine whether injection of 8-pCPT 240 min after training alters memory consolidation. Due to the high freezing levels displayed by control group (saline), I was unable to detect any changes in the 8-
pCPT group. With an optimized handling and injection protocol, this experiment is worth repeating.

I also attempted to measure Rap1 activity in the Ep2bV injected mice (data not shown) but without success. For one thing, basal Rap1 activity is relatively low by itself and attempts to detect further reduction is difficult. More importantly, viral injected brain tissue was more difficult to homogenize than intact tissue, which resulted in lysate containing less Rap1 to work with. Elongation of homogenation time or stronger lysing buffer increase the speed of Rap1-GTP hydrolyzation and resulted in loss of Rap1 activity signaling. In future studies, a separate batch of injected mice could be used to measure the Rap1 activity 30 min after fear conditioning, when the basal Rap1 activity is higher.

The reduction in Epac2 protein levels that we observed after Ep2bV injection is not as high as initially expected. It is possible that I did not identify the time window when Epac2shRNA was expressed at maximum levels and Epac2 was reduced to its minimum levels. It looks like repeated injections give better reductions, and the virus needed less time to express than expected. In future studies, further optimizing of injection protocol
will be necessary. Alternatively, a transgenic mouse line with reduced Epac expression is
needed to further analyze the role of Epac in synaptic plasticity and explore other targets
of Epac signaling involved in the memory consolidation. Recently, an Epac2KO line was
reported from Graybiel’s lab at MIT recently. This line could be a good tool for these
studies.

In conclusion, the reduction of Epac2 expression in the hippocampus impairs long-term
memory, suggesting Epac-Rap1 signaling is required for memory consolidation. Since
reduction of Epac expression is linked with various psychiatric disorders including
Alzheimer’s (McPhee et al. 2005), Further studies of mice with reduced Epac expression,
caused by either viral injection or transgenic expression, will help us not only understand
the molecular mechanism underlying learning and memory, but also the pathology and
potential treatments to the cognitive aspect of those psychiatric disorders.
Figure 3.1 Fear conditioning enhances hippocampal Rap1 activity.

Eight male BL6 mice from in-house breeding were randomly divided into two groups, one group was fear conditioned (as described in the Methods) when the other group were left in the home cage undisturbed. 30 minute after the conditioning, the hippocampal Rap1 activity was significantly increased in the fear conditioning group (FC) compared with home cage control group (HC) (* p=0.01). Data are expressed as means ± SEM and n represents the number of mice.

Rap1 activity was measured by the relative amount of Rap1-GTP, which was pull down by the Rap1-GTP-binding-peptide-conjugated agarose beads, blotted with anti-Rap1 and normalized with the total Rap. A sample of bolts is shown, the top panel is the blot from the Rap1 pull-down assay (Rap1-GTP) while the bottom panel is from the lysate before the pull-down (total Rap1) to control for loading differences during the Rap1 pull down assay.
Figure 3.2 Epac2shRNAb AAV virus (Ep2bV) significantly reduced Epac2 protein level.

Three groups of male BL6 received repeated hippocampal injection of two types of Epac2-shRNA AAV virus (Ep2aV and Ep2bV) and negative control virus (control), respectively. The hippocampal expression of Epac2 was significantly down-regulated with Ep2bV injection (54.4%±11.2%, * p=0.023, student’s t-test). Ep2aV injected group also showed decreased Epac2 expression levels (71.4%±9.9%), but not as low as Ep2bV. Ep2bV were selected for the follow-up experiments. Epac2 expression levels were normalized with Tubulin. A sample blot is shown. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.2

![Graph showing relative Epac protein levels after viral injection.](Diagram)

- **Control** n=15
- **Ep2bV** n=15

p=0.012

**Western Blot Analysis**

- **Epac2**
- **β-Tubulin**
Figure 3.3 Hippocampal Epac2 expression levels were significantly down-regulated six weeks after Ep2bV injection.

Epac2 expression levels were significantly down-regulated six weeks after Ep2bV injection. (83.9%±4.4%, * p=0.012, student’s t-test), Epac2 expression levels were normalized with Tubulin. A sample blot is shown. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.3

![Graph showing relative Epac protein levels after viral injection. The graph compares control (n=15) and Ep2bV (n=15) groups. The p-value is 0.012.](image)

**Legend:**
- Control
- Ep2bV

**Protein Levels:**
- Epac2
- β-Tubulin
Figure 3.4 Ep2bV injected mice showed normal locomotor activity in the open field.

Four weeks after hippocampal injection of Ep2bV and control virus, mice were placed in the open field. Compared with control group, the Ep2bV group showed similar activity levels both in the central part of the arena and the peripheral part of the arena. The total movement activities (Total) are similar between the two groups as well. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.4

Index of movement in the open field

- Control n=16
- Ep2bV n=15
Figure 3.5 Ep2bV injected mice exhibited no deficits during the rotarod learning task.

Five weeks after viral injection, Both Ep2bV and control group were trained with rotarod task for three consecutive days, each day for three trials. The learning curve (measured by latency to fall off the rotarod during each trial) of the Ep2bV group showed no differences with the curve of the control group. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.5

![Graph showing latency to fall off the rotarod (sec) for Control n=16 and Ep2bV n=15 over trials and days.](image)
Figure 3.6 Mice with Ep2bV injection showed normal anxiety related behavior in the elevated zero maze.

Four weeks after the viral injection, mice were placed on an elevated zero maze to measure anxiety levels. No differences of the number of entries to open quadrant (top) or the time spent exploring open quadrant (bottom) were observed between Ep2bV and control group. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.6

A

Number of entries to open quadrant of the zero maze

Control n=16
Ep2bV n=15

B

Exploring time in open quadrant of the zero maze (s)

Control n=16
Ep2bV n=15
Figure 3.7 The viral injection of Ep2bV impaired hippocampal-dependent memory.

Six weeks after the viral injection, the mice were trained with contextual fear conditioning paradigm. The Ep2bV group showed similar freezing levels before (pre-shock) and after (post-shock) the shock was delivered during the training session. In the 24 testing, the mice in the Ep2bV group exhibited significant lower freezing levels (36.4%±2.53%, p=0.003, student’s t-test). Data are expressed as means ± SEM and n represents the number of mice. * P<0.05
Figure 3.7

The graph shows the percentage of freezing behavior in two groups: Control (n=16) and Ep2bV (n=15) across three time points: pre-shock, post-shock, and 24h. The x-axis represents the time points: Training and Testing. The y-axis represents the percentage of freezing. The black bars represent the Control group, and the white bars represent the Ep2bV group. There is a significant difference (*) between the groups in the 24h post-shock time point.
Figure 3.8 Ep2bV injected mice exhibit normal hippocampal-independent memory.

Six weeks after the viral injection, mice were trained with cued fear conditioning paradigm. The Ep2bV group showed similar freezing levels before (pre-shock) and after (post-shock) the shock was delivered during the training session. In the 24 h testing session, the Ep2bV group showed no differences before (pre-CS) or after (post-CS) the cue was played. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.8

- Control n=14
- Ep2bV n=15

% freezing

pre-shock | post-shock | pre-CS | CS

Training | 24h Testing
Figure 3.9 Infusion of the Epac-specific agonist does not change phosphorylation levels of MAPKs in the hippocampus.

Phospho-MAPK44 (ERK1) and phospho-MAPK42 (ERK2) levels do not change 15 min after unilateral infusion of 8-pCPT (1 μg) relative to the saline-treated side. All values were normalized to β-tubulin levels within individual samples to control for differences in total protein loaded. Data are expressed as mean ± s.e.m.
Figure 3.9

![Graph showing relative phosphor-MAPKs activities for MAPK 44 and MAPK 42. The graph compares saline (n=5) and 8-pCPT (1 ug) (n=5) treatments.](image-url)
Figure 3.10 Hippocampus Rap1 activity increases 4 h but not 2 h after fear conditioning.

Fifteen male BL6 mice from in-house breeding were randomly divided into four groups, two groups were fear conditioned (FC) (as described in the methods) while the other two group were left in the home cage (HC) undisturbed. Two hours after the conditioning, one HC group and one FC group were assayed for Rap1 activity and no differences were detected. Four hours after the conditioning, the remaining mice were assayed for Rap1 activity. The hippocampal Rap1 activity showed a strong trend towards increase (#,p=0.053, student’s t test) in the FC group compared with HC. Data are expressed as means ± SEM and n represents the number of mice.
Figure 3.10
Chapter 4 Rap1 activity and sleep deprivation

Abstract

Sleep deprivation impairs cAMP signaling and the reduction in cAMP levels following sleep deprivation appears to cause memory deficits. Here I show that the activity of Rap1, a target of Epac signaling, decreases after sleep deprivation, suggesting the memory deficits caused by sleep deprivation may be related to reductions in Epac signaling.

4.1 Introduction

In the early years of 20th century, the hypothesis that sleep favors memory consolidation was proposed (Jenkins and Dallenbach 1924). More and more compelling evidence to support this hypothesis has been obtained in the years since then. In human studies, participants with a period of post-learning sleep perform better in word recognition and word pair association tasks (Benson and Feinberg, 1975; Schoen and Badia, 1984; Drosopoulou et al. 2006; Gais et al., 2006), indicating improved memory consolidation.
In rodent studies, sleep deprivation impairs LTP (Vecsey et al. 2009), memory retention performance in Morris water maze (Guan et al., 2004) and different forms of associative learning tasks (Graves et al. 2003).

Researchers have focused on electrophysiological properties of the sleeping brain and proposed different mechanisms through which sleep facilitates memory consolidation: (Tononi and Cirelli, 2006; Marshall and Born, 2007). However, little is known about the molecular mechanism underlying the interaction between sleep and memory consolidation. Our lab demonstrated that sleep deprivation reduces cAMP signaling and impairs cAMP-and PKA-dependent forms of LTP and hippocampus dependent memory. Furthermore, treatment of mice with phosphodiesterase inhibitors rescued those sleep-deprivation-induced deficits (Figure 4.1) (Vecsey et al. 2009). Therefore, it is possible that sleep influences memory consolidation by modulating cAMP signaling cascades. Because Epac-Rap1 signaling is involved in memory consolidation, I explore the impact of sleep deprivation on Rap1 activity.

4.2 Materials and Methods
4.2.1 Animals

C57BL/6J adult male mice (2–4 months of age) were housed individually on a 12 h/12 h light/dark schedule with lights on at 7:00 (ZT0). Food and water were available ad libitum throughout the experiment. The sleep deprivation protocol was described before (Vecsey et al. 2009). Each animal was handled daily for 6 days before sleep deprivation. Starting at approximately ZT0, mice were sleep-deprived (SD) in their home cages for 5 h by gentle handling. Non-sleep-deprived (NSD) mice were left undisturbed in their home cages. All experiments were approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with all National Institutes of Health guidelines.

4.2.2 Biochemistry

Hippocampi were dissected from SD and NSD mice and immediately frozen in a dry-ice and ethanol slurry. Samples were sonicated in Rap1 activation buffer (Upstate, freshly added protease inhibitor and phosphatase inhibitor cocktails, 1:100 respectively). The lysate containing 1500 μg of protein were then assayed with Rap1 activation kit (Upstate). Briefly, lysate were incubated with glutathione-agarose beads slurry pre-coupled with glutathione-S-transferase (GST) fusion protein for 45 min at 4 degree. The GST fusion
protein precipitates active Rap1 (GTP-Rap1) from the extracts. Next, the beads slurry was washed with Rap1 activation buffer for three times. Then the active form of Rap1 were eluted from the beads by adding 4x protein loading buffer and water bathing in 95 degree for 5 min. Samples were then analyzed by western blotting using a Rap1 antibody (Upstate).

4.2.3 Sample quantification

The eluted Rap1 from either SD or NSD mice was resolved using NuPAGE 4–12% Bis-Tris gel and NuPAGE MOPS SDS running buffer (Invitrogen) for 2 h at 200 V. A separated gel was loaded with 5 ug of unprocessed lysate as control for total Rap1. The separating proteins were transferred to PVDF membrane (Invitrogen) overnight at 10 V using NuPAGE transfer buffer. The membranes were blocked in TBST blocking reagent (Thermos) for at least 1 h with gentle shaking. Anti-Rap1 (1:1,000 upstate) were then added in the blocking reagent and incubated at 4 °C for two nights. The membranes were washed 3 times for 15 min in TBST. Peroxidase-conjugated anti-rabbit (Santa Cruz) were added 1:2,000 in the blocking reagent and incubated for 2 h at room temperature. Membranes were washed as before, then incubated in Pierce ECL plus Western Blotting
Substrate (Thermo Scientific) for 60 s and the signal was detected using film (Kodak, Carestream Health, Inc.). Densitometry was performed using Image J.

For each gel, the amount of active Rap1 in each sample was first normalized to the optical density of corresponding levels of total Rap1. Then, the normalized active Rap1 levels in the SD group were presented as a percentage of the average of the NSD group normalized levels of active Rap1 levels. Then the results of 3 different gels were combined together.

4.3 Results and discussion

4.3.1 Hippocampal Rap1 activity decreases after sleep deprivation.

To investigate whether sleep deprivation interferes with Rap1 activity, we performed 5 h sleep deprivation (SD, n=10) on a group of single housed male BL6 mice starting at ZT0 with a control group undisturbed in the home cage (NSD, n=9). Hippocampal Rap1 activity was then assayed for both groups. Similar to its effect on cAMP activity, 5 h sleep deprivation significantly decreased Rap1 activity to 67.7±9.7 % (p=0.02 student’s t
test) (Figure 4.2). These findings are consistent with the hypothesis that sleep modulates the molecular cascades involved in memory consolidation and therefore regulates memory consolidation through these cascades.

Compared with changes in cAMP levels (Figure 4.1), sleep deprivation appears to have a greater effect on basal Rap1 activity levels (Figure 4.2). There are two possibilities; one is that the cAMP activity changes were measured in CA1 region only whereas Rap1 activities were measured in whole hippocampi. Different regions of hippocampus may respond differently to sleep deprivation. A cAMP assay from the whole hippocampi could investigate this possibility. Another possibility is that Epac signaling might be more sensitive or vulnerable to sleep deprivation. Because cAMP-dependent LTP is impaired in sleep deprived mice, it is possible that Epac-dependent LTP is also impaired by sleep deprivation. In addition, like the PDE inhibitor rolipram, Epac-specific agonist 8-pCPT could be used to prevent the memory deficits caused by sleep deprivation.

Interestingly, I did not detect a similar down regulation pattern in PKA activity (data not shown), which is consistent with the hypothesis I discussed in Chapter 3 that Epac
signaling response is slower but more persistent to changes in cAMP as compared to
PKA signaling. Because animals with reduced PKA activities exhibit abnormal
sleep/wake patterns (Hellman et al., 2010), it would be interesting to characterize the
sleep/wake patterns in the Epac2KO mice and compare that with the sleep/wake patterns
R(AB) mice. These experiments may also help explore the functional differences
between Epac signaling and PKA signaling.
Figure 4.1 Sleep deprivation (SD) impairs cAMP activity, synaptic plasticity, and memory consolidation, which could be rescued by treatment of PDE inhibitor rolipram (ROL).

A) LTP induced by the adenylyl cyclase activator FSK was impaired in sleep-deprived mice relative to NSD controls (P=0.007). B) Sleep deprivation decreased baseline cAMP levels in CA1 regions of vehicle treated slices (P=0.02). C) Rolipram (ROL) treatment rescued deficits in spaced 4-train LTP due to sleep deprivation (P=0.003). VEH, vehicle. The black bar represents the time of rolipram treatment. D) Sleep deprivation significantly impaired context-specific memory (P=0.02), and treatment with rolipram rescued this deficit (P=0.0009). Data are expressed as means ± SEM and n represents the number of mice. Modified from Vecsey et al, 2009.
Figure 4.1

A

IEPSP slope (% baseline)

-20 0 20 40 60 80 100 120

Time (min)

SD NSD (n = 6) SD (n = 5)

FSK

B

cAMP relative to NSD control (%)

NSD n=25

SD n=25

*

C

IEPSP slope (% baseline)

-50 0 20 50 100 150

Time (min)

SD + ROL (n = 6) SD + VEH (n = 6)

 rol

 D

 Content-specific freezing (trained - altered)

NSD + VEH (n=20) SD+VEH (n=20) NSD + ROL (n=20) SD + ROL (n=20)

*

Modified from Vecsey et al., 2009
Figure 4.2 Five hours of Sleep deprivation decreased basal Rap1 activity levels in the hippocampus.

Hippocampal Rap1 activities in the sleep deprived group (SD) is significantly lower than that of non-sleep deprived group (NSD) (P=0.02, student’s t test). A sample blot is shown.

Data are expressed as means ± SEM and n represents the number of mice.
Figure 4.2

Relative Rap1 activity fold changes after 5h SD

- NSD n=9
- SD n=10

* Significant difference
Chapter 5 Conclusions and future directions

5.1 Conclusions

In this dissertation, I have demonstrated a role for Epac/Rap1 signaling in memory consolidation and sleep deprivation. In Chapter 2, I provide a direct link between Epac signaling and memory consolidation by showing that activation of hippocampal Epac with the specific Epac agonist 8-pCPT enhanced long-term contextual fear memory. Furthermore, 8-pCPT increased the activity of the direct target of Epac, Rap1, but not PKA. 8-pCPT also ameliorated PKA-related memory deficits. These findings are the first to exhibit that Epac signaling can modulate memory consolidation, which broadens our understanding of cAMP signaling in memory formation and provides the basis for the development of novel cognition-enhancing drugs. In Chapter 3, the necessity of Epac signaling in memory consolidation was addressed. First, I showed that hippocampal Rap1 activity was elevated after fear conditioning, indicating that Epac signaling is required for fear memory formation. Next, I showed that mice injected intrahippocampally with Epac2shRNA AAV exhibited low hippocampal Epac2 expression and specific hippocampus-dependent memory deficits. However, locomotor activity, anxiety levels and motor learning and memory were unaffected. These findings suggest that Epac
signaling is required for hippocampus-dependent memory consolidation. Since reduced Epac expression is linked with various psychiatric disorders including Alzheimer’s disease (McPhee et al. 2005), my findings may help to understand the pathology of the cognitive deficits associated with those psychiatric disorders. In Chapter 4, I examined the effect of sleep deprivation on Epac signaling. Previously, sleep deprivation was reported to impair cAMP signaling and the reduction in cAMP levels following sleep deprivation appears to cause memory deficits (Vecsey et al, 2009). Likewise, Rap1 activity is also impaired after sleep deprivation, suggesting reductions in Epac signaling may be related to memory deficits caused by sleep deprivation.

Figure 5.1 illustrates how cAMP/Epac together with other cAMP targets modulates memory formation. First, cAMP activates both PKA and Epac. PKA then phosphorylates various downstream kinases (MAPKs) and transcription factors (CREB) that are required for memory formation. Epac offers a separate signaling pathway whereby small G proteins (Rap1) are activated, which then leads to the activation of transcription and translation. As a result, target proteins required for long-term memory formation are generated. In contrast, inhibition of either PKA or Epac signaling is known to cause memory deficits. In conclusion, I show that memory consolidation can be modulated by
the cAMP-Epac signaling pathway. This is a novel in that cAMP was previously thought to regulate memory only through PKA. Furthermore, this signaling pathway is potentially involved the mechanism by which sleep deprivation impairs memory.

5.2 Future directions: Epac signaling versus PKA signaling

My work and other studies have demonstrated that the function of Epac signaling is similar to that of PKA signaling in synaptic plasticity and memory formation (Gelinas et al. 2008; Ma et al. 2009) where activation and inhibition of either pathway enhances and impairs memory formation, respectively. However, what functional differences, if any, are there between these two pathways? To answer this question, the function of Epac in memory needs further investigation. For instance, what are the downstream targets of Epac/Rap1 signaling? Does Epac positively regulate other forms of memory where different brain regions are involved? Even in the hippocampus, is the function of Epac the same at the pre-synaptic region as the post-synaptic region? In addition, it is possible that sleep has different effects on PKA signaling from Epac signaling. These follow-up investigations on those aspects will help us understand the complexity of cAMP/Epac and PKA signaling modulating memory formation.
5.2.1 What are the downstream targets of Epac/Rap1?

I have demonstrated that Epac signaling modulates memory consolidation, a process that requires transcription and translation. However, how Epac signaling regulates transcription and translation on the molecular level is unknown. Cellular studies suggest some links between Epac and transcription and translation (Yarwood et al. 2008; Misra and Pizzo, 2009) Epac appears to activate transcription factor C/EBPβ in umbilical vein endothelial cells. Recently, a study in human prostate cancer cells reported that Epac-mediated cellular effects require activation of mTOR signaling cascades. But no one is able to provide such links in the hippocampus. The fact that Epac appears to function independent of CREB (Delghandi et al. 2005; Chaudhuri et al. 2007) suggests that this pathway activates other transcription factors involved in memory consolidation. By performing microarray on transgenic mice with reduced Epac activity (by a dominant negative form of Epac or Epac knock-outs), we are able to identify the mRNA that are sensitive to Epac signaling during memory consolidation and then associates corresponding transcription factors to those mRNA.

5.2.2 Does Epac positively regulate other forms of memory?
It is worth noting that cAMP-dependent PKA signaling does not have uniform effect on memory formation within all brain regions. Instead of enhancement, PKA activation impairs the working memory (Arnsten et al., 2005). Likewise, the effects of Epac on different forms of memory require further investigation where different brain regions, including the amygdala or prefrontal cortex, instead of hippocampus, are involved in modulation. This problem can be addressed by evaluating the performance of transgenic mice with reduced forebrain Epac activity in various memory tasks.

5.2.3. Does pre-synaptic Epac modulates memory formation?

The focus of this study was on the overall effect of hippocampal Epac activity, but further studies are needed to separate the post-synaptic function and pre-synaptic function of Epac. A study in cultured hippocampal neuron showed that Epac2 regulated neurotransmitter release at the pre-synaptic sites (Gekel and Neher, 2008). Because pre-synaptic neurotransmitter release regulates synaptic plasticity and memory formation, it is possible that pre-synaptic Epac2 alone is able to modulate memory consolidation. This hypothesis could be tested by determining if mice with pre-synaptic Epac2 deletion exhibit memory deficits. To test this hypothesis a knockout mouse line could be
generated using a loxp/cre recombination system to constrain the deletion of Epac2 specifically in the CA3 region of the hippocampus.

5.2.4 Does sleep affect Epac signaling differently from PKA signaling?

In Chapter 4, I described that the Rap1 activity decreased following sleep deprivation. Interestingly, I did not detect a similar down regulation pattern in PKA activity, suggesting sleep may have different effects on Epac signaling from PKA signaling. Since animals with reduced PKA activities exhibit abnormal sleep/wake patterns (Hellman et al., 2010), the sleep/wake patterns of the mice with reduced Epac activities need to be characterized. Additionally, my findings suggest that the memory deficits followed by sleep deprivation may be prevented by Epac activation. It would be interesting to test this indication as well because it could help to find treatment of sleep-deprivation related cognitive dysfunction.
Figure 5.1 The cAMP/Epac signaling pathway regulates molecular components underlying long-term memory.

Like the two other targets of cAMP, PKA and HCN, Epac regulates the molecular components underlying long-term memory. When binding to cAMP, Epac activates Rap1, which then modulates corresponding transcription factors and leads to synthesis of memory-related regulators and effectors. Inhibition of Epac expression blocks this pathway and leads to memory deficits.
Figure 5.1
References


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attenuated by drugs that enhance the cAMP signaling pathway." *Proc Natl Acad Sci U S A* **96**:5280-5285.


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