KINASE INHIBITORS AS MEDIATORS OF NEURONAL DIFFERENTIATION

by

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For Mom and Dad

and

For Peter

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LIST OF ABBREVIATIONS

8-CPT-cAMP = 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate

AKAP = A-kinase anchoring protein

AP = Anterior-posterior

Ascl1/Mash1 = Mammalian achaete-scute homolog 1

BDNF = Brain-derived neurotrophic factor

bHLH = Basic helix-loop-helix

BMP = Bone morphogenic protein

bp = Base pair

C = PKA catalytic subunit

CaM = Ca^{2+}/calmodulin-dependent kinase

CaMKII = Ca^{2+}/calmodulin-dependent kinase II

CBP = CREB binding protein

CDK = Cyclin-dependent kinase

ChIP = Chromatin immunoprecipitation

CKI = CDK inhibitor

CNS = Central nervous system

CRE = cAMP-response element

CREB = CRE binding protein

CS = Calf serum

Dll = Delta-like

DMEM = Dulbecco's modified Eagle's medium

Dox = Doxycycline

DPBS = Dulbecco's phosphate buffered saline

DSL = Delta/Serrate/Lag

DV = Dorsal-ventral

EB = Embryoid body

EC = Embryonic carcinoma

EGFP = Enhanced green fluorescent protein

ES = Embryonic stem

FBS = Fetal bovine serum

FGF = Fibroblast growth factor

Gadd45 = Growth-arrest and DNA-damage inducible protein 45

GFAP = Glial fibrillary acidic protein

HCG = Human chorionic gonadotropin

HEKT = Human embryonic kidney 293 cells stably expressing the SV40 large T antigen

HOX = Homeobox

Id = Inhibitor of differentiation

JNK = Jun N-terminal kinase

kb = Kilobase

LC-MS/MS = Liquid chromatography coupled with tandem mass spectrometry

LIF = Leukemia inhibitory factor

Map2 = Microtubule-associated protein 2

MEKK4 = MAPK/ERK kinase kinase 4

MEMα = Minimal essential medium alpha

MPF = M-phase promoting factor

NBM = Neurobasal medium

NCID = Notch intracellular domain

NES = Nuclear export signal

NF-L = Neurofilament-L

NGF = Nerve growth factor

NSC = Neural stem cell

P19T1A2 = Ascl1-inducible P19 cell line

P19T3GIE2 = Gadd45γ-inducible P19 cell line

PBS = Phosphate buffered saline

PKA = cAMP-dependent protein kinase

PKG = cGMP-dependent protein kinase

PKI = Protein kinase inhibitor

ProtA = Protein A of *Staphylococcus aeurus*

Ptc = Patched

R = PKA regulatory subunit

RA = Retinoic acid

RACE = Rapid amplification of cDNA ends

RAR = Retinoic acid nuclear receptor

RARE = Retinoic acid response element

RT-PCR = Quantitative real-time polymerase chain reaction

RXR = Retinoid X nuclear receptor

SGZ = Subgranular zone

Shh = Sonic hedgehog

Smo = Smoothened

SR = Serine/arginine-rich

SVZ = Subventricular zone

TAP = Tandem affinity purification

TEV = Tobacco etch virus

TORC = Transducer of regulated CREB activity

TRE = Tetracycline response element

TuJ1 = Monoclonal antibody that recognizes neuronal-specific β-III-tubulin

VASP = Vasodilator-stimulated phosphoprotein

VPA = Valproic acid

CHAPTER I

INTRODUCTION

Neurogenesis is the process by which functionally integrated, post-mitotic neurons are generated from pools of neural stem cells (NSCs). This elegant process involves the proliferation and cell fate specification of NSCs into discrete phenotypes, as well as the maturation and integration of the neuronal progeny into functional neuronal circuits. During early development of the central nervous system (CNS), embyronic NSCs are located in the ventricular zone of the neural tube. These multipotent cells can give rise to all the cell types required for the formation of the CNS. Contrary to the long-held dogma that neurogenesis only occurs during development, research has shown that neurogenesis occurs throughout life in mammalian brains in two key locations: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus. The processes of neuronal differentiation and determination are regulated at many levels by a very complex network of extrinsic cues such as cell-cell interactions and secreted factors, and intrinsic genomic and proteomic programs. These intrinsic programs include, but are not limited to: 1) specific transcriptional regulatory pathways in which transcription factors increase or decrease the expression of target genes, 2) protein interaction pathways where protein-protein binding can alter the function or stability of other proteins, and 3) kinase/phosphatase pathways where the phosphorylation state of proteins can alter their function. Understanding the regulatory

mechanisms for these intricate molecular networks is crucial for understanding the function and plasticity of the CNS.

