PRO- AND ANTI-MITOTIC ACTIONS OF PACAP IN THE DEVELOPING CORTEX: POTENTIAL MEDIATION BY DEVELOPMENTAL SWITCH OF RECEPTOR mRNA ISOFORMS

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ABSTRACT OF THE DISSERTATION

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The multi-functional neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has been associated with different physiological processes. The actions of PACAP signaling in neurogenetic regulation are complex: the peptide functions in cell cycle progression, differentiation and precursor survival. In this thesis, I focused on its functions in neurogenesis, including proliferation, differentiation, cell death and different signaling pathways in which it is involved. PACAP has been well established to act as an anti-mitogenic signal in neuronal precursors at embryonic day (E) 13.5 onward. Furthermore, PACAP exhibits trophic effects in cerebellum and hippocampus postnatally. PACAP is present at the onset of neurogenesis (E9.5 rats) (Zhou et al., 1999), PACAP well-defined proliferation inhibitory effects seem inconsistent with the very robust proliferation. Since the majority of the cells are proliferating in the environment that is
rich in mitogens in early neurogenesis. Due to the extremely tiny embryos (~3000μm in length at E10 rats) and lack of young cortical precursor culture models, the roles of PACAP in early corticogenesis has not been studied thoroughly and therefore are unclear. Defining the role of PACAP in brain development is important because recent studies suggest PACAP signaling abnormalities may contribute to schizophrenia (Hashimoto et al., 2007) and post-traumatic stress disorder (PTSD) (Ressler et al., 2011). Thus I decided to define the function of PACAP during early neurogenesis in the developing cerebral cortex.

PAC1 receptors, especially hop and short isoforms, differentially couple to and activate distinct pathways that produce pro- or anti-mitogenic actions in neuronal precursors, respectively. Previously we found that the anti-mitogenic activity in older cortical precursors, both in culture and \textit{in vivo}, is caused by activated cAMP signaling through the short isoform. Hop isoform, however, is linked to pro-mitogenic activity through phospholipase C (PLC)/protein kinase C (PKC) pathway. Furthermore, ectopic over-expression of hop isoform transformed the anti-mitotic effects of PACAP in E14 cortical precursors into a pro-mitogenic signal (Nicot and DiCicco-Bloom, 2001). These results suggest that expression of distinct PAC1 isoforms is essential for regulating neural precursor mitosis.

E9.5 PACAP-/- mice exhibited a decrease in BrdU labeling index, suggesting that PACAP normally promotes proliferation at this time. I established a culture model of
young precursors (E10.5 rat; E9.5 mouse) viable for up to 48h, overcoming previous limitations of studying this early age precursors in regular media. In contrast to older precursors, I found in this model at 24h that PACAP exhibits mitogenic effects stimulating S-phase entry and proliferation of E10.5 precursors without affecting cell survival, evokes intracellular calcium fluxes and increases phospho-PKC levels. The opposing effects of PACAP during development are due to age-dependent receptor isoform (hop to short) switch. Significantly, I found the expression of the hop isoform is 24-fold greater than short at E10.5, a ratio that is reversed at E14.5 when short isoform expression is 15-fold greater. In addition, both the hop expression and PACAP mitogenic activity are maintained by retinoic acid exposure.

These observations suggest that PACAP elicits temporally specific effects on cortical neurogenesis determined by developmentally regulated expression of PAC1 receptor isoforms.
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CHAPTER 1  INTRODUCTION

1.1 Early Development of cerebral cortex

The mammalian cerebral cortex is a complex laminated structure that contains different types of neurons and has rich local and extrinsic connectivity. The cortical neurogenesis is a highly regulated process that is composed of several key steps, including cell proliferation, migration, cell death and differentiation. In all mammals, the basic principles of cortical neurogenesis are similar: the neocortex forms at the outer surface of the embryonic cerebral vesicles, through the migration of neurons from proliferative regions next to the cerebral ventricle (Bystron et al., 2008). Ectoderm gives rise to the entire nervous system: starting from the columnar shaped dorsal ectoderm, the neural plate (Gilbert). The neural plate bends, and then folds to form the U-shaped neural groove in the center of the plates. The neural groove will later close to form neural tube starting at embryonic day 8.5 (E8.5) at the cervical and hindbrain boundary and finishes at E10.5 at sacral level in mouse (Ybot-Gonzalez et al., 2007). In rat cerebral cortex, the rostral portion of the neural tube is completely fused by E10.5, producing the prosencephalic neuroepithelium. The rostral end of neural tube then differentiates into three primary vesicles - prosencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain). The prosencephalon will further subdivide into the telencephalon and diencephalon. The telencephalic vesicles first appear on E12 due to the growing neuroepithelium (Bayer et al., 1991), which are the beginning of the cerebral cortex. On E10.5 the future brain consists of a single layer of ventricular zone (VZ) cells, the majority of which are dividing in the VZ to increase the surface area and the thickness during neurogenesis (Qian et al., 1998). Before the onset of neurogenesis, the
proliferative cells in the VZ constitute a homogeneous pseudo-stratified epithelium, meaning that while the nuclei appear to be at different levels in reality each cell maintains attachments to both the ventricular and pial surfaces (Nowakowski and Hayes, 1999). The nuclei move towards the ventricular surface before metaphase and cytokinesis, and migrate toward the pial surface during DNA synthesis (S phase), which is a dynamic process named interkinetic nuclear movement (Sauer, 1935). Neurogenesis occurs through a combination of different modes of cell division. VZ cells have radial processes and divide symmetrically at the onset of neurogenesis to expand the pool of neuronal precursor cells, which generate two daughter cells with the same fate. Following expansion of proliferative precursor pool, neuroepithelial cells, which can be considered stem cells, down regulate their epithelial characteristics and begin to switch to an asymmetrical mode of cell division at approximately E10 in mice (Shen et al., 2002). One daughter cell remains a precursor, and the other is a postmitotic cell that is destined to become a neuron or a glial cell (Chenn and McConnell, 1995). The cell fate determination and cell divisions are determined by both intrinsic determinants and extrinsic signals. Intrinsic determinants, such as Notch and Numb are dispensable for the proper ordering of symmetric and asymmetric cell division, which is an evolutionarily conserved mechanism for regulating cell fate in the developing nervous system from drosophila to mammals (Zhong, 2003). The differentiated neurons start to exit the cell cycle and migrate out of the VZ to form cortical layers following “inside-out” sequence from E15 through E21 in rat (Bystron et al., 2008) and around E12-13 in mouse (Qian et al., 1998) to form a well-laminated cerebral cortex.
1.2 Cell cycle in the ventricular zone

Neurogenesis is a highly regulated process in which cycling neural precursor cells are dividing in the cell cycle, and they exit the cell cycle and differentiate into post-mitotic neurons. A cell reproduces by performing a sequence of events in which it duplicates and divides in two. The major function of the cell cycle is to accurately duplicate the DNA in the chromosomes and segregate into two genetically identical daughter cells. The eukaryotic cell cycle is composed of mitosis (M) phase, first gap (G1) phase, synthesis (S) phase, and second gap (G2) phase (Alberts). Eukaryotic cells have a complex network of cell-cycle control system that governs progression through the cell cycle. These regulatory proteins consist of cyclins, cyclin-dependent kinases (Cdks) and cyclin-dependent kinase inhibitors (CKIs). The interactions between cyclins, Cdks and CKIs have been studied extensively, and four classes of cyclins have been defined by the stage of the cell cycle. Here we focus on G1-S transition process, through which protein interactions are precisely regulated by mitogens and mitotic inhibitors (Sherr, 1993, 1994b).

In mammals, G1 cyclins and their associated kinases are key regulators of the cell cycle. The levels of cyclins are controlled largely by the extracellular environment. G1 cyclin levels are induced by mitogens and their levels decline when mitogens are absent or when anti-mitogens are present (Matsushime et al., 1991). Thus, they integrate mitogen-induced signals to drive G1 progression and initiate DNA replication. G1 cyclins consist of D-type cyclins (D1, D2, and D3) and E-type cyclins (E1 and E2). D-type cyclins can bind, in different combinations, to their catalytic binding subunits, Cdk4 and Cdk6 in early G1 phase, while E-type cyclins govern the activity of Cdk2 in late G1
Once G1 cyclins bind Cdns, they allosterically activate Cdns, respectively, to trigger G1/S progression (Sherr, 1993, 1994a). Cyclin levels undergo dynamic changes during cell cycle: they become accumulated at G1 and they rapidly decay during mitosis to regulate Cdns activity. Activated Cdk4/6 can phosphorylate retinoblastoma tumor suppressor protein (Rb), thus inactivating Rb, thereby leading to release of E2F transcription factors and promotion of G1/S progression (Ferguson and Slack, 2001; Sherr and Roberts, 1999). When Rb is underphosphorylated, Rb is active and able to bind to and repress the E2F family of transcription factors, which can activate downstream genes that drive cell cycle progression (Ferguson and Slack, 2001).

On the other hand, Cdk activity is negatively regulated by CKIs. Two major classes of CKI are identified in mammals including CIP/KIP family and INK4, and the first class is more critical for G1/S progression (Sherr and Roberts, 1999). CIP/KIP family consists of p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, which can act on all G1/S cyclin-Cdk complexes and therefore results in inactivation of the kinase activity without releasing cyclins (Sherr and Roberts, 1999). The interactions between cyclin-Cdk complexes and CKIs are still under investigation since it is an extremely complicated process. For example, in contrast to CKIs inhibitory roles, CKIs are able to facilitate the activation of cyclin-Cdk complex activation through assembly promotion or stabilization (LaBaer et al., 1997).

Recent evidence indicates that CKIs also regulate other aspects of neurogenesis besides inhibiting cell cycle progression. For example, p57^{Kip2} is involved in the development of dopamine neurons (Joseph et al., 2003) and retinal amacrine cells (Dyer and Cepko, 2000). Specifically, p57^{Kip2} is required for midbrain dopamine neuronal cells development in vivo, it promotes dopamine neuron maturation through a mechanism
independent of cell cycle inhibition (Joseph et al., 2003). A novel subpopulation of amacrine interneurons expresses p57\textsuperscript{Kip2} postnatally, at the time beyond the window that p57\textsuperscript{Kip2} regulate proliferation. Loss of p57\textsuperscript{Kip2} alters the amacrine subtype/subpopulation, suggesting p57\textsuperscript{Kip2} is an essential regulator of neuronal differentiation during retina development in addition to CKI (Dyer and Cepko, 2000). Similar to p57\textsuperscript{Kip2}, independent of cell cycle regulation, p27\textsuperscript{Kip1} controls cortical neuron radial projection and promotes neuron differentiation through Neurogenin2 in developing rodent cortex (Nguyen et al., 2006). Our group recently found p57\textsuperscript{Kip2} to be expressed more abundantly during early corticogenesis and differentially regulates cortical neuroglial fate depending on different developmental stages and extrinsic signals (Tury et al., 2011). Furthermore, we found that p57\textsuperscript{Kip2} controls precursor pools by regulating the rate of cell cycle progression during G1 and the balance of cell cycle re-entry: p57\textsuperscript{Kip1} controls radial glial cells (RGC) and intermediate precursor cells (IPCs) cell cycle dynamics while p27\textsuperscript{kip1} only regulates IPCs. In addition, the CKIs regulate precursor proliferation in a spatiotemporal manner during corticogenesis (Mairet-Coello et al. unpublished data).

The complexity of cyclin/Cdk interactions is still not fully defined especially at the G1 to S boundary in mammalian cells. Major approaches include genetic manipulation of different cyclins (knock-out, knock-down, overexpression, knock-in, to name a few) and developing optical probes that report on the cell cycle kinetics and localization of protein phosphorylation and methylation (Murray, 2004; Sherr and Roberts, 2004).

1.3 Proliferation control in developing cerebral cortex

1.3.1 Patterning genes

Sophisticated functioning of the mammalian cerebral cortex depends on the division of
the cortical sheet into different regions of the cortex, and further subdivision into more specialized areas. A classic model proposed that the mammalian frontal cortex is divided into different cytoarchitectonic areas early in neurogenesis. These areas are defined as regions of the cortex with distinct cellular, biochemical, connectional, and physiological characteristics. As a result, different cytoarchitectonic areas incorporate specific sensory information, direct precise motor activity, and are in charge of different cognitive functions (Rakic, 1988). However, the developmental molecular mechanisms that generate the area map are elusive, in part, because of the lack of markers that are able to distinguish the regions clearly. Increasing evidence supports this “protomap” model in the proliferative cell layer of neocortex: the neocortical areas are regionalized by transcription factor expression and other regulatory genes, which results in a patterning strategy of many signaling proteins secreted from nearby signaling centers. The regulatory genes that control neocortical arealization are assumed to be expressed in graded patterns especially during embryonic stages. These candidate sources include members of the fibroblast growth factor (FGF), Wingless-Int (WNT), bone morphogenetic protein (BMP) families, homeodomain transcription factor Emx2 and the paired-box-containing transcription factor Pax6 (O’Leary et al., 2007). Both Emx2 and Pax6 are expressed in the dorsal telencephalic neuroepithelium [Introduction figure-1].
Introduction figure-1 Patterning genes and secreted factors during neurogenesis

A lateral–superior view of the two cerebral hemispheres of the embryonic mouse, sitting above the midbrain and hindbrain (broken lines). The anterior–lateral extent of Pax6 gene expression is indicated by circles. The posterior–medial domain of Emx2 expression is indicated by stripes. The genes exhibit continuous gradients of expression that decrease as they extend to opposite poles. In contrast, Emx2 is expressed in low rostral to high caudal and low lateral to high medial gradients, whereas Pax6 is expressed in low caudal to high rostral and low medial to high lateral gradients. Based on these expression patterns, people predicted that Emx2 imparts the caudal and medial identities while Pax6 imparts the rostral and lateral identities, which was confirmed to be true using KO mice in combination of molecular and connectional markers. Emx2 and Pax6 act and are independently regulated to control neocortical arealization (Bishop et al., 2000). The signaling factor
FGF8 is produced by and released from mesenchymal tissue in the anterior neural ridge, which regulates *Pax6* and *Emx2* expression. In the midline, BMPs and Wnts are secreted from other signaling centers, including the roof plate and the roof plate-vesicle junction, the cortical hems (DiCicco-Bloom, 2009). The combinations of these genes and factors result in unique embryonic subdivision in both neocortex and spinal cord.

1.3.2 Extracellular signaling/growth factors

Area patterning is a critical event during neurogenesis, which is governed by secreted molecules. Morphogens and signaling molecules expressed in early patterning centers help establish transcription factor expression patterns that correlate with morphologic boundaries in the telencephalon (Puelles and J.L.R, 2004) as mentioned earlier. Next we discuss three important molecules that contribute to both area patterning and cell cycle in CNS: fibroblast growth factor (FGF), sonic hedgehog (Shh), and retinoic acid (RA). The reason why we choose them is because PACAP has been reported to interact with all three. PACAP antagonizes FGF and Shh mitogenic effects (Lu and DiCicco-Bloom, 1997; Nicot et al., 2002), and RA can upregulate PACAP and VIP receptor expression (Waschek et al., 1997).

Fibroblast growth factor (FGF)

FGF family is a large group of secreted proteins, now at least more than 22 membranes have been identified in mammalian FGF family. They function from specifying regional cell fate to promoting precursor proliferation, differentiation and survival (Mason, 2007). FGF signaling is implicated in the induction of posterior neural tissue in the early neural ridge. Several FGF members are expressed during neural plate maturation with distinct
expression patterns after neurulation (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997).

FGF has been characterized as a strong mitogen in cortical precursor both in vitro and in vivo (Temple and Qian, 1995; Vaccarino et al., 1999) by promoting the proportion of dividing cells and increasing the absolute number of total cells (Ghosh and Greenberg, 1995). Some research shows FGF delays differentiation processes of multipotent neural stem cells to become neurons and astrocytes in a dose-dependent manner in cultures (Temple and Qian, 1995), although it is still under debate. FGF regulation of precursor proliferation is required from embryonic cerebral cortex morphogenesis since missing this molecule decreases all cortical layers and injection of FGF increased the total cortex volume in adulthood (Vaccarino et al., 1999). Fgf2 (also called basic FGF or bFGF) plays significant roles in blood vessel formation (Gilbert) and now is widely used in basic culture media. Morphogenesis in telencephalon is mainly governed by FGF8 and FGF17, which positively regulates transcription factors (Erm, Er81, Foxg1, Nkx2.1, Pea3 and Sp8) and negatively regulates (CoupTF1, Emx2 and Wnt8b) (Cholfin and Rubenstein, 2008; Fukuchi-Shimogori and Grove, 2001, 2003).

FGF8 has been studied extensively in CNS besides its significant roles in limb bud induction. The dorsal part of the neuroepithelium secretes FGF8 at the boundary of midbrain and hindbrain, while the ventral part secretes sonic hedgehog (Shh). As a result, the crest-derived mesenchyme between the dorsal and ventral epithelia receives both signals in a gradient, and different gene combinations are turned on to induce tissue specific differentiation (Gilbert, 2002).
The interactions between FGF members are interesting since different members exhibit distinct or even antagonist roles. For example, FGF15 is expressed in E9.5 anterior forebrain neuroepithelium where FGF8 is excluded. FGF15 and FGF8 have opposite effects on patterning genes: FGF8 is proliferation promoting while FGF15 blocks neural proliferation, and FGF 15 is repressed by FGF8. In addition Shh can promote FGF15 expression (Borello et al., 2008).

**Sonic hedgehog (Shh)**

Shh is a soluble signaling protein that belongs to the *Hedgehog* secreted protein family, which regulates many developmental processes in both vertebrates and invertebrates (Machold et al., 2003). Earlier studies of neural plate patterning have shown the importance of Shh signaling in ventralizing the neural tube. Whereas in the limb, Shh is crucial in the zone of polarizing activity that establishes the anterior polarity in limb bud (Ruiz i Altaba et al., 2002). Shh signaling pathway is triggered by the interaction of Shh molecule and its heterodimeric receptor complex: the transmembrane protein is called Patched, which originally binds to repress another transmembrane protein, Smoothened. When Shh binds Patched, Smoothened is relieved and it activates members of Gli proteins, which enter the nucleus and induce expression of target genes, such as *HNF3β* and *PTC* (Huangfu and Anderson, 2006). Shh is secreted by both the notochord and floor plate, resulting in an activity gradient over the ventral half of the neural tube. Shh gradient contributes to the patterning process in the spinal cord. The exposure of neural tube cells to Shh gradient will form floor plate cells, motor neurons and ventral interneurons from ventral to dorsal (Ericson et al., 1997), probably dependent on a dose-dependent activation (class II genes: Nkx6.1 and Nkx2.2) or repression (class I
genes: Pax6, Irx3, Dbx2) of a diverse group of homeodomain-containing transcriptional regulators (Briscoe et al., 2000). The forebrain (telencephalon and diencephalon) is devoid of motor neurons, and it has no floor plate expect the most posterior, nor is it underlain by the notochord. As a result, it was expected that Shh is absent in the forebrain at first. However, Shh expression extends in the telencephalon and diencephalon and specifies cell fates in the forebrain (Lumsden and Graham, 1995). Interestingly, in addition to ventral neural tube patterning, Shh has a general role controlling progenitor cell number and cell fate in the developing dorsal brain and cerebellum (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001), suggesting that deregulation of Shh/Gli pathway may lead to brain tumorigenesis.

Shh elicits strong, dose-dependent proliferative responses in neural stem cells and causes quiescent neural stem cell to self-renew for more than a year and to generate multiple cell types over time (Ahn and Joyner, 2005; Lai et al., 2003). Some research implicates the cyclic AMP-dependent protein kinase A (PKA) as a negative regulator of the Shh signal transduction pathway, and the antagonistic actions between PKA and Shh contribute to cell fate determination at multiple regions (Epstein et al., 1996; Hynes et al., 1995). PKA has been reported to phosphorylate Gli protein in the Shh signaling pathway. Phosphorylation inactivates Gli, so Shh targeting gene activation is abolished (Ingham, 1998). The discovery of the PKA antagonistic actions provides therapeutic directions during Shh signaling deregulation which has been thought to cause brain tumors such as medulloblastoma (Waschek et al., 2006).

Retinoic acid (RA)
Retinoic acid (RA) belongs to a class of steroid-like molecules expressed by cells surrounding the organizer region. RA is produced by neural crest-derived frontonasal mesenchyme between the olfactory placode and the ventrolateral forebrain (LaMantia et al., 1993). Recent work has shown that RA is also produced by the middle meningeal layer that can regulate cortical neuron generation (Siegenthaler et al., 2009). Uniquely, RA is derived from vitamin A and is able to pass through the plasma membrane by binding to specific carrier proteins. The reason why RA signaling pathway contributes to many decisions in neurogenesis is partially because of the complex receptor system. There are two classes of RA-binding transcriptional regulators belonging to the nuclear receptor superfamily, the retinoic acid receptors (the multiple isoforms of the RAR$\alpha$, $\beta$, and $\gamma$ isotypes), and the retinoid X receptors (the multiple isoforms of the RXR$\alpha$, $\beta$, and $\gamma$ isotypes). There are two major isoforms for RAR$\alpha$ ($\alpha1$ and $\alpha2$) and for RAR$\gamma$ ($\gamma1$ and $\gamma2$), and four major isoforms for RAR$\beta$ ($\beta1$ to $\beta4$). Similarly, at least two isoforms have been identified for RXR$\alpha$ ($\alpha1$ and $\alpha2$), RXR$\beta$ ($\beta1$ and $\beta2$) and RXR$\gamma$ ($\gamma1$ and $\gamma2$) (Mark et al., 2006). RAR/RXR controls the transcription of RA-targeted genes through retinoic acid response elements (RAREs), which locate within the promoters of certain genes, such as $Hox$ (Rao, 2005) to exhibit multiple biological functions. Similar to Shh, RA functions as a morphogen, in a gradient highest at the posterior end of the neural plate. This gradient (conserved from fish to mammals) appears to be controlled by the expression of RA-synthesizing enzymes in the posterior and RA-degrading enzymes in the anterior parts of the embryo (Gilbert, 2006). Exposure of *Xenopus* gastrula embryo to RA leads to generation of posterior neural plate tissue at the expense of anterior neural cells (Tanabe and Jessell, 1996). This discovery established the early anteroposterior (AP) axis,
which also builds AP patterning in rodent through regionalizing stem cells (Temple, 2001). Experiments in mouse demonstrated that disturbed RA gradient resulted in the loss of anterior structures and markers and RA can also influence differentiation during several critical steps along the AP axis (Rao, 2005; Simeone et al., 1995). RA can induce differentiation in multiple cancer cell lines which correlates with up- or down-regulation of PACAP and VIP receptor expressions (Waschek et al., 1997). Recently, its neural differentiation roles have been linked to a sequential inducer of forebrain development, which established that RA is a potent and bioactive signal that controls the neuronogenic decision (Siegenthaler et al., 2009).

1.4 General G Protein Coupled Receptor (GPCR) Signaling

G proteins are composed of three protein subunits: α, β, and γ. When ligand is absent, G protein is inactive, GDP binds to α subunit, clamping β and γ subunits to form the trimer [Introduction figure-2A]. Stimulated by an activated receptor, α subunit releases GDP, leaving GTP to bind its place [Introduction figure-2B]. Binding GTP causes α undergo conformation change, which leads to dissociation of the trimer into two activated components- an α subunit and βγ complex [Introduction figure-2C]. The conformational change of α subunit allows it to interact with its target protein. The surface of βγ complex previously covered by α subunit is available to interact with a second set of target proteins. The target proteins include enzymes and ion channels and they relay the signal onward. The α subunit is a GTPase, which can hydrolyze GTP to GDP. α reassociates with βγ complex to reform the trimer-inactive G protein once GDP is formed. The active time of G protein, when α and βγ complex are separate, is usually short and depends on how fast α can hydrolyze its bound GTP (Alberts, 2002).

1.4.1 Cyclic AMP Signal
Cyclic AMP (cAMP) was identified as a small mediator. All receptors that act via cAMP are couple to a stimulatory G protein (Gs), which activates adenylate cyclase and increases intracellular cAMP concentration. Downstream targets of cAMP include certain ion channels and cAMP-dependent protein kinase A (PKA). PKA has various target proteins in different cell types. PKA consists of two catalytic subunits and two regulatory
subunits in inactive state. When cAMP binds to the regulatory subunits, it causes the
dissociation of regulatory subunits. Free catalytic subunits become activated to
phosphorylate specific substrate molecules, which will cause specific downstream
signaling responses. Some PKA mediated responses are rapid while others may take
hours to complete and involve several transcription gene changes. For example, cAMP
can activate the gene that encodes a peptide hormone, somatostatin. The regulatory
region of the somatostatin gene has a short DNA sequence, named the cyclic AMP
response element (CRE). CRE can be also found in the regulatory region of other cAMP
target genes. CRE-binding (CREB) protein specifically recognizes CRE. When CREB is
phosphorylated by PKA, it recruits a transcriptional coactivator-CREB binding protein
(CBP), which stimulates downstream gene transcription [Introduction figure-3] (Alberts,
2002).
Introduction figure-3 cAMP/PKA activates gene transcription. Increased cAMP concentration activates PKA, which further phosphorylates CREB gene regulatory protein. Once phosphorylated, CREB is active and recruits the coactivator CBP, causing downstream gene transcription (Alberts, 2002).

1.4.2 Inositol Phospholipid Signaling Pathway.

Another group of G-protein-linked receptors is called Gq. Gq mainly functions via phospholipase C-β (PLC). PLC is a plasma-membrane-bound enzyme, once activated, it cleaves an inositol phospholipid-phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to generate two products: inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). DAG remains in the membrane while IP₃ diffuses through the cytosol and engages IP₃-gated
Ca\(^{2+}\) release channels in the endoplasmic reticulum (ER) membrane, quickly raising cytosol Ca\(^{2+}\) concentration. The initial rise in cytosolic Ca\(^{2+}\) causes protein kinase C (PKC) translocate from cytosol to plasma membrane, where PKC is activated by DAG. Once activated, PKC can phosphorylate different target proteins depending on the cell type [Introduction figure-4] (Alberts, 2002).

**Introduction figure-4 The inositol phospholipid pathway.** In the presence of ligand, Gq is activated. PI(4,5)P\(_2\) is hydrolyzed by the activated PLC into IP\(_3\) and DAG. Ca\(^{2+}\) is released from ER once IP3 engages IP3 receptor. Ca\(^{2+}\) and DAG activate PKC (Alberts, 2002).

1.5 **PACAP background**

1.5.1 **PACAP system and PACAP receptors**

Pituitary adenylate cyclase-activating polypeptide (PACAP) (ADCYAP1) was first isolated in bovine hypothalamus on the basis of its ability to stimulate adenylate cyclase
to increase cAMP in anterior pituitary cells (Miyata et al., 1989). Hypothalamic neurons containing PACAP project toward the median eminence and terminate in the vicinity of capillary loops of hypothalamopituitary system (Arimura, 2007; Miyata et al., 1989). However, PACAP has been discovered in numerous locations besides hypothalamic region and it is known that PACAP signaling exhibits various biological functions (Vaudry et al., 2009). PACAP has two biologically active forms, PACAP-38 and PACAP-27, sharing the same biological activity. The two active forms are both C-terminally α-amidated (Eipper et al., 1992). PACAP-38 and PACAP-27 are processed alternatively from the 176-amino acid precursor prepro PACAP and they share the identical 27 amino acids N-terminus (Miyata et al., 1990). The primary structure of PACAP is highly conserved through evolution, from fish to mammals. PACAP is a member of the VIP/glucagon/growth-hormone-releasing hormone/secretin superfamily because PACAP-27 displays 68% identity with vasoactive intestinal polypeptide (VIP) and 37% with secretin.

PACAP is widely expressed in CNS and other peripheral organs (Vaudry et al., 2009). Consistent with its broad distribution pattern, this novel peptide exhibits multifunctional biological effects. PACAP acts on three heptahelical GPCRs in mammals. There are two classes of PACAP binding sites characterized by their relative affinity to PACAP: type I (PAC1), which was originally detected in pituitary and hypothalamus region, shows high affinity to PACAP38 and PACAP27 (Kd ≈ 0.5 nM) and low affinity to VIP (Kd > 500 nM) (Cauvin et al., 1990; Gottschall et al., 1990; Suda et al., 1992); type II (VPAC1 and VPAC2), which was abundant in peripheral tissues, exhibits similar affinity to PACAP and VIP (Kd ≈ 1 nM) (Gottschall et al., 1990).
PAC1 receptor has been cloned in human, rat, mice, bovine and other nonmammalian species. In rat five splice variants of PAC1 from alternative splicing have been isolated, including the short, hop1, hop2, hip, and hip-hop1 [Introduction figure-5,-6]. These variants are characterized by the absence (short) or presence of either one (hop1, hop2 and hip) or two (hip-hop1) 84-bp insertions at the C-terminal end of the third intracellular loop (Spengler et al., 1993). These splicing variants trigger different signaling pathways, which we will discuss in detail in the biological function section. The diversity of PACAP receptors or splicing variants suggests the wide spectrum of functions related to this neuropeptide, and the distinct distribution of receptor classes suggests different transduction signaling pathways are tissue specific. The diversity of the PACAP receptor variants and the versatility of the signaling that it can trigger depends on its cell types. Since PAC1 is generally more abundant in CNS compared with VPAC1 and VPAC2, and the affinity to PACAP is >1000 times than VIP, I will mainly focus on PAC1.

**Introduction figure-5 Alternative splicing generates two major PAC1 variants:**

**short and hop**. If the spliced mRNA does not contain the insert, it is the relatively short mature mRNA: short. The insert containing mature mRNA is called the hop.
Introduction figure-6 Variants of PAC1 receptor. At protein level, hip, hop1/2 or hip-hop cassettes are located at the third intracellular loop showing in different colors, whereas short variant has no insert.

1.5.2 Distribution of PACAP system in the CNS

PACAP and its receptors are highly expressed in multiple brain regions both embryonically and postnatally. The mRNAs can be detected as early as E8 in mouse and the expression level increases throughout embryogenesis and reaches a peak at birth (Watanabe et al., 2007). PACAP38 and PACAP27 are both present in CNS. However, PACAP38 is the predominant form, while PACAP27 counts for less than 10% of the total peptide content in brain tissue (Arimura et al., 1991).

In situ hybridization and immunohistochemistry show the PACAP mRNA is localized in cerebral cortex, amygdale, hippocampus, hypothalamus (paraventricular and supraoptic nuclei), striatum, the nucleus accumbens, brain stem (locus ceruleus, pontine nucleus and
vagal complex), medulla oblongata (solitary nucleus, nucleus ambiguous, raphe nuclei) and cerebellum (Purkinje cells) (Skoglosa et al., 1999). Particularly in cerebral cortex, regional distribution studies demonstrate that PACAP is just outside the VZ in E10.5 mice and more intense PACAP signal is found in upper VZ and intermediate zone (IZ), where cortical precursors exit the cell cycle at E14.5 (Suh et al., 2001).

In the neural tube of mouse and rat embryos, PAC1 mRNA expression starts approximately at E9, during primitive streak stage, suggesting the peptide system regulates neural patterning (Waschek et al., 1998; Zhou et al., 1999). In E10.5 mouse cortex PAC1 mRNA is abundantly expressed through the neuraxis, especially in VZ and rhombencephalon (Waschek et al., 1998; Watanabe et al., 2007). The mRNA signal was found in the neuroepithelia of the mesencephalon at E11 and of the telencephalon at E13 (D’Agata et al., 1996; Jaworski and Proctor, 2000; Sheward et al., 1996). The expression is enriched in the developing cortex and spinal cord at E16 and E18 (D’Agata et al., 1996; Sheward et al., 1996). A strong level of hybridization signal was detected in the rostral portion of the lateral ventricle in the region where the rostral migratory stream starts, and in the olfactory bulb from E18 (Jaworski and Proctor, 2000). After birth, PAC1 mRNA is intensively expressed in the olfactory bulb, the hippocampus, and the cerebellum. However, the signal markedly decreases from P8 to P25 in the cerebellum (Jaworski and Proctor, 2000). Significant levels of PAC1 mRNA expression were almost co-localized with PACAP in multiple brain regions as listed above. The major splice variant of PAC1 receptor in the rat brain is the short isoform, which does not contain any cassette (Zhou et al., 2000). VPAC1 and VPAC2 mRNAs are found mainly
in the olfactory bulb, cerebral cortex and hippocampus. The PAC1 receptor transcript level is much denser than VPAC1 and VPAC2 transcripts in CNS (Basille et al., 2000). Embryonic stem cells culture models demonstrate the expression of PACAP and PAC1 mRNA correlate with neuronal and glial differentiation. Both PACAP and PAC1 mRNA are upregulated when stem cells are differentiated into neurons. In contrast, they both markedly decrease after glial differentiation, suggesting that this signal plays different roles in neurogenesis and gliogenesis. In addition, PACAP shows self-renewal inhibition and differentiation induction in neuronal progenitor cells (Hirose et al., 2006; Hirose et al., 2005). In contrast, other studies show that PACAP increases neural stem cell proliferation and maintains the undifferentiated state dependent on PKA activation (Ohta et al., 2006; Scharf et al., 2008). These findings raise the possibility that PACAP system has a critical role in embryonic development at a very early age and studies of PACAP signaling in stem cells may shed light on therapeutic replacement.

1.5.3 Association of PACAP signaling with neuropsychiatric disorders

Using multidisciplinary approaches in both humans and rodents, PACAP and PAC1 genes have been demonstrated to have possible association between several neuropsychiatric disorders recently. Here we discuss three of them.

1 Post-traumatic stress disorder (PTSD)

PTSD symptoms significantly correlate with PACP38 peripheral blood levels in females, but not in males, with markedly increased startle reflex responses in fear potentiation tests only in females. Notably, one PAC1 receptor single nucleotide polymorphism (SNP) rs2267735 is specifically associated with PTSD psychological and physiological phenotypes and has no correlation with other disorders, such as Alzheimer’s disease,
bipolar and schizophrenia. SNP rs2267735 is strikingly correlated with PTSD and is positioned in a putative estrogen response element, which predicts PTSD diagnosis and symptoms in females only. Rodent models demonstrate that fear increases PAC1 mRNA in amygdala and estrogen induces PAC1 mRNA in extended amygdala area. Although further analysis is warranted, these studies clearly illustrate that dynamic PACAP signaling is tightly correlated with central areas mediating fear, stress and estrogen responsiveness. In addition, the data may provide insights to explain sex-specific differences in PTSD diagnosis, symptoms and fear physiology, which may identify potential therapeutic targets for PTSD (Ressler et al., 2011).

2 Schizophrenia

Schizophrenia affects 0.5-1% of the general population and has some intermediate phenotypes including neurocognitive dysfunction, abnormal brain morphology and deficits in prepulse inhibition (PPI). PACAP -/- mice exhibit profound behavioral abnormalities such as hyperactivity and explosive jumping in an open field, increased novelty-seeking behavior and deficits in PPI (Hashimoto et al., 2001). Together with location of the PACAP gene, since the locus may be associated with schizophrenia, PACAP gene could be a good candidate gene for schizophrenia. The major allele of SNP3 rs1893153 and the minor allele of SNP5 rs2856966 in the PACAP gene were in excess in schizophrenia patients compared to controls. PAC1 gene SNP2 rs2302475 is also tightly associated with schizophrenia, suggesting PACAP signaling may contribute to pathophysiology of schizophrenia. Alleles of SNP3 in PACAP gene also exhibit hippocampal volume reductions and poorer memory performance, which may have
diagnostic application. Finally, an atypical antipsychotic, risperidone can rescue PACAP-
/- mice hyperlocomotion and abnormal social behavior (Hashimoto et al., 2007).

3 Major depressive disorder (MDD)

MDD is a common and highly prevalent mental disorder and its symptoms include
deficits in a range of cognitive, psychomotor and emotional processes. This disorder is
heterogeneous involving genetic and environmental contributors (Wong and Licinio,
2001). The MDD research is facing several obstacles. Specifically, many groups expose
the wild type animals to various acute or chronic stress in order to mimic MDD
symptoms since there is an absence of adequate animal models. The psychiatric medicine
is still under investigation and the majority of efforts in drug discovery have been
relatively unsuccessful because of the following reasons. CNS is the most complex
system, especially related to emotion, so each MDD patient symptoms and progression
vary. In addition, MDD is lacking defined pathology, has no direct tissue accessibility
and has a variety of behavior symptoms (Agid et al., 2007).

Clinically, doctors use some behavioral changes for more than two weeks as the possible
onset of MDD. In neuroscience research, animal models especially genetically modified
mice, exhibit similar behavior abnormalities that can provide some insights to MDD
therapy. PACAP-/ mice exhibited novelty-induced hyperactivity and deficits in prepulse
inhibition (Hashimoto et al., 2001; Tanaka et al., 2006), suggesting this gene correlates
with some fundamental mental processes. Recent studies showed PACAP-/ mice
exhibited depression-like behavior in the forced swim test, impaired circadian
corticosterone rhythm, reduced mRNA expression of glucocorticoid receptor in the
hippocampus and the low and flattened body temperature (Hashimoto et al., 2009). These
phenotypes are all related to clinical features of depression, implying that PACAP contributes to MDD. Furthermore, the atypical antipsychotic risperidone (a combined dopamine D2 and serotonin receptor antagonist) and the selective serotonin antagonist ritanserin normalized immobility behavior in forced swim test in PACAP-/- mice without affecting in wild type (Hashimoto et al., 2009). These observations suggest PACAP deficiency leads to some depressive conditions that might be due to perturbed serotonin signaling. Recently, analysis with depression patients and controls demonstrated the association with MDD with PACAP SNP3 (Hashimoto et al., 2010). Recent research has shown PACAP to be up-regulated by selective serotonin reuptake inhibitors and has shown the inducible effect of PACAP on brain derived neurotrophic factor (BDNF) mRNA expression in both rat cortical and hippocampal neurons. These data provide evidence of involvement of BDNF in antidepressant action and possible regulatory role in the process of neurogenesis (Reichenstein et al., 2008).

1.6 Biological functions of PACAP system in Central Nervous system

1.6.1 PACAP signaling transduction pathways

PACAP exhibits multiple functions, including modulating cell proliferation, promoting cell survival, inhibiting cell migration and stimulating cell differentiation, depending on its receptor splice variants. PACAP was named on the basis of its ability to stimulate cAMP. Binding of PACAP and PAC1 short variant activates Gs, which causes the increase of cAMP and causes the phosphorylation of CREB. cAMP then activates PKA signaling pathway, which further induces phosphorylation of ERK through Rap1 and Ras. In contrast, binding of PACAP and PAC1 hop variant also activates Gq, which activates
PLC and triggers PKC pathway besides activation of Gs. Similar results were obtained for hop1 and hop2, so I will use hop to refer to these two isoforms. Interestingly, the hop cassette encodes a consensus motif for phosphorylation by PKC, which may contribute to the fine-tuned regulation of receptor function (Spengler et al., 1993). In contrast, the presence of the hip cassette impairs both adenylate cyclase stimulation and PLC stimulation while hip-hop is less potent for both transduction pathways and unable to cause PLC stimulation (Spengler et al., 1993). Thus, short and hop are the major active isoforms in CNS that can couple with either PKA or PLC/PKC. Both PKA and PKC are essential kinases and involved in different biological processes. Although the original study has shown that short and hop isoforms are able to be coupled with cAMP and inositol phosphates (Spengler et al., 1993). More recent research showed short is unable to trigger calcium influxes while hop isoform can cause robust calcium influxes and PKC translocation to nucleus (DiCicco-Bloom et al., 2000; Nicot and DiCicco-Bloom, 2001).

### 1.6.2 Actions on neuronal precursor proliferation and differentiation

In rat, E13.5 dissociated cerebral cortical cultures, PACAP elicited a 43% decrease in thymidine incorporation and a 25% decrease in BrdU labeling index without affecting cell survival. Taken together with PACAP blockage of stimulation through combination of mitogens (IGF, FGF, EGF), PACAP appears to induce precursors to leave the cell cycle in general. PACAP caused robust intracellular cAMP increases and cAMP analog and activator in the same experimental paradigm also decreased DNA synthesis, suggesting PACAP elicits cell cycle withdrawal through cAMP pathway. At the same time, PACAP increased trkB immunoreactive cells by 30%, one widely expressed BDNF receptor to enhance differentiation. In addition, PACAP increased the number of cells
with neurites 2-fold, probably through cAMP elevation since PACAP raised P-CREB nuclear immunoreactivity and cAMP activator or analog exhibited similar results (Lu and DiCicco-Bloom, 1997). These results were confirmed and strengthened in vivo, that endogenous PACAP is a strong anti-mitogenic signal in the developing cerebral cortex (Nicot and DiCicco-Bloom, 2001). Using E13.5 and onward cortical neural precursors, PACAP inhibits proliferation by decreasing mitotic cell population through short variant (Lu et al., 1998; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001), which is mediated through p57^{kip2}-dependent CDK2 activity (Carey et al., 2002; Tury et al., 2011).

On the other hand as mentioned earlier, through hop variant, PACAP is coupled to inositol phosphate turnover and one downstream effector, increased intracellular calcium in several culture systems (DiCicco-Bloom et al., 2000; Nicot and DiCicco-Bloom, 2001; Spengler et al., 1993). Specifically, in E14.5 cortical precursors, where short PACAP isoform is present and normally an anti-mitogenic signal, overexpression of hop converts anti-mitotic effects of PACAP into a pro-mitogenic signal, through PLC activation, increased calcium level and translocation of PKC (Nicot and DiCicco-Bloom, 2001).

In the developing cerebellum (postnatal day 1 in rat), when PAC1 mRNA co-localizes with Shh receptor Patched 1 and the target gene Gli1, it significantly reduces the effects of Shh on proliferation, but does not affect the stimulation induced by other mitogens (insulin and B27). On the other hand, in the absence of Shh, PACAP is a significant pro-mitogenic factor in granule cell cultures. Again, PLC mediated pathway is pro-mitogenic while cAMP is anti-mitogenic in cerebellum. These data suggest that environmental context may determine PACAP signaling pathway (Nicot et al., 2002).
1.6.3 Effects on cell survival and neurite growth

PACAP’s trophic effects are mainly studied at cerebellar granule cells in different culture conditions and in vivo. Initially, PACAP was found to reduce the number of dying cells by morphological examination, while VIP is approximately 1000 times less potent in identical cultured conditions. Interestingly, KCl significantly increases PACAP survival-promoting effects, which can be accounted for the calcium influxes through voltage-operated L-type calcium channels (Gonzalez et al., 1997). The survival promoting effects are also confirmed by in vivo administration of PACAP in rat cerebellum. Specifically, PACAP injection increases the number of granule cells in the molecular layer and the internal granule cell layer, so overall PACAP induces a significant increase in the volume of the cerebellar cortex. However, it does not change the number of Purkinje cells and no effect on the medulla, suggesting PACAP may have regional specificity for survival promotion. The overall effect of PACAP in cerebellum is the significant increase in laminar thickness, which may result from proliferation promotion apoptosis inhibition or both (Vaudry et al., 1999).

PACAP has also been shown to rescue cerebellar granule cells from some deleterious actions of toxic molecules such as ethanol, which may provide therapeutic value for alcohol related mental problems. PACAP totally suppressed the effects of ethanol on DNA fragmentation and morphological changes while, again, VIP has no effect on ethanol-evoked cell death. Pharmacological inhibitors together with biochemical essays demonstrate that caspase-3 and caspase-6 are involved in the neurotoxic effect of ethanol. PACAP’s neuroprotective effects are primarily mediated through PKA/PKC pathways in cerebellum based on two pieces of evidence. First, both dibutyryl-cAMP (dbcAMP) and
phorbol-12-myristate-13-acetate mimic PACAP inhibitory effects on caspase-3. Second, PKA and PKC inhibitors (H89 and chelerythrine) abrogate PACAP action on caspase-3. (Vaudry et al., 2000). However, caspase-6 pathway is not clear and the inactivation may be due to PKA dependent phosphorylation or PACAP may activate endogenous caspase-6 blocker (Vaudry et al., 2002b).

Interestingly, incubation of PACAP (10^{-10} to 10^{-6} M) exhibits a dose-dependent increase in the length of neurites and cell survival in P8 rat granule cells (Gonzalez et al., 1997) as well as in PC12 cell line (Vaudry et al., 2002a), which may be mediated through activation of the adenylate cyclase pathway. On the other hand, ethanol causes deleterious effects on granule cells with the direct morphological changes resulting in the inhibition of neurite outgrowth. In contrast, nanomolar concentrations of PACAP increase the total length of neurites and the number of processes per cell, and may serve as a neurotrophic supportor suppressing apoptosis (Vaudry et al., 2002b).

Cardiac arrest is a commonly used model to study ischemia. The circulation is interrupted completely by compressing the major cardiac vessels to cause transient global ischemia. In this model, PACAP protects hippocampal neurons from apoptosis by inhibiting c-Jun N-terminal kinase 1/stress activated protein and p38 pathway (Dohi et al., 2002). Notably, PACAP exhibits long term effects preventing cell death up to several hours (Reglodi et al., 2000) after ischemia. Administration of this peptide prior to stroke (Reglodi et al., 2002) reduces the infarct size, and endogenous PACAP can protect brain from neuronal damage and decrease infarct volume (Ohtaki et al., 2006). In sum, studies of PACAP in cell survival provide a potential therapeutic target.
PACAP inhibits apoptosis, stimulating cAMP and polyphosphoinositol hydrolysis. Activation of ERK contributes to long-lasting inhibition of caspase-3 activity. In addition, PACAP regulated c-fos expression, and c-fos stimulates Bcl-2 expression, an important anti-apoptosis gene. PACAP also prevents cytochrome c release and inhibits caspase-9 activation. PACAP anti-apoptotic effects after ischemia is indirect from STAT3 by generation of IL-6, whereas PACAP directly regulates ERK (Ohtaki et al., 2006).

1.7 RNA splicing

1.7.1 RNA splicing background
Alternative pre-mRNA splicing is a complicated process that controls many eukaryotic genes’ protein output and contributes to proteomic diversity (Black, 2000). First we discuss the general splicing to demonstrate this process.

General Splicing
Unlike other steps of RNA production, RNA splicing is performed mainly by RNA molecules (less than 200 nucleotides each) instead of proteins. RNA molecules are able to recognize intron-exon borders to initiate splicing events, and they usually form different complexes with several protein subunits to form a snRNP (small nuclear ribonucleoprotein). There are five of these RNA molecules (U1, U2, U4, U5, and U6), known as snRNAs (small nuclear RNAs). General splicing apparatus includes ribonucleoprotein (RNP) complex called spliceosome (core) and auxiliary proteins. The assembly of the spliceosome has been characterized using several in vitro systems. U1 snRNP forms base pairs with the 5’ splice junction after the branch-point is recognized
by the branch-point binding protein and U2AP, a helper protein. U2 snRNP is recruited by U2AP and displaces U2AP to form complementary base pairs with the branch-point lying upstream of the 3’ splice site, which is an ATP-dependent step. The mechanisms underlying the assembly are still poorly understood, however, these factors form a complex named E complex or commitment complex before U2 joins, which bends the intron and brings two splice sites, that are ready to be cleaved next, close. Once the pairing of U2 snRNP at the branch-point, the complex is called pre-spliceosomal A complex. At this point, U4-U5-U6 snRNAs enter the spliceosome and form a larger complex, which is also a step driven by ATP. In this triple snRNP, the U4 and U6 snRNAs are held firmly by base-pair interactions and U5 is relatively loose. Several RNA-RNA rearrangements occur to eject U4 snRNP from the splicesome and allow U6 snRNP to displace U1 at the 5’ splice site [Introduction fig.-2]. These steps create the active site of the splicesome and place the appropriate portions of the pre-mRNA substrate for the splicing reaction to occur. Finally, the reaction produces the ligation of the exons and the excision of the intron, in the form of a lariat RNA that has 5’phosphate joined to the 2’ hydroxyl at the branch point. Excised intron RNA will be degraded in the nucleus and snRNPs will be recycled for the next reaction (Alberts) (Li et al., 2007; Shin and Manley, 2004).

Now many questions remain to be answered, for example we are still unable to predict, from sequence alone, the location of splice sites to determine what exons will be generated (Li et al., 2007).
Introduction figure- 7 Spliceosome assembly pathway (Li et al., 2007).

1.7.2 RNA splicing in nervous system

Alternative pre-mRNA splicing is found widely in nervous system, and the output proteins that are products after splicing exhibit significant biological activities including cell-fate determination (proliferation or differentiation), axon guidance, synaptogenesis, migration, and neuronal connectivity (Lee and Irizarry, 2003; Revil et al., 2010). This is a complicated, dedicated, dynamic and multiple-layer regulation process in neuronal cell biology. Errors in splicing events lead to several neurological and neuromuscular
diseases such as frontotemporal dementia and spinal muscular atrophy (Licatalosi and Darnell, 2006; Ranum and Cooper, 2006).

Now it is known that a substantial proportion of human genes (35-60%) undergo alternative splicing to produce transcripts (Johnson et al., 2003) and regulatory splicing decisions can be found at almost every step in neuronal development, which reinforces the idea that the control of splicing is an important area in gene regulation. Recently, PACAP signaling has been shown to be one of the genes that undergoes a splicing event at the E8.5-E11.5 window in mouse (Revil et al., 2010). However, the mechanisms of splicing regulation, especially the regulatory proteins and signaling transduction during spliceosome assembly, are not well understood (Li et al., 2007; Shin and Manley, 2004). Recently, several examples have emerged that link cell signaling and splicing control in neuronal cell biology.

Many different splicing events happen during neuronal development, presumably as a result of expression of splicing regulators. These regulatory decisions are critical in neurogenesis, since splicing could be found at any step in neuronal development starting at neuroblast commitment. Next, we will describe examples of three splicing events in CNS development.

**FGF8 during neural tube patterning**

As we have described earlier, FGF8 is secreted at the midbrain-hindbrain boundary and the gradient contributes to the patterning of the neural tube. There are two FGF8 isoforms in this region, FGF8a and FGF8b, which exhibit distinct affinity for FGF receptors. The alternative 3’ splice site in the second exon generates the additional 11 amino acid at N terminus in FGF8b. The two isoforms elicit remarkable patterning
difference in neural tube. Ectopic expression of FGF8a expands the midbrain, and ectopic expression of FGF8b transforms the midbrain tissue into cerebellum, suggesting their regional specificity. Alternative splicing is also happening in the developing forebrain in other FGF members, including FGFs 2, 5, 9, 17 (Li et al., 2007).

Synaptogenesis
Proteins assemble to form different pre- and post-synaptic complexes at the synaptic cleft during synaptogenesis. Two key adhesion molecules are neurexins on the presynaptic membrane and the neuroligins on the postsynaptic membranes. They are both encoded by multiple genes in mammals, each have alternative splicing sites, and the combination of these splicing positions can potentially result in more than 1000 isoforms (Ullrich et al., 1995). Coordination of the appropriate excitatory or inhibitory synapses is governed by the splicing of both neurexins and neuroligins. Specifically, glutamatergic synapses have neuroligin 1 (+B) and β-neurexin (-S4) while the GABA mediated synapses are neuroligin2/neuroligin 1(-B) and α/β-neurexin (Li et al., 2007).

Regulation of ion channels
Exons in calcium- and voltage-gated potassium channels (BK channels) are alternatively spliced to control neuronal activity. BK channels are widely expressed in CNS and different splicing results in diverse electrical properties. Intracellular contents are also involved in the splicing events, such as high-potassium media represses expression of the exons and stress hormones regulate the stress-axis-regulated (SRTEX) exon specifically. Presence of STREX alters the calcium and voltage sensitivity and also changes modulation by PKA. The process is mediated by an L-type calcium channel-and calcium/calmodulin-dependent protein kinase (CaMK). A CaMK responsive RNA
element is also identified that can repress a heterologous exon based on the activated CaMK (Li et al., 2007; Shin and Manley, 2004). Many components in the process are unknown, but importantly it outlines a signaling pathway involved in regulating ion channel properties.

CHAPTER 2 RATIONALE

Although PACAP signaling pathways have been well studied in later central nervous system development and play essential roles in regulating neural precursor mitosis at E13.5 and onward (Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001), its function during early neurogenesis, are rarely addressed. Both short and hop mRNA isoforms are present as early as primitive stage at E9 (Basille et al., 2000; Waschek et al., 1998; Watanabe et al., 2007; Zhou et al., 1999), when the neuroepithelium consists of a single cell layer (Qian et al., 1998). The majority of neuroepithelial cells are actively proliferating at this time to enlarge neuronal precursor pools in the presence of several morphogens such as FGF and Shh. PACAP anti-mitogenic effects on proliferation seem contradictory to the proliferation promoting environment at the onset of neurogenesis. On the other hand, PACAP has been shown to stimulate neural stem cell proliferation and promote neurogenesis both in vitro and in vivo in hippocampus, subventricular zone (SVZ) and spinal cord (Fang et al., 2010; Mercer et al., 2004; Ohta et al., 2006; Scharf et al., 2008). The underlying mechanisms of PACAP effects on proliferation are still under debate, but hop isoform mediated PLC/PKC pathway can stimulate mitotic activity (DiCicco-Bloom et al., 2000; Lu et al., 1998; Nicot and DiCicco-Bloom, 2001). Ectopic overexpression of hop in
E14.5 cortical precursors converts PACAP inhibition to stimulation (Nicot and DiCicco-Bloom, 2001). Given that the hop isoform exhibits pro-mitogenic effects, PACAP is a good candidate mitogen during this critical developmental period.

The purpose of the project is to identify the relative expression levels of short and hop isoforms during corticogenesis to further define the function of PACAP system at specific development times. Here we tested the hypothesis that PACAP exhibits distinct mitogenic activities during neurogenesis, depending on PAC1 receptor isoforms. We found E10.5 precursors predominantly express the hop isoform, while the short mRNA is up-regulated and becomes dominant at E14.5. Consistent with this age-dependent isoform expression, PACAP stimulates proliferation, evokes calcium fluxes and increases phospho-PKC levels at E10.5 but not at E14.5 precursors, suggesting control of mRNA isoform expression contributes to neurogenetic regulation. I will describe these results in details in the following chapters.

CHAPTER 3 MATERIALS AND METHODS

3.1 Animals

Time-mated pregnant Sprague Dawley rats were obtained from Hilltop Lab Animals (Philadelphia, PA). Breeding pairs of PACAP mice on a C57BL/6 background were derived by Waschek as described (Colwell et al., 2004). The animals were managed by Robert Wood Johnson Animal Facility. Animal maintenance, husbandry, transportation, housing and use were in compliance with the Laboratory Animal Welfare Act (PL 89-544;
PL-91-579) and NIH guidelines (NIH Manual Chapter 4206). Food and water were available ad libitum. The day of plug was considered as E0.5.

### 3.2 Cell cultures

**Culture preparation and conditions**

Embryos of E10.5, E11.5, E12.5 and E14.5 *Sprague Dawley* rats or E9.5, E10.5 and E13.5 C57BL/6 mice were dissected under a dissecting microscope. E10.5 rat and E9.5 mouse telencephalic vesicles were incubated with trypsin (0.25 mg/ml, Sigma-Aldrich, St. Louis, MO) at 37°C for 12 min to aid in removing overlying epidermal ectoderm using dissecting forceps. An equal volume of trypsin inhibitor (1 mg/ml) was added to stop the enzyme activity. The cortices were then mechanically dissociated using a fire-polished glass pipette. Cells were plated on 2 µg/ml poly-D-lysine-coated 96-well plates (10,000 cells/well), 24-well plates (25,000 cells/well) or 35 mm dishes (80,000 cells/dish) in defined medium consisting of a 1:1 mixture of DMEM and F12, as described previously (Lu and DiCicco-Bloom, 1997; Mairet-Coello et al., 2009) containing insulin (10 µg/ml), bFGF (10 ng/ml) and BDNF (30 ng/ml, Peprotech, Rocky Hill, NJ) with vehicle (0.01 N acetic acid) or PACAP (10 nM). To study the PLC pathway, cells were plated into medium containing PLC specific antagonist U-73122 (2 µM, Enzo, Farmingdale, NY) or inactive isomer U-73343 (2 µM, Enzo), and after 30 min we added either vehicle (0.01 N acetic acid) or PACAP (10 nM). For RA experiments, cells were incubated with either RA (3 x 10⁻⁸ M) or ethanol.

**Immunocytochemistry**

For triple immunofluorescence, cells were fixed in 4% paraformaldehyde (PFA) for 20 min at 2 h, 24 h or 48 h, washed with PBS three times, incubated with anti-RC1 (1:1000,
40E-C, Developmental Studies Hybridoma Bank, San Francisco, CA), anti-nestin (1:400, Chemicon, Billerica, MA), anti-Tuj1 (1:1000, Convance, Princeton, NJ), anti-phospho-PKC (1:200, Cell Signaling) or anti-phospho-ERK (1:1000, Cell Signaling) in PBS containing 0.3% Triton X-100 and 2% NGS overnight at 4°C. Antibodies in PBS containing 0.3% Triton-100 overnight at 4°C. Images were acquired with fluorescence microscope (Axiovert 200; Carl Zeiss MicroImaging) using 20X objective from 10 random fields per dish. Fluorescence analysis was processed with the computer program AxioVisionLE software (Carl Zeiss MicroImaging) to determine mean fluorescence intensity.

### 3.3 DNA synthesis and cell division in vitro

DNA synthesis was assessed using [³H] thymidine incorporation. 1μCi/ml [³H] thymidine (Amersham/GE Healthcare, Pittsburgh, PA) was added to 24-well cell plates 4h before harvest for scintillation spectroscopy. Alternatively, BrdU (10µM, final) was added 4h before culture termination to define BrdU labeling index at 24h, by counting BrdU immunolabeled cells over total cells under phase, using 10X objective from 10 randomly selected fields in each of three dishes per group.

To determine whether cells entering S phase in vitro subsequently underwent division between hours 24 and 36, a cohort of cells in each group was labeled using a 4h BrdU pulse from hour 20 to 24. After cells were washed twice with PBS, one set of cultures was fixed immediately to define numbers of cells in S phase. A parallel set received culture media and were incubated another 12h, then they were fixed at 36h. The absolute number of BrdU-labeled cells per field at both times was determined by the average of
BrdU immunolabeled cells using 10X objective from 10 randomly selected fields per dish. The total numbers of both groups at 24h and 36h are determined by the sum of 10 random fields per dish at 10X objective. Control and PACAP groups at 24h and 36h have two dishes per experiment.

3.4 Cell survival analysis

At 2h and 24h, 15μg/ml FDA (green fluorescence in living cells) and 15μg/ml propidium iodide (red fluorescence in dead cells) were incubated with cells for 10min as described previously (Mairet-Coello et al., 2009; Vaudry et al., 2003). Percentage cell survival was determined as the ratio of total green cells at 24h over total green cells at 2h.

3.5 Measurement of intracellular calcium

Culture medium was removed from 96-well plates and cortical precursor cells were washed with Balanced Salt Solution (pH=7.2) (Pan et al., 2000; Wang et al., 2005). Cells were incubated with Ca$^{2+}$ indicator Fluo-4 AM (3μM, Molecular Probes, Eugene, OR) for 30min at 37°C. Following vehicle, PACAP (10nM) or KCl (40mM) addition, fluorescence intensity was measured using excitation wavelength at 488±5nm and emission wavelength at 515±5nm using BD Pathway 855 BioImager with 20x objective (Olympus Plan fluo 0.75 NA). Images were acquired at 1s intervals and the recording lasted for 110 s after administering reagents. Recordings were performed at 37°C in a 5% CO$_2$ atmosphere. The mean time-dependent fluorescence intensity of individual cells was measured using Image J software after background subtraction.
3.6 BrdU labeling of PACAP WT and KO mice

To maximize comparability, we performed heterozygous PACAP mating to produce WT and KO littermate embryos for analyses. BrdU 100µg/g was injected into E9.5 pregnant mice 1h before sacrifice. Embryo brains were immersion fixed in 4% PFA for 30min at 4°C and processed for paraffin embedding. Brains were cut into 6µM sections on a microtome (Leica). Sections were immunostained for BrdU incorporation and counterstained with propidium iodide as previously reported (Mairet-Coello et al., 2009). Images were obtained from a Zeiss LSM 510-META confocal microscope. BrdU positive nuclei and total nuclei were counted within a 100µm wide sector based on the ventricular surface and to the ventricular zone/intermediate zone boundary. BrdU labeling indices were calculated as the BrdU positive cells over total as described (Suh et al., 2001). Tissues from each embryo were used to determine the genotype using touchdown PACAP PCR described previously (Colwell et al., 2004). The sequences of the primers:

omPCPF1+: 5’- GTT ATG TCG GTG CGG AGG AGT TTC-3’
omPCPF2-: 5’-TTC AAG CAG CCT GCC CCA GAC TCA-3’
omPCPD2-: 5’- GCT GGA TAG TAA AGG GCG TAA-3’

3.7 Immunohistochemistry

Animals were perfused with PBS and then 4% paraformaldehyde (PFA). After that, brains were dissected and further fixed in 4% PFA overnight and dehydrated by 30% sucrose in PBS until they have sunk. Tissue was then embedded in OCT and stored in -80 degrees until use. Frozen tissue sectioning was performed on a cryostat at 12µm thickness. Section was then immunostained with Cleaved Caspase-3 (1:200, Chemicon). After exposure to biotinylated secondary antibody (1:200 dilution; Vector) and
Vectastain ABC kit (Vector), signal was visualized using DAB. Sections were air-dried and mounted on gelatin-coated slides and coverslipped.

3.8 Quantitative reverse transcription-PCR (Q-RT-PCR)

Total RNA was extracted from E10.5 and E14.5 rat cerebral cortices using RNeasy Mini Kit (QIAGEN. Valencia, CA). DNA was digested by 15min DNase I treatment. 0.5μg RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, Madison, MI). Quantitative reverse transcription PCR was performed using PCR Master Mix (Applied Biosystems, Foster City, CA) and reactions were performed in an ABI Prism 7000 Sequence Detection system (Applied Biosystems) as described (Mairet-Coello et al., 2009). The sequence of the primers:

PAC1 short forward 5’- AGTCGAGCATCTACTTACGCGC-3’
PAC1 short reverse 5’-TTCCCTCTTGCTGACGTTCTC-3’
PAC1 hop forward 5’-ACTTCAGCTGCGTGCAGAAATGC-3’
PAC1 hop reverse 5’-GACGTTCTCTGGAGAGAAGGCAA-3’

Both primers worked with high specificity and non-specific PCR was ruled out by a clear peak in the melting curves. cDNAs encoding PAC1 short or PAC1 hop1 were provided by Dr. Laurent Journot, Montpellier, France and purified as reported previously (Nicot and DiCicco-Bloom, 2001).

3.9 Western blot

Four to nine animals from 2 to 3 different litters were examined at different ages for each genotype. The mixture was centrifuged and the supernatants (50-100μg/lane) were electrophoresed on 12% SDS-PAGE, transferred to a PVDF membrane, immunoblotted
and revealed by chemiluminescence. Cleaved caspase-3 antibodies are from Chemicon and used at 1:1000. (Mairet-Coello et al., 2009)

3.10 Statistical analysis

Data are expressed as mean±SEM. Statistical comparisons were made by unpaired Student’s t test or one-way ANOVA using Excel (Microsoft) and GraphPad Prism (GraphPad Software, La Jolla, CA).

CHAPTER 4 EXPERIMENTAL RESULTS

4.1 Deletion of the PACAP gene (ADCYAP1) results in decreased proliferation in the early embryonic ventricular zone (VZ)

Previous studies indicate that PACAP inhibits cortical precursor proliferation at E13-18 (Lu and DiCicco-Bloom, 1997; Suh et al., 2001; Waschek et al., 1998). To begin defining effects on early neurogenesis, we compared the number of cells in S phase in the VZ of the WT and PACAP-/- mice at E9.5 using BrdU immunohistochemistry [Fig. 1]. At E9.5, the embryo is around 3500μm in length and 1500μm in width, the rostral neural tube has closed to form the three primitive brain vesicles, and the telencephalic vesicles appear from E9.5 to E10.5 in mouse. The prosencephalic vesicle consists of only the VZ and exhibits a 4-5 cell thick layer. The PACAP-/- prosencephalon exhibited a 21% decrease in BrdU labeling index (LI) compared to the WT, suggesting PACAP has a pro-mitogenic role at this age. To further analyze this possibility and to manipulate PACAP in various conditions, we established a culture model of young precursors to define PACAP effects during early neurogenesis.
Figure 1  
Reduced BrdU labeling in the telencephalic vesicles of E9.5 PACAP -/- mice.

BrdU immunohistochemical staining of E9.5 wild type (WT) (a-c) and PACAP-/- (d-f) ventricle zone (VZ). BrdU-positive cells (green) and total cells (propidium iodide; red) were counted in the VZ. (g) Quantification of BrdU labeling index (LI). PACAP-/- exhibited a 21% decrease in BrdU LI. n=3 for each genotype. Results are expressed as mean ± SEM, **P<0.01. Scale bar, 10μm.
4.2 Cell survival is increased in PACAP -/- animals

Since PACAP has been well-documented to have neuroprotective roles, we conducted the following experiments to test whether PACAP deletion affects cell survival. We first performed cleaved-caspase 3 western blotting using E12.5 to E14.5, E18.5 and P0 wild type and PACAP-/- cortices: KO animals exhibited more total caspase-3 level compared to WT [Fig. 2]. Next we conducted caspase-3 immunohistochemical staining and found KO animals have 3-fold more positive cells in E12.5 cortices (WT=3.1±0.7; KO=14.3±4.4). Increased caspase-3 immunostaining suggests cell survival is affected after E12.5, consistent with total protein levels in western. To distinguish PACAP impacts on proliferation from survival promoting, we need to generate a better animal system that cell death is prevented. We purpose to cross PACAP heterozygous line to Bcl-2 overexpression mice to distinguish PACAP anti-apoptotic effects to its developmental dependent mitosis impact in vivo. Decreased BrdU labeling index in KO may be a result of increased cell death, so we are now collecting E9.5 WT and KO tissues to carry out TUNEL staining. Next, using the newly established cell culture system, we confirmed PACAP effects on cell cycle are independent of cell survival.
Figure - 2 Cell death is increased in E12.5 PACAP-/- cortices.
(a,b) Capase-3 positive cells (DAB, brown), and total cells (toluidine blue, blue), LV: lateral ventricle.
(c) Quantification of cleaved caspase-3 immunohistochemistry using three animals in each
genotype*P<0.05. Scale bar, 10μm. (d) Total cleaved caspase-3 protein expression in WT and KO
cortices.

### 4.3 Characterization of precursor cell culture at E10.5

At E10.5 (similar to E9.5 mouse; Fig. 1a), the rat cerebral cortex consists primarily of
VZ precursors, with the majority proliferating [Fig. 3a]. To maintain precursor survival,
we used defined media containing multiple trophic factors including insulin, bFGF and
BDNF. In the absence of trophic factors, early precursors were unable to survive for 20h
even when incubated in Neurobasal plus B27 media. Leukemia inhibitory factor (LIF)
and neurotrophin-3 (NT-3), which are essential for embryonic stem cell growth, seem not
to enhance young precursor survival in our preliminary studies. In the presence of LIF
(30ng/ml) or NT-3 (30ng/ml), >95% E10.5 precursors (E9.5 mice) die at 20h, at the time
[3H] thymidine is added. After 4h incubation, [3H] thymidine reading is lower than
100cpm at 24h, similar to control media lacking growth factors. In this model,
98.1±5.4% of the E10.5 precursors expressed RC1 and 96.2±3.2% expressed nestin at
24h, both markers of neural precursors [Fig. 3b, c]; 15.6±2.2% of the cells expressed β
III tubulin (TuJ1), an early marker of postmitotic neocortical neurons [Fig. 3d]. At 48h,
the percentage of β III tubulin increased to 19.8±2.9% at 48h, suggesting the
differentiation potential of these precursors. In contrast, neither 24h nor 48h cultures
exhibited glial antigens, including markers of astrocytes, glial fibrillary acidic protein
(GFAP), nor oligodendrocyte progenitors, NG2 (not shown). E11.5 and E12.5 cultures at
24h exhibited a decrease in precursor markers compared to younger age (E10.5) (92.1% and 88.4% expressing RC1; 87.9% and 86.2% expressing nestin, respectively) and an increase in neuronal protein (18.1% and 43.9% β III tubulin, respectively). At 48h, the percentage of β III tubulin increased in both E11.5 and E12.5 cultures (32.2% and 48.0%, respectively) [Tabel-1; Figure-4], suggesting age dependent differences in developmental capacity at the time of plating, as reported previously (Romito-DiGiacomo et al., 2007).

In addition, cell shapes vary at 24h

**Table-1 Characterization of E10.5-E12.5 cell cultures at 24h and 48h**

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Percentage (%)</th>
<th>RC1</th>
<th>Nestin</th>
<th>Tuj1</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5 24h</td>
<td>96.2</td>
<td>98.1</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>E10.5 48h</td>
<td>100</td>
<td>100</td>
<td>19.8</td>
<td></td>
</tr>
<tr>
<td>E11.5 24h</td>
<td>92.1</td>
<td>87.9</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>E11.5 48h</td>
<td>90.2</td>
<td>72.5</td>
<td>32.2</td>
<td></td>
</tr>
<tr>
<td>E12.5 24h</td>
<td>88.4</td>
<td>86.2</td>
<td>43.9</td>
<td></td>
</tr>
<tr>
<td>E12.5 48h</td>
<td>85.1</td>
<td>82.7</td>
<td>48.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure - 3 Characterization of a new precursor culture model and age-dependent effects of PACAP.

(a) Photomicrograph of a rat embryo at E10.5. White box indicates the telencephalic region dissected for culture. (b-d) Cortical cultures consist of neural precursors and differentiating neurons at 24h and 48h. Immunocytochemical analysis of cell type specific markers: (b) RC1 (c) nestin (d) β III tubulin (e) merged. Arrowheads indicate RC1 and nestin double positive cells, double arrowheads indicate β III
tubulin positive cells, and arrows indicate triple positive cells. (d) Scale bar, 10\(\mu\)m. Data were obtained from three experiments, 100-150 cells analyzed per staining. (f, g) Effects of PACAP on DNA synthesis at 24h in rat (f) and mouse (g) precursors from different ages. DNA synthesis was assessed using \([^3]H\) thymidine incorporation. Data were obtained from five experiments for each species, using three wells (25,000 cells/well) per group for each experiment. Data are expressed as percent control: control values ranged from 4000-12000cpm, *P<0.05, **P<0.01.

**Figure - 4 Characterization of E10.5-E12.5 rat precursors at 24h.** Examples of immunocytochemical staining, typical morphology at different ages and percentage of neuronal precursor markers and immature neuron marker.
4.4 PACAP exhibits age-dependent effects on DNA synthesis in both rat and mouse.

To define PACAP neurogenetic activity, we examined effects of exogenous PACAP using rodent cortical precursors isolated at different embryonic ages under identical culture conditions. Initial studies employed rat, rather than mouse, precursors based on more extensive previous work in the former model. In E10.5 rat precursors incubated for 24h, PACAP exposure elicited a 33% increase in $[^{3}H]$ thymidine incorporation [Fig. 3f]. However, just one day later, at E11.5, PACAP elicited no effects. Subsequently, at E12.5 and E14.5, PACAP inhibited mitosis by 23% and 39% respectively, suggesting mitogenic effects are developmental stage dependent [Fig. 3f]. The anti-mitogenic effects elicited at older ages are consistent with previous studies that used media lacking the panel of trophic factors (Lu and DiCicco-Bloom, 1997; Suh et al., 2001).

To allow comparison to genetic deletion mutants, we extended study to the mouse, examining comparable developmental stages, E9.5, E10.5 and E13.5. In E9.5 mouse precursors. We first tested several culture conditions because of limited previous experiences. We found in the presence of insulin (10μg/ml), BDNF(30ng/ml) and FGF (0.2ng/ml) E9.5 precursors are able to live up to 72h in defined media, so we conducted all the mice precursor cultures in this condition. Notably, FGF concentration is much lower compared to rats, suggesting the species sensitivity difference to some growth factors. PACAP exposure increased $[^{3}H]$ thymidine incorporation by 23% compared to vehicle, a stimulation comparable to that observed in E10.5 rat. Again PACAP stimulation is a result from G1/S progression not survival promoting, which is confirmed by BrdU labeling and total cell number counting. Similarly, PACAP elicited no effect at
E10.5 (E11.5 in rat) and induced a 30% decrease on E13.5 (E14.5 in rat), suggesting mitogenic effects are developmental stage dependent in both species [Fig. 3g]. Interestingly, we found PACAP is a strong anti-mitogenic signal in E14.5 mouse precursors (E15.5 in rat) in all culture conditions that we used at 24h [Fig. 5].

4.5 PACAP promotes G1/S progression without altering cell survival at E10.5.

Since PACAP elicited similar effects in both species, we continued investigations using rat precursors, because they are more convenient to isolate and maintain, and allow
comparision with extensive previous studies (Carey et al., 2002; DiCicco-Bloom et al., 2000; Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001). While PACAP increased DNA synthesis by 33% in E10.5 cultures [Fig. 3f], this effect may be attributable to either promoting G1/S progression, or alternatively, preventing precursor cell death, since PACAP has well documented neurotrophic activity (Ohta et al., 2006; Vaudry et al., 2009; Waschek, 2002). To begin addressing this question, precursors engaged in S phase were labeled with a 4h BrdU pulse to define the labeling index at 24h. After 24h PACAP exposure, the BrdU LI was increased by 25% [Fig. 6a-c] from 36% in control to 45% in PACAP, consistent with PACAP increasing G1/S progression. To determine whether cells engaged in S-phase were able to complete mitosis and divide, we labeled a cohort of precursors by BrdU exposure from 20h to 24h, and following PBS washes, cells were either fixed immediately, to establish numbers of cells in S phase, or alternatively incubated them another 12h in control or PACAP containing medium. If cells engaged in S phase (incorporated BrdU) at 24h subsequently underwent division, the absolute number of labeled cells would increase from 24h to 36h. Indeed, at 36h, both control and PACAP groups demonstrated an increase in BrdU+ cells, indicating that cells successfully underwent division, with the PACAP group producing more new cells than control [Fig. 6d].

At 24h, when the BrdU LI was increased by PACAP [Fig. 6c, d], total cell numbers were no difference between control and PACAP exposed cultures [Fig. 6e, 24h], consistent with PACAP promoting S phase entry instead of survival promoting. To examine the role of cell survival, we assessed the FDA/PI assay. Further, there was no difference in cell survival among groups compared to cells plated at 2h [Fig. 6f], suggesting that PACAP did
not exhibit trophic activity during this period. On the other hand, at 36h, while there was no change in cell number in control media, the PACAP treated group displayed a 61% increase in total cells compared to 24h (Fig. 6e, 36h), suggesting that enhanced S phase entry induced by PACAP at 24h resulted in more neurogenesis 12h later. In aggregate, these data suggest that PACAP promotes precursor proliferation by increasing the proportion of cells that enter S-phase and complete cell division.
Figure - 6  Effects of PACAP on cell cycle progression and cortical precursor cell number in vitro.
(a, b) BrdU positive cells and total cells were counted in 10 randomly selected fields. (c) PACAP exposure increased the BrdU LI by 25%. (d) Precursors in S phase were BrdU labeled from hour 20 to 24, then after PBS washes, were fixed at 24h or incubated in control or PACAP medium for another 12h and were fixed at 36h. At 24h, 59% more cells were in S phase in the PACAP exposed group, similar to results in 3c. At 36h, the number of BrdU+ cells increased in both groups, indicating ongoing cell division. (e) Quantification of total cell number in control and PACAP-treated cultures at 24h and 36h. The y-axis corresponds to the total number of cells counted in 10 random fields. Whereas no difference in cell number among groups was observed at 24h, a 61% increase was detected in the PACAP group at 36h. Note there was no change in control between 24h and 36h. (f) Effects of PACAP on cell survival and cell death. PI and FDA were used to distinguish dead cells (arrowheads) and living cells (arrows) respectively. Scale bar, 10μm. Percentage cell survival was determined as the ratio of FDA-stained cells at 24h over 2h. Results are expressed as mean ± SEM, *P<0.05, **P<0.01. Data were derived from three different experiments, two or three dishes per group per experiment.

4.6 Transition of PAC1 receptor isoforms during neurogenesis.

Since PACAP elicited opposing effects on proliferation of precursors at different ages, we speculated the effects may reflect expression of distinct PAC1 isoforms. Thus, we examined expression of short and hop isoforms in E10.5 and E15.5 rat cortices using traditional RT-PCR [Fig.6a] and found that both variants were present, consistent with previous studies (Waschek et al., 1998; Zhou et al., 1999). To quantify stage-dependent expression of the short and hop isoforms, we performed quantitative real-time PCR. Using two standard curves for short and hop [Fig. 6b], we obtained both short and hop mRNA expression levels at E10.5 and E14.5. There was 24-fold greater expression of the hop than
the short isoform at E10.5 [Fig. 6c, left panel]. In contrast, by E14.5 the short mRNA was up-regulated markedly, while the hop isoform changed little, reversing the ratio, so that short was 15-fold greater than hop [Fig.6c, right panel]. The transition of the PAC1 isoform mRNA suggested that different signaling pathways may underlie the developmental stage specific mitogenic effects of PACAP.
Figure 7: Transition of PAC1 receptor isoform mRNAs during corticogenesis.
(a) Both short and hop isoforms are present at E10.5 and E15.5 rat cortices. (b) Construction of the real-time PCR standard curves using serial dilutions of the two plasmids. Each standard dilution and sample was analyzed in duplicate. The curves are highly linear (R2>0.99). (c) At E10.5, hop is 24-fold greater than short. At E14.5, short is 15-fold greater than short. Values presented as fold difference when compared with short mRNA levels. Values are representative of three experiments, three to eight animals per group for each experiment.

4.7 The role of PLC activation in PACAP’s pro-mitogenic effects.

In previous studies, PACAP mitogenic stimulation has been associated with activation of PLC that triggers the PKC pathway (Lu et al., 1998; Nicot and DiCicco-Bloom, 2001; Spengler et al., 1993). Since hop is the dominant receptor isoform at E10.5, we hypothesized that PLC activation may underlie PACAP signaling. To investigate the role of PLC, we employed a membrane-permeable PLC specific inhibitor, U-73122 and the inactive form U-73343 as a negative control. We incubated E10.5 cultures for 16h only to avoid overall cell toxicity. In the presence of inactive drug, PACAP elicited a 22% increase in [³H] incorporation whereas PACAP elicited no change when the PLC inhibitor was present, suggesting that pro-mitogenic effects were mediated via the PLC pathway [Fig. 5]. Nonetheless, because of possible cell toxicity even using shortened incubation, we turned to more direct methods to detect cellular responses.
Figure - 8 The role of PLC activation in PACAP’s pro-mitogenic effects at E10.5.

In the presence of the inactive analogue U-73343 (2μM) for 16h, PACAP increased DNA synthesis by 22%, while the peptide-induced increase in DNA synthesis was blocked by PLC antagonist U-73122 (2μM). Four groups were analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. Data were derived from four experiments, three wells per group for each experiment, **P<0.01, ***P<0.001.

PACAP induces intracellular calcium oscillation in E10.5 but not E14.5 cortical precursors.

Activated PLC can hydrolyze phosphatidylinositol 4,5-bisphosphate to generate two second messengers, diacyl glycerol (DAG) and inositol 1,4,5 tris-phosphate (IP3). The latter leaves the plasma membrane and diffuses rapidly through the cytosol to engage the IP3 receptor in the endoplasmic reticulum (ER). Ca^{2+} stored in the ER then is released
through the IP3-gated Ca\textsuperscript{2+} channels, quickly raising the concentration of Ca\textsuperscript{2+} in the cytosol. As the foregoing evidence raised the possibility of PLC pathway involvement in PACAP mitogenic activity, we next examined this downstream PLC target, intracellular Ca\textsuperscript{2+}. Ca\textsuperscript{2+} signaling has been well documented to increase proliferation in multiple systems (Berridge et al., 2000; Hogan et al., 2010; Weissman et al., 2004). We compared simultaneously E10.5 and E14.5 cortical precursor cells by monitoring intracellular Ca\textsuperscript{2+} changes upon PACAP activation using Ca\textsuperscript{2+} fluorescence indicator Fluo-4 AM. PACAP triggered an increase of cytosolic Ca\textsuperscript{2+} in most E10.5 cortical precursors, suggesting the peptide activated the inositol phospholipid signaling pathway (Nicot and DiCicco-Bloom, 2001; Spengler et al., 1993) [Fig. 8 a, d]. Interestingly, the majority E10.5 cells exhibited Ca\textsuperscript{2+} oscillations [Table-2], consistent with PAC1 hop isoform being involved in PACAP mitogenic effects [Fig. 8 a, d]. In contrast, PACAP did not elicit Ca\textsuperscript{2+} changes in E14.5 cortical precursors [Fig. 8 b, e]. Significantly, both E10.5 and E14.5 precursors were able to respond to 40mM KCl [Fig. 68 c, f, g], suggesting that depolarization induced Ca\textsuperscript{2+} release was maintained by cells at both stages in vitro, and that the absent PACAP effects at E14.5 were not due to a defect of endoplasmic reticulum Ca\textsuperscript{2+} storage or release machinery. We did note that PACAP induced complex Ca\textsuperscript{2+} oscillations, especially compared to those elicited by KCl [Fig. 8 d, f, g], but did not pursue this further as others have defined the complex mechanisms of IP3 induced Ca\textsuperscript{2+} oscillations (Berridge et al., 2003; Hogan et al., 2010).
Figure – 9 PACAP induces intracellular calcium increase in E10.5 but not E14.5 cortical precursors.
Temporal patterns of Ca$^{2+}$ indicator fluorescence following reagents addition. Examples of single cell intracellular fluorescence intensity are represented as $\Delta F/F_0$ traces. PACAP (10nM) exposure increased intracellular calcium levels in E10.5 (a, d) but not E14.5 precursors (b, e) at 2h cultures. KCl (40mM) elicited increased intracellular Ca$^{2+}$ in both E10.5 (f) and E14.5 (c, g). Cells were monitored by BD Pathway BioImager program after being exposed to Fluo-4 AM. Data were derived from three experiments using parallel cultures of E10.5 and E14.5 precursors in 96 well plates, 100-150 cells per group were monitored in each experiment.

**Table-2 Calcium fluxes and oscillations in E10.5 and E14.5 cortical precursors.**

<table>
<thead>
<tr>
<th>Calcium Fluxes</th>
<th>E10.5</th>
<th>E14.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACAP responsive cells</td>
<td>201/220 (91%)</td>
<td>7/306 (2%)</td>
</tr>
<tr>
<td>PACAP induced oscillations</td>
<td>172/201 (86%)</td>
<td>0/306 (0%)</td>
</tr>
<tr>
<td>KCl responsive cells</td>
<td>137/168 (82%)</td>
<td>398/412 (97%)</td>
</tr>
</tbody>
</table>

**4.8 PACAP activates PKC but not MAPK during early neurogenesis.**

Another signal downstream of PLC production of DAG and Ca$^{2+}$ elevation is PKC (Nishizuka, 1984). Phosphorylation of PKC is an initial step in its activation, which may serve as a molecular marker (Pearce et al.) and these phosphorylation must be processed before it is competent to trigger downstream signaling pathways (Keranen et al., 1995). We chose the general form of phosphor-PKC antibody, which can detect $\alpha$, $\beta$ I, $\beta$ II, $\delta$, $\epsilon$, $\eta$ and $\theta$ isoforms at Ser660. PACAP treatment (30 min) produced two-fold increases in phosphorylation of PKC in E10.5 cortical precursors, a response nearly identical to that elicited by the potent PKC agonist 12-O-tetradecanoylphorbol-13-acetate (TPA) [Fig. 9]. In contrast, PACAP did not elicit changes in phoso-PKC levels of E14.5 precursors [Fig. 10].
Figure - 10 PACAP activates PKC in early cortical precursors (E10.5).
Patterns of phospho-PKC staining in E10.5 cortical precursors 30min after treatment with vehicle (a), PACAP (10nM) (b) and PKC agonist TPA (200nM) (c). PACAP exposure increased phospho-PKC by 2-fold compared to vehicle in 2h cultures, comparable to the PKC agonist. Positive cells are indicated by arrows and negative cells by arrowheads. (d) Quantification of phospho-PKC immunostaining. Data are expressed as total phospho-PKC cells per field and were derived from three experiments, three dishes per group, ***P<0.001. Scale bar, 10μm.
Figure - 11 PACAP does not activate PKC in old cortical precursors (E14.5).

Patterns of phospho-PKC staining in E14.5 cortical precursors 30min after treatment with vehicle (a), PACAP (10nM) (b) and PKC agonist TPA (200nM) (c). PACAP did not change phospho-PKC+ cells
compared to vehicle in 2h cultures while the agonist TPA increased phospho-PKC+ by 3-fold. Positive cells are indicated by arrows and negative cells by arrowheads. Data are expressed as total phospho-PKC+ cells per field and were derived from three experiments, ***P<0.001. Scale bar, 10μm.

We also expected the PKC specific agonist TPA and antagonist bisindolylmaleimide I (BIM), which exhibits strong stimulation or inhibition effects on several PKC isoforms in cancer cells (Toullec et al., 1991) to promote or block PACAP pro-mitogenic effects in young precursors, respectively. Since the lack of experience in neuronal precursors, especially for vulnerable young precursors (E9.5 mouse or E10.5 rat), we first did dose response curve at 24h starting at nanomolar concentration for both drugs. For TPA, it did not cause proliferation changes in E10.5 precursors as expected, since it is possible that E10.5 precursors are insensitive to this agonist in long incubation. For BIM, however, similar to PLC antagonist, precursors were extremely sensitive to this antagonist and >80% were unable to survive to 24h. Therefore, we used 16h pulse, the same paradigm as PLC antagonist incubation, to test bisindolylmaleimide I, and 4μM seemed to be the highest concentration that did not affect cell survival. However, in the presence of the antagonist, [3H] thymidine incorporation results in both PACAP and control groups varied from time to time and the inconsistency of its effects on TPA induced PKC phosphorylation precluded this approach. There are two possible reasons: first, PKC is involved in several key cellular responses including proliferation, and thus blocking its function may impair proliferation; second, PKC is critical for cell homeostasis, and
without it, cells cannot maintain health status, consequently they would not respond to mitogen stimuli appropriately.

Limited cell number at this age (400,000-500,000 cells/litter) prevents the western blotting assay to test whether this concentration is working in young neuronal precursors at protein level.

In addition, since PACAP has been shown to activate MAPK pathways in several culture systems (Barrie et al., 1997; Ohtaki et al., 2006; Vaudry et al., 2002c), we performed similar studies as phospho-PKC and assessed phosphorylated ERK. However, PACAP did not elicit increased phospho-ERK in E10.5 or E14.5 [Fig. 11], while bFGF, a well-defined mitogen, elicited robust ERK activation in parallel dishes in both ages, suggesting independence of ERK pathway to PKA, consistent with previously published work (Gerdin and Eiden, 2007), and ERK activation may mainly contribute to neuritogenesis.
Figure – 12 PACAP did not activate phospho-ERK while bFGF exposure led to ERK activation in both E10.5 and E14.5 precursors. (a) Examples of phospho-ERK
staining in E10.5 and E14.5 10 min after treatment with vehicle (Con), PACAP (30 nM) and bFGF (10 ng/ml). (b) Quantification of phosphor-ERK immunostaining. Cells were plated in 35mm dishes in defined media without growth factors, and reagents were added at 2h for 10 min. Data are representative of three experiments, three dishes per group. **P<0.01, ***P<0.001. Scale bar, 10 μm.

4.9 Retinoic acid maintains hop isoform expression and PACAP mitogenic activity

The foregoing data suggest the hop isoform and PLC pathways contribute to PACAP mitogenic stimulation in early precursors. Indeed this mechanism was established using transfection mediated hop isoform overexpression in E14.5 precursors (Nicot and DiCicco-Bloom, 2001). While a requirement for the hop isoform in E10.5 precursors may be assessed by transfecting sh RNA, the very limited number of precursors obtainable at this age (400-500,000/litter) precluded this approach. Instead we examined whether a stimulus increasing hop expression would result in PACAP mitogenic stimulation. We explored several hormone pathways known to affect early neurogenesis including steroids and retinoic acid (RA) (Haskell and LaMantia, 2005; Studer et al., 1994). To examine RA, E10.5 precursors were incubated in RA vehicle (ethanol) or in RA (3x10⁻⁸M) for 24h, and real time PCR was performed. Hop was upregulated 3-fold by RA treatment, whereas the short form was only enhanced by 51% [Fig. 12a]. However, it would be difficult to test RA enhancement on PACAP mitogenic stimulation at 24h, since the majority cells in PACAP group may have entered cell cycle. So we chose to assess
proliferation 24h later, from 24h to 48h, when precursor proliferation may be slowed down in the absence of PACAP stimulation. In preliminary studies, we found that when E10.5 precursors were incubated 24h, the addition of PACAP for another 24h elicited no stimulatory effect at 48h [Fig. 12b], suggesting the developmental maturation observed in vivo [Fig. 3f, E11.5] was recapitulated in vitro. Now we plated E10.5 precursors in four dishes. Two dishes first were incubated with RA vehicle and the other two were incubated with RA for 24h, since we know RA is able to upregulate hop isoform during the first 24h. Then PACAP vehicle or PACAP was added to each group at 24h and incubated for another 24h. [3H] thymidine was added at 44h and precursors were harvested at 48h to measure DNA synthesis. In the presence of RA, PACAP exposure increased DNA synthesis by 34% at 4h [Fig.12c, right panel], whereas it had no effect in the RA vehicle [Fig. 12c, left panel], suggesting this stimulation may have depended on the increased hop to short ratio in the presence of RA.
Figure – 13 Retinoic acid maintains hop isoform expression and mitogenic stimulation.
(a) RA increased differentially the short and hop isoforms at 24h detected using real time PCR. (b) E10.5 precursors were incubated for 24h and then either PACAP vehicle (Con) or PACAP were added, DNA synthesis was assessed at 48h. (c) E10.5 precursors were incubated for a total of 48h with either vehicle (ethanol) or RA (3x10^{-8}M). At 24h, cultures received either PACAP vehicle (Con) or PACAP and DNA synthesis was assessed at 48h. In the presence of RA, PACAP increased precursor DNA synthesis, while there was no change in the RA vehicle group. Data represent three experiments, two dishes per group, N=6 (200,000 cells/dish) for RNA quantification; 3 wells per group (25,000 cells/well) in each experiment for [³H] thymidine incorporation. *P<0.05, ***P<0.001.
CHAPTER 5  DISCUSSION

5.1 Summary of results

Effects of PACAP on cortical neurogenesis have been studied mainly in cortical precursors after E13.5 in rodent. However, the functions of this multi-functional neuropeptide in early neurogenesis remain elusive. To address this question, we chose E9.5 PACAP KO and WT embryos to start. We found BrdU labeling exhibits a 21% decrease in the KO, suggesting PACAP can stimulate proliferation at the onset of neurogenesis. We then successfully built a young age neuronal precursor dissociated culture (E9.5 in mouse and E10.5 in rat). Consistent with in vivo results, exogenous PACAP causes an increase in DNA synthesis (33% in rat, 23% in mouse measured by [3H] thymidine incorporation) in the presence of trophic factors. In contrast, only one day later (E10.5 in mouse and E11.5 in rat), PACAP does not change DNA synthesis. In later neurogenesis (after E13.5), PACAP exhibits inhibitory effects on proliferation, which is consistent with previous publications in which experiments were performed in the absence of growth factors. We also found that PACAP increases DNA synthesis through stimulation of G1/S progression rather than serving as an anti-apoptotic factor. Since PACAP shows bi-directional impacts on proliferation based on its receptor isoform: short or hop, we further investigated whether the isoform expression contributes to the developmental pro-mitogenic to anti-mitogenic switch. We used realtime PCR to quantify the actual amount of short and hop at different developmental ages. Significantly, expression of hop isoform is 24-fold greater than short at E10.5, a period of development when PACAP is stimulatory. In contrast at E14.5, when PACAP is inhibitory, short expression is 15-fold greater than the hop. Taken together these results
suggest that the transition of receptor isoforms contributes to PACAP developmental effects on neuronal precursor proliferation. Different receptor isoforms trigger different transduction pathways and result in pro- or anti-mitogenic signals. Next we examined signaling pathways using E10.5 and E14.5 rat neuronal precursors: PACAP evokes calcium fluxes and increased phospho-PKC levels at E10.5 but not at E14.5. In addition, pharmacological inhibitor experiments show PLC is involved in PACAP pro-mitogenic effects.

We next tried to explore any component that can affect isoform expression, such as retinoic acid (RA). In preliminary studies, we found that RA (3x10^{-8}M) incubation (0-24h) increased hop by 3-fold and short was up regulated by 51% in E10.5 precursors. This upregulation of hop expression results in a 34% increase in DNA synthesis when E10.5 precursors are exposed to PACAP from 24h to 48h.

Our observations provide compelling evidence that PACAP contributes to neuronal precursor proliferation through age-dependent and bi-directional functions during development. PACAP exhibits mitogenic effects during early neurogenesis, while the peptide restrains proliferation after E12.5. The temporally specific effects on cortical neurogenesis correlate with developmental expression of PAC1 receptor isoforms: hop and short. PACAP signaling provides a novel example in the developing nervous system that alternative splicing of receptor isoforms (Spengler et al., 1993) can precisely control cortical proliferation.
5.2 Potential application of the new culture system

At the onset of neurogenesis (E9.5 mice; E10.5 rat) the telencephalic vesicles consist of 4-5 cell layer thick VZ, in which the majority are neuronal precursor cells/radial glia cells (Fishell and Kriegstein, 2003; Gotz and Barde, 2005; Gotz and Huttner, 2005). These precursor cells will proliferate and differentiate to give rise to the entire laminated neocortex.

The currently published early neural precursor/stem cell culture models include adherent cell cultures, neurosphere cultures, mixed cell co-cultures, and optimized serum-free suspension cultures (Qian et al., 1998; Shen et al., 2004; Shen et al., 2006; Watanabe et al., 2005). These culture systems are delicate and have established routine procedures based on previous method development. For example, cell isolation requires either shaking and centrifugation (Qian et al., 1998), preplating with endothelial cells (Shen et al., 2004), or incubation with different soluble factors (Watanabe et al., 2005). The procedures of the dissociated culture system we have established are relatively less complicated and easy to perform. Further, the precursors are ready for use immediately after isolation, an attribute that may preserve comparability to their original state in vivo, and that may mitigate against emergence of traits reflecting adaptation to the culture environment. In addition, the defined culture medium we employ contains only insulin, bFGF and BDNF, a relatively restricted panel of growth factors and hormones especially when compared to the commonly used Neurobasal medium plus B27 (Invitrogen). While this simplified medium may not support certain developmental events, we were in the position to test the effects of important factors of direct developmental significance, such as steroids and retinoic acid, studies precluded by complex media that includes these factors. Our immunocytochemical staining from 24h to 48h demonstrated the
differentiation potential of these young age precursors: the expression of β III tubulin (Tuj1) increases from 16% to 20%. Also, percentage of β III tubulin at 24h was 16%, 18% and 44% for cells derived from E10.5, E11.5 and E12.5 cortex respectively. In addition, when E10.5 cells were incubated for 24h, the cells response to PACAP exposure for another 24h: an effect virtually identical to that of cells obtained from E11.5 embryos [Fig.3f]. The parallel effects in the two ages suggest that cells underwent, in culture, the normal developmental transition in receptor isoform that occurs *in vivo*. The retinoic acid induced upregulation of the PAC1 hop receptor isoform with maintained pro-mitogenic activity, indicated these cells are plastic and are able to respond to normal environmental cues. Our culture system provides a different platform in which to elucidate intrinsic and extrinsic connectivity of neuronal stem cells and may provide insights into stem cell division patterns, stem cell potency and windows of plasticity (Qian et al., 1998; Shen et al., 2006; Watanabe et al., 2005).

5.3 Different signaling pathways are coupled with short or hop isoforms

Understanding regulation of PACAP pro-mitogenic activity could provide a potential strategy for neural stem cell based brain regenerative therapy since this peptide has been well-documented to stimulate neural stem cell proliferation and promote neurogenesis both *in vitro* and *in vivo* in postnatal mouse brains and spinal cord (Fang et al., 2010; Mercer et al., 2004; Ohta et al., 2006; Scharf et al., 2008). It remains uncertain whether the short (Ohta et al., 2006; Scharf et al., 2008) or hop (Mercer et al., 2004) isoform mediated the proliferative stimulation since both forms were detected and both cAMP and PLC/PKC pathways were activated in the adult subventricular zone and
dentate gyrus. This suggests that there is likely both a regional and temporal specificity involved. Our study demonstrates that PACAP pro-mitogenic activity in young neuronal precursors is mediated through the hop isoform and that the developmental upregulation of the short isoform restrains precursor proliferation in the developing cortex.

It is known that the hop isoform stimulates cAMP (Spengler et al., 1993) and that cAMP can initiate ERK activation in a PKA independent fashion, leading to neuritogenesis in PC12 cells (Gerdin and Eiden, 2007). However, we did not observe ERK activation in E10.5 precursors at 2h, at the time PKC is activated, suggesting that hop mediated PLC/PKC pathway activation is ERK independent at the beginning of our culture incubation. However, this is the only time that we tested phospho-ERK using immunocytochemistry, we cannot exclude the activation of ERK pathway in our culture systems. While the signal transduction network is complex, especially regarding PKA and PKC interactions, our observations suggest the hop isoform stimulates young neuronal precursor proliferation through the PLC/PKC pathway. On the other hand, we cannot exclude the possible involvement of other signaling mechanisms in the PACAP pro-mitogenic effects mediated through hop.

5.4 Temporal effects on precursor proliferation correlate with receptor expression

During early neurogenesis, when the majority of the precursors are proliferating to enlarge the precursor pool, PACAP enhances proliferation through hop isoform: it increases DNA synthesis, and promotes G1/S entry and cell division without affecting cell survival. In contrast, at later developmental stages, PACAP inhibits proliferation
through short isoform, restraining precursor pool expansion, and promoting the transition from proliferation to differentiation (Lu and DiCicco-Bloom, 1997; Tury et al., 2011). During early neurogenesis when hop predominates, PACAP exposure activates PLC and intracellular calcium flux, enhancing PKC phosphorylation. Although we were unable to downregulate hop signaling at this age, previous ectopic hop overexpression converted PACAP inhibition to stimulation (Nicot and DiCicco-Bloom, 2001), suggesting the relevance of this developmental switch. Indeed, the maintenance of an elevated hop/short ratio, produced by RA treatment, sustained PACAP promitogenic activity beyond this early developmental window. However, with increasing age and upregulation of short isoform, PACAP promoted cell cycle exit, as reported previously (Carey et al., 2002; Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Suh et al., 2001). PACAP induces cell cycle exit by selective increases in cyclin dependent kinase inhibitor p57kip2 (but not p27kip1), inducing increased levels of p57 mRNA, its protein and association with cyclin dependent kinase 2/cyclin E complex (Carey et al., 2002; Tury et al., 2011). In future studies, we could relate PACAP pro-mitogenic activity to symmetrically dividing neuroepithelial cells and relate PACAP anti-mitogenic activity to asymmetrically dividing radial glial cells.

The model of PAC1 splice isoform transition provides an important target for future exploration of mRNA splicing mechanisms because the receptor is expressed in both peripheral and central nervous systems for the majority of developmental neurogenesis (Jaworski and Proctor, 2000; Spengler et al., 1993; Vaudry et al., 2009). The developmental control of neurogenesis through RNA splicing of receptors that regulates proliferation is not commonly reported in the nervous system. However, alternative
isoforms of cytosolic adaptor protein NUMB regulate proliferation and differentiation in P19 carcinoma cells (Verdi et al., 1999) and mouse cortical precursors (Bani-Yaghoub et al., 2007). Alternative receptor splicing has been reported in cancer cells: The predominant fibroblast growth factor receptor 3 (FGFR3) elicits stimulation, whereas a normal FGFR3 splice variant (\(\Delta 8-10\)) inhibits proliferation. As we observe here, it is the ratio of different splice isoforms that underlies mitogenic regulation. However, in cancer cells the result is uncontrolled proliferation (Tomlinson et al., 2005). In the adult cortex, the activities of neurotransmitters that act via GPCRs depend on control of alternative receptor splice isoforms: the dopamine D2 receptor gene encodes two isoforms, D2L and D2R (Picetti et al., 1997), which are generated by alternative splicing of a 29 amino acid insert within the third intracellular loop. D2L and D2R have antagonistic functions in the synapse \textit{in vivo} (Usiello et al., 2000). Control of pre-mRNA splicing is a complex process that involves many layers of regulation including the structure of alternative promoters, levels of small nuclear ribonucleoproteins and auxiliary proteins in the spliceosome, and mRNA subcellular localization and stability, to name a few (Greenberg et al., 2009; Li et al., 2007). Recent research indicates alternative splicing is frequent during embryonic development (E8.5-E11.5 in mouse embryos) including the PACAP system, and the alternatively splicing is disproportionately involved in important developmental processes (Revil et al., 2010). Thus defining normal as well as abnormal splicing regulation may provide insights into neuropsychiatric disorders, such as schizophrenia and PTSD (Hashimoto et al., 2007; Ressler et al., 2011).

5.5 Possible mechanism of PACAP age-dependent mitogenic effects.
Our results suggest bi-directional mitogenic effects correlate with receptor isoform switch: hop and short. At this time, we do not fully understand whether hop and short are expressing in the same cell or they express in different cells before we conducted single cell PCR or single cell in situ hybridization. It is also possible that only short changes: it upregulates from E10.5 to E14.5 whereas hop remains constant. At E14.5, its anti-mitogenic effects become dominant at a later time, when precursors need signals to start differentiation and migration. The mitogenic effects of PACAP result from potential interactions with different signaling pathways, one candidate is Shh. The studies of Shh mainly focus on the cerebellum, however, recent research indicated that Shh is essential to govern neocortex, since Shh is produced in the cortex and Gli-1 is expressed in the ventricular zone (Ruiz i Altaba et al., 2002). Recent studies demonstrated the interaction between Shh signaling and Ca\(^{2+}\) dynamics in the developing spinal cord: Shh acutely increases Ca\(^{2+}\) spikes and IP3 transients through Smoothened activation in neurons. When Smoothened is activated, it recruits G\(\alpha_i\)\(\beta\gamma\). G\(\alpha_i\)\(\beta\gamma\) inhibits adenylate and hence inhibits PKA, while it triggers PLC activation and increased IP3 levels [Fig.14] (Belgacem and Borodinsky, 2011). This study is consistent with Ca\(^{2+}\) spikes and oscillation promotes cell proliferation as previously published (Berridge et al., 2003; Hogan et al., 2010).

In contrast, cAMP/PKA can specifically inhibit IP3-induced Ca\(^{2+}\) elevation, through inhibition of Ca\(^{2+}\) release not by stimulation of Ca\(^{2+}\) removal from the cytoplasm (Tertyshnikova and Fein, 1998). cAMP and cAMP dependent PKA regulates cell proliferation by multiple mechanisms. Distinct mechanisms result in either stimulation or inhibition in various cell types. Most mechanisms suggest cAMP/PKA is inhibitory,
including in neuronal precursors (Lu and DiCicco-Bloom, 1997; Lucchi et al., 2011; Schmitt and Stork, 2002; Waschek et al., 1998). cAMP inhibition of ERK plays an important role in these anti-proliferative effects, as well as in other signaling pathways. PKA, Raf-1 and Rap1 are required in cAMP inhibition of ERK (Cook and McCormick, 1993; Sevetson et al., 1993). PKA phosphorylates certain sites (serine 621, serine 259, serine 43) of Raf-1, which is another upstream molecule in ERK signaling. Once Raf-1 is phosphorylated, it is unable to bind Ras and cause downstream pathway activation (Cook and McCormick, 1993; Wu et al., 1993). Another possible mechanism involves Rap1, a GTPase. Rap1 directly inhibits Ras-dependent signaling to ERK, once activated by PKA (Altschuler et al., 1995; Cook et al., 1993). Additionally, cAMP inhibits cell cycle progression through increasing p21cip1, p27kip1, and decreasing cyclin D1 and cyclin D3 levels (L’Allemain et al., 1997; Lee et al., 2000; van Oirschot et al., 2001).

One simple model may be PACAP accounts for Shh-dependent proliferation in corticogenesis. In early neurogenesis, PACAP may exert pro-mitogenic activity through hop mediated PLC/PKC pathway, Shh signaling upregulates cyclin D, E and B, maintaining phosphorylation of Rb, for its proliferative effects in neuroepithelial cells. At later time, when short becomes dominant form, PACAP acts mainly through cAMP/PKA pathway, which can antagonize Shh mediated proliferation stimulation through Gli-1 inhibition (Nicot et al., 2002; Ruiz i Altaba et al., 2002; Waschek et al., 1998). As a result, the mitotic radial glia cells may exit cell cycle and start to differentiation. However, we could not exclude other mechanisms involved in this age-dependent bi-directional proliferation effects. Our findings provide another example of PLC/PKC and cAMP/PKA complexity.
**Figure 14 Molecular mechanisms of Shh induced Ca\(^{2+}\) spikes.** Shh activates Smoothened, leading to activate PLC and increased IP3 levels. Both opening of IP3 receptor (IP3R) and transient receptor potential cation channel (TRPC1) results in increased Ca\(^{2+}\) spikes. In contrast, activated Smoothened inhibits adenylate cyclase and PKA (Belgacem and Borodinsky, 2011).

In conclusion, PACAP signaling influences precursor mitosis during brain development. The developmental switch in PAC1 hop and short isoforms converts PACAP mitogenic stimulation to inhibition. It is likely that the PAC1 receptor is but one of many signals governing corticogenesis that is subject to regulation by mRNA splicing mechanisms.
CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 On-going research

In this dissertation, PACAP has been shown to exhibit pro-mitogenic activity in early neurogenesis and later it becomes anti-mitogenic, which is potentially mediated through the switch of hop to short mRNA expression. We now know that alternative splicing occurs in PACAP RNA in an age-dependent fashion. Next we would like to take one step further: to figure out the mechanism of why alternative splicing happens during neurogenesis to control precursor proliferation, as a normal developmental process. What mechanisms are involved in how the brain determines the precise timing of proliferation to differentiation? What factors or genes are in charge of receptor isoforms expressed during development? How does PAC1 receptor splicing happen? What signaling pathways are involved? To answer those questions in this elusive field, there are a few approaches.

Identify other genes that interact with PAC1 receptor mRNA

mRNA-Seq can discover and profile the entire universe of mRNA without probes or primers. In collaboration with genome or transcriptome groups, we can extract total RNA from rat cortices at different ages and prepare databases. Differentially expressed genes can also be detected to compare whether different genes are interacting with PAC1 receptor mRNA at developmental ages or same genes can function and result in the right amount of mRNA products at each age. To detect transcriptional regulation, mRNA-seq can be analyzed with Cufflinks to identify all transcription start sites associated with the primary transcript. Cufflinks can also identify post-transcriptional regulation by looking for changes in relative abundances of mRNA spliced from the same primary transcript to
detect alternative splicing. In addition, we can investigate whether splicing patterns share the same transcription site at different developmental ages. Although it seems overwhelming, the outcomes are exciting if we could identify any new interactions with PACAP signaling. Now whole-exome sequencing can identify any specific gene expression and any alternative splicing pattern, which provides new molecular basis of brain development and generate a rich source of new hypotheses about functional and transcriptional relationships between gene and brain malformation (Bilguvar et al., 2010; Johnson et al., 2009).

To further explore the alternative splicing in PAC1 receptor isoforms, other approaches may also be considered including measuring promoter usage and characterizing subcellular localization of short and hop mRNAs. Now scientists usually use chemiluminescence-based assays (to test β-galactosidase reporter vector, β-glucuronase, alkaline phosphatase reporter vector, and luciferase reporter vector) to quantify gene elements (Sandelin et al., 2007). We can make constructs using these vectors to PAC1 gene and test the promoter usage using luciferase assay.

Since RA can upregulate the isoform expression, identifying RA or other gene response elements (RAREs) is informative. Initial sequence search for published RARE sequences are not found in PAC1 gene, however, integrated bioinformatics analysis is necessary before we draw to any conclusions.

Discontinuity of the developmental studies *in vivo*

The decrease in BrdU LI at E9.5 in PACAP KO mice suggests either PACAP is normally a pro-mitogenic signaling at this age or that it plays a survival promoting role. That is the
reason why we continued our study in vitro to distinguish the two possibilities. Our in vitro data indicate PACAP promotes G1/S entry instead of affecting cell survival in young precursors, and the observation in vitro is: at the onset of neurogenesis, PACAP signaling produces mitotic stimulation and later it becomes mitotic inhibition. If this model works in vivo, we should detect decreased BrdU LI at E9.5, and at E11.5 and onward, BrdU LI increases in the KO. Two reasons preclude this developmental age related experiments. First, PACAP KO animals may have unexpected abnormalities, since PACAP is critical to maintain glucohomeostasis and normal adrenal catecholamine level (Hamelink et al., 2002), therefore missing PACAP after fertilization, may result in severe or fatal neurotransmitter and glucose deficiency in the embryo which may result in unexpected outcomes. Cultures of PACAP KO precursors could be used in place of whole PACAP KO animals. However, these cells, which have never been exposed to PACAP, may not display the same receptor isoforms as their wild-type equivalents. In addition to unexpected results due to any number of metabolic disorders, these cultures may not react to PACAP the as wild-type cells. Therefore, we decided not to continue these studies in PACAP KO cultures since it is not be a comparable model. Rat or mouse precursors that are used in our cultures normally secrete PACAP since they are born and the effects of exogenous PACAP may occur at different ages. Second, as PACAP exhibits anti-apoptotic functions in several systems (Ohta et al., 2006; Vaudry et al., 2009; Waschek, 2002), PACAP may serve as a trophic factor during neurogenesis in vivo, which is confirmed by our E12.5 studies. However, the increased cell death in KO animals may also results in decreased BrdU positive cells, especially at later ages.
To address the first reason, conditional KO is a good assessment. E9.5 and any other embryonic stages can be a good start. We can use the conditional KO and turn off PACAP gene only at the designated ages at telecephalon and perform BrdU immunohistochemistry staining to see whether PACAP has temporally distinct proliferation roles. We conducted individual cultures to test the responses of PACAP KO precursors. Since PACAP KO lines are unable to survive to adulthood, we are using the PACAP heterozygous line. In the individual cultures, precursors are isolated from each embryo, dissociated and plated, vehicle or PACAP are added to cultures. At the same time, PCR is performed to determine the genotype. We initially thought the precursors from PACAP KO mice may not respond properly to environmental cues. However, the individual cultures from E15.5 cultures at 24h showed PACAP KO precursors are able to respond to exogenous PACAP, it caused a >40% decrease in DNA synthesis, similar to WT precursors. These results show that PACAP KO precursors are able to survive to 24h, and the receptor expression is not affected.

To answer the second possibility, we propose to cross PACAP heterozygous line to a Bcl-2 overexpressed line to generate a model that prevents apoptosis. We did some preliminary experiments in vitro, in the presence of caspase inhibitor Z-VAD-FMK (20μM), PACAP elicited a 16% increase in DNA synthesis in E10.5 rat precursors at 24h, suggesting that PACAP cell cycle progression promotion is independent from its anti-apoptotic roles.

Test other signaling pathways involved in PACAP pro-mitogenic activity
As mentioned earlier in the discussion section, 2h is the only time that we tested phospho-ERK activity. We could not exclude the involvement of MAPK pathway since it is a classic mitogen pathway. We plan to conducted 4h, 6h, 8h, 16h, and 24h to see whether ERK is activated at E10.5 using immunocytochemistry staining, since we are unable to perform western blot using the limited cells.

In addition, we are unable to administer PKC activator or inhibitor to mimic PACAP pro-mitogenic effects at E10.5, however, we may not find the working concentration and time in this specific culture system. Also, other molecules may be involved in this process, such as arachidonic acid, we will use pharmacological agonist and antagonist to continue this research.

6.2 Conclusions

In this study, I used PACAP KO mice as a start to study the function of PACAP on proliferation during early neurogenesis, and I established a novel culture system for young neuronal precursors. During early neurogenesis, I found that PACAP promotes proliferation through G1/S progression, acting through hop mediated PLC/PKC pathway, while during late neurogenesis, PACAP is anti-mitogenic and promotes differentiation through short mediated cAMP/PKC pathway. The temporally specific effects correlate with the developmental expression of hop and short. Because of incapability of down-regulating hop expression in young neuronal precursors, we tested a stimulus (retinoic acid) that can up-regulate hop and maintains PACAP pro-mitogenic activity. In sum, these results provide a novel example that alternative splicing of receptor isoforms contributes to neuronal precursor proliferation in central nervous system.
References


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