The neurogenic basic helix-loop-helix (bHLH) proteins play critical roles in the intrinsic genetic program responsible for neuronal differentiation and are sufficient to initiate a neurogenic program of differentiation in many cells, resulting in the formation of mature neurons both *in vivo* and *in vitro*. The goal of this thesis is to examine the gene regulatory networks involved in neuronal differentiation using a model system of P19 embyronic carcinoma (EC) cells. The P19 cell line has many properties in common with embryonic stem (ES) cells and is widely used as a model system for neuronal differentiation. After induction of neurogenic bHLH proteins, the homogeneous population of P19 cells undergoes neuronal differentiation, as evidenced by the expression of neuronal marker proteins, appropriate morphological changes, and spontaneous electrical activity. This dissertation characterizes the differentiation process in P19 clonal cell lines with respect to the transcriptional changes that occur following induction of bHLH protein expression. These changes can then be used to construct and test models of gene regulatory networks.

By examining the regulation of gene expression that is fundamental to coordinating responses to intracellular and extracellular cues, an understanding is gained of key components of molecular pathways necessary for neuronal differentiation. This is especially important in light of several studies that have shown that neurogenesis can be recapitulated *in vitro*, generating particular classes of neurons from ES cells through a series of discrete steps aimed at guiding cells through each stage in this process. Of particular interest, human ES cells offer the promise of treating many neurological

diseases such as Alzheimer's, Parkinson's, stroke, and spinal cord injuries. Whereas ES cells can be propagated with high efficiency and even generated from somatic cells, the ability to generate the variety of neurons required for replacement therapy of neurological diseases is inefficient at this time. This inefficiency is largely due to the lack of information concerning the molecular events required for specific neuronal differentiation. Therefore, defining the cellular and molecular mechanisms involved in the process of controlling neural cell fate determination is of direct benefit to efforts to harness neural stem cells for repair of the diseased and injured brain.

From shared lineage to distinct functions: the development of the vertebrate CNS

Neural induction and early regional patterning

The birth of a neuron involves sequential steps precisely orchestrated by intrinsic and extrinsic signaling events (Wilson and Edlund, 2001). The first decisive step of neural development, neural induction, is commonly thought to arise from a "default" pathway of embryonic differentiation, which has been most extensively studied in *Xenopus*. In this default model, neural fate represents the default state of the ectoderm of the early embryo that is normally repressed by factors of the bone morphogenetic protein (BMP) family. The overexpression of BMP proteins prevents neural induction and promotes the formation of ectoderm, while the expression of the BMP inhibitors, such as chordin and noggin, promote neural induction at the expense of the ectodermal fate (Weinstein and Hemmati-Brivanlou, 1999). The ultimate result of neural induction is the specification of the neural plate from the ectoderm on the dorsal side of the embryo and

subsequent formation of the neural tube from the neural plate. However, recent findings challenge this "default" model and implicate some positive instructive factors, such as fibroblast growth factors (FGFs) and Wnt morphogens, in neural induction. FGF signaling has been shown to act in concert with BMP inhibition to promote induction and survival of neural progenitors from embryonic stem cells (Delaune et al., 2005; Varga and Wrana, 2005; LaVaute et al., 2009; Marchal et al. 2009), and is thought to antagonize the BMP signaling pathway by directly inducing specific transcription factors which then determine neuroectoderm induction and inhibit mesoderm formation (Bertrand et al., 2003). Interference with FGF and Wnt signaling abolishes neural induction at an early stage in chick embryos (Wilson et al., 2001). Hence, a balanced view of neural induction needs to include both instructive and inhibitory factors, leading to a coherent model whereby BMP, FGF, and Wnt morphogens coordinately control neural induction.

The central nervous system (CNS) forms directly from the neural tube, whereas the peripheral nervous system (PNS) forms from cells derived from the neural crest, a transient population of cells that migrates just as the neural tube closes dorsally. Further neural tube development occurs through the process of neurulation, which involves changes in cell division, cell migration, and cell-cell contacts, ultimately resulting in the formation of post-mitotic neurons and glia (Copp et al., 2003). Classical views of neurulation imply that the neural tube is patterned along its anterior-posterior (AP) and dorso-ventral (DV) axes to establish a grid-like set of positional cues (see Figure 1.1A; reviewed in Altmann and Brivanlou, 2001). The neural tube initially acquires a rostral character, and is eventually posteriorized by exposure to FGF, Wnt, BMP, and retinoic acid (RA) signals to establish the main divisions of the CNS: the forebrain, midbrain,

hindbrain, and spinal cord (Munoz-Sanjuan and Brivanlou, 2002; Agathon et al., 2003; Melton et al., 2004). The synthesis of RA by retinaldehyde dehydrogenases in the underlying posterior mesoderm and the degradation of RA by retinoic acid hydroxylases in the anterior mesoderm results in the formation of a gradient of RA across the developing embryo. This gradient is responsible for the anterior-posterior specific expression of Homeobox (HOX) genes, which defines regions of the neural tube known as rhombomeres that ultimately develop into the hindbrain (Maden, 2001; Bel-Vialar et al., 2002).

Similarly, the general mechanisms of DV neural patterning is also dependent on different concentrations of morphogens inducing specific expression of transcription factors in successive discrete domains. Along the dorso-ventral axis, the neural tube is patterned into more subdivisions in an antagonistic interaction of two signals: sonic hedgehog (Shh) ventrally from the notochord, and BMP dorsally from the roof plate (Jessell, 2000; Altmann and Brivanlou, 2001; Lee and Pfaff, 2001). Exposure to a unique set of morphogens at specific concentrations results in distinct subpopulations of progenitors acquiring the competence to generate types of neuronal and glial cells in a region-specific manner (Osterfield et al., 2003). Thus, for the purpose of engineering human embryonic stem cells for differentiation, it will be crucial to imprint *in vivo* positional information into neurons generated *in vitro* to achieve their full potential for cell replacement therapies.

A network of growth and transcription factors controls neuronal differentiation in the developing nervous system

The brain is the most complex organ in the human body, containing a rich array of diverse cell types, with traditional estimates of a few hundred mammalian neuronal subtypes considered to be overly conservative (Stevens, 1998). Collectively, cells that form the nervous system express 80% of genes in the genome (Lein et al., 2007). This cellular diversity is what underlies the remarkable information processing capacity of the CNS. Complexity within the brain continues through adulthood, where cells continue to undergo phenotypic changes in response to environmental cues and neuronal signaling (Li and Pleasure, 2010). This plasticity underlies higher cognitive functions, such as those involved in learning and memory. However, the relative contribution of the intrinsic and extrinsic cues that dictate neuronal diversity as a function of cell type and developmental time generally remains elusive.

After the neural tube has been patterned along its AP and DV axes, subsets of progenitor cells undergo differentiation, and these new neurons are thought to feedback and inhibit neighboring progenitor cells from adapting a neuronal fate. This process, called lateral inhibition, serves to regulate the numbers of neurons born at a given time and to maintain a pool of progenitor cells. Prominent examples of lateral inhibition include the formation of neuroblasts in fruit flies (Skeath and Thor, 2003) and formation of sensory hair cells in the inner ear of vertebrates (Riley and Phillips, 2003). Lateral inhibition is mediated at the molecular level by the intercellular Notch signaling pathway (Lewis, 1996; Lowell, 2000). Notch signaling is composed of a cell-surface bound ligand from the Delta/Serrate/Lag (DSL) gene family that binds to its cognate cell-surface bound receptor, Notch, on a neighboring cell. The membrane-bound Notch receptor then

undergoes proteolytic cleavage by gamma secretase to release its intracellular domain. The Notch intracellular domain (NICD) is then translocated to the nucleus where it interacts with the Mastermind-like protein to convert the required transcriptional co-factor of Notch, RBP-J, from a transcriptional repressor to an activator (Radtke et al., 2005). This switch subsequently regulates transcription of downstream target genes, including those in the proneural bHLH family (Artavanis-Tsakonas et al., 1999; Kageyama et al., 2005).

During embryonic development, Notch signaling is a universally utilized fate signal integrator in stem cells (Yoon and Gaiano, 2005). However, its role in post-natal neurogenesis is unclear, primarily because Notch plays so many other roles in maturation, neuroplasticity, and even survival (Carlson and Conboy, 2007; Corbin et al., 2008). The first study to inducibly alter Notch signaling in post-natal neural stem cells generated two lines of mice to either knockout Notch or over-express the active domain of Notch in stem cells and their progeny (Breunig et al., 2007). Loss of Notch signaling from stem cells and their progeny increased the incidence of cell cycle exit, shifting cells from the stem cell-like phenotype to a neuronal phenotype. The overactivation of Notch signaling led to persistent stem cells, thereby increasing the progenitor pool and reducing the number of neurons in the population. These results established that post-natal Notch signaling recapitulates embryonic Notch signaling. However, these studies also raise the question of whether the microenvironment dictates the potency of adult neural stem cells. Understanding how Notch signaling regulates the choices neural stem cells make during development into neurons paves the way for controlling neural stem cells both *in vivo* and *in vitro*, which is critical for therapeutic applications.

Neural differentiation does not depend solely on intrinsic factors such as those triggered by the Notch signaling pathway, but also on extrinsic signals. Extrinsic signals pattern the neural tube spatially and temporally, such that distinct types of neurons are formed at defined places and times (McConnell, 1995; Jessell, 2000; Anderson et al., 2001). Recent advances in developmental biology have begun to uncover the molecular mechanisms that underlie patterning of the DV and AP axes of the spinal cord and other brain regions. A major mechanism underlying this patterning results from the activity of several secreted molecules that provide positional information to neural progenitor cells, including Shh, Wnts, BMPs, and RA (Figure 1.1).

BMPs are members of the TGF-β family of secreted ligands and bind to type-I and type-II receptor kinases. After binding of a BMP to at least one type-I and one type-II receptor, the type-II receptor phosphorylates the type-I receptor (Massague et al., 1992; Wrana et al., 1994), which leads to activation of Smad DNA binding factors (Nohe et al., 2004). BMPs are instructive for autonomic neuron precursors (Howard et al., 2000; Liu and Niswander, 2005). Shh is a secreted glycoprotein that plays a crucial role in patterning the ventral midline structure of the neural tube during development (Yamada et al., 1991; Echelard et al.,1993), and induces cells at this location—the floor plate—to express Shh. In this environment, Shh acts as a morphogen, forming a gradient in the ventral neural tube, to which cells differentiate in a concentration-dependent manner (Roelink et al., 1995; Briscoe et al., 1999). The canonical Shh signaling pathway involves two transmembrane proteins: Patched (Ptc), which is the Shh receptor, and Smoothened (Smo), which initiates the intracellular signaling. In the absence of Shh ligand, Ptc blocks Smo activity. Binding of Shh to Ptc relieves its inhibition of Smo, ultimately leading to

Ci/Gli protein entering the nucleus and acting as a transcriptional activator for the same genes it represses when Ptc is inhibiting Smo (Drossopoulou et al., 2000). The combined graded signaling of BMP and Shh are translated into transcription factor codes that delineate different progenitor domains along the DV axis (Jessell, 2000; Wilson and Maden, 2005).

The Wnt signaling pathway is extremely complex, following several possible transduction pathways (Logan and Nusse, 2004). To date, however, the Wnt signals involved in neural crest development are thought to act through binding to cell surface receptors of the Frizzled family. This initiates signaling transduced through Dishevelled, resulting in stabilization of β-catenin. β-catenin translocates to the nucleus, where it regulates transcription by binding to TCF/LEF DNA binding proteins. Wnts emanate from the dorsal aspects of the neural tube, and like BMPs antagonize the actions of the Shh pathway to induce dorsal identities (Hari et al., 2002; Lee et al., 2004; Ille and Sommer, 2005). Research has shown that simply reducing BMP signaling in *Xenopus* is insufficient for neural crest induction without the activity of the Wnt signaling pathway (LaBonne and Bronner-Fraser, 1998), and BMP has been shown to stimulate transcription of *WNT1*. Inhibition of *WNT1* results in reduced expression of BMP-regulated genes, suggesting that neural crest delamination is regulated via BMP-dependent Wnt activity (Burstyn-Cohen et al., 2004). These findings have led to the development of a "two-step" model of induction, whereby BMPs act as a competence factor for subsequent signals such as Wnt (Kleber et al., 2005).

RA—like BMPs, Wnts, and Shh—is an extracellular molecule which acts in a concentration-dependent fashion. The developing neural tube contains the highest levels

of endogenous RA (Maden et al., 1998), and a role in posterior patterning of the nervous system has been well established (Maden, 2002). RA crosses the cell membrane and mediate their effects through the retinoic acid and retinoid X nuclear receptors (RAR and RXR, respectively), which form RAR-RXR heterodimers as well as RXR homodimers (Giguere et al., 1987; Brand et al., 1988; Krust et al., 1989). These complexes move into the nucleus, where they can regulate gene expression through interaction with a specific sequence in the promoters of target genes called the retinoic acid response element, or RARE. Considerable evidence has shown that *in vivo*, RA is an overall modulator of HOX gene expression (Gavalas and Krumlauf, 2000; Gavalas, 2002). Excess RA causes a transformation of neural and mesodermal segments towards a posterior identity, accompanied by an anterior shift in HOX gene expression boundaries (Conlon, 1995).

It is important to note that not all neuronal progenitor cells progress to fully differentiated neurons. Depending on the neuronal population, 25-75% of progenitor cells will undergo apoptosis or programmed cell death (Becker and Bonni, 2005). The loss of progenitor cells occurs at all stages of differentiation and is prevented by neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) via activation of their cognate receptors TrkA and TrkB. Neuronal survival is also enhanced by insulin and insulin-like growth factors, cytokines, and target-derived factors. Finally, a small, but very important population of neural progenitor cells never differentiates or undergoes cell death. These neural stem cells never exit the cell cycle and retain the potential to proliferate and differentiate into functional neurons (Gage, 2000). Adult neurogenesis from stem cells that populate the SVZ of the lateral ventricles and the SGZ of the hippocampal dentate gyrus have become focus of intense research (Parent et al.,

1997; Parent et al., 2002). These adult neural stem cells have been implicated in the remodeling that occurs following brain injury (Miles and Kernie 2008; Kernie and Parent, 2010), but in order to fully harness their potential for therapeutic strategies, further mechanistic insight must be gained into the molecular mechanisms regulating neurogenesis (Eriksson et al., 1998; Lie et al., 2004; Curtis et al., 2007).

Roles of bHLH transcription factors in neural development

In addition to growth factors, the development of the nervous system is brought about by the coordinated action of transcription factors, which act in combination to specify neural gene networks and determine cell fate. The mammalian genome encodes for about 1500 transcription factors that contain known DNA-binding motifs (Gray et al., 2004). In the developing mouse nervous system, over 350 transcription factors have been identified that show spatially and/or temporally restricted expression (Gray et al., 2004). Transcription factors are expressed in multiple brain regions, and graded expression of transcription factors is thought to underlie the genetic basis for the topographical organization of the brain (Albright et al., 2000).

The analysis of actions of transcription factors has begun to clarify some of the ways in which intrinsic signals control neural cell differentiation, but there are many unresolved issues: first, it remains unclear whether there are common transcriptional programs that control the expression of generic neuronal properties shared by diverse classes of neurons. Second, whether the subtype identity of individual neuronal cell types requires the convergent activities of many genes, or if it can be achieved through the

actions of a single, dedicated subtype-specific factor. And third, there is uncertainty about the mechanisms used to coordinate the assignment of generic and subtype-specific neuronal properties to individual classes of neurons.

Neurons all share a common set of features: they express pan-neural markers, have elaborate dendritic and axonal processes depending on cell type, form synaptic connections, and have the ability to generate and transduce electrical signals. Specification of these generic neuronal features is at least in part controlled by a group of neuronal determination genes that encode for bHLH transcription factors (see Figure 1.2; Anderson et al., 1997; Lee, 1997; Schuurmans and Guillemot, 2002). Proteins of the bHLH class play an integral role in the acquisition of a neural fate and the determination of neural lineage, and also in the specification of the phenotypes of terminally differentiated neurons (Brunet and Ghysen, 1999; Bertrand et al., 2002; Ross et al., 2003; Kageyama et al., 2005).

In the nervous system, bHLH factors are functionally classified into two families of proteins: the repressor-type bHLH factors, which promote maintenance of neural stem cells and the differentiation of glial cells, and the activator-type bHLH factors, which induce production of neurons. bHLH proteins bind DNA as heterodimeric complexes that are formed with widely expressed bHLH proteins, or E proteins, encoded by one of three mammalian genes: E2A (with its two alternative products E12 and E47), HEB, and E2-2 (Johnson et al., 1992; Massari and Murre, 2000). Crystal structure analysis has shown that bHLH dimers are formed by interactions between the two helices of each partner to form a four-helix bundle (Ellenberger et al., 1994; Ma et al., 1994). Because heterodimerization is a prerequisite for DNA binding, factors that interfere with

dimerization effectively act as passive repressors of proneural gene activity. For example, the vertebrate inhibitor of differentiation (Id) genes have an HLH domain, but lack an adjacent basic motif for DNA binding. These proteins have a high affinity for E proteins, so they can compete with bHLH proteins, forming heterodimers that can no longer bind DNA. Specifically, bHLH factors bind to DNA sequences that contain a core hexanucleotide motif, CANNTG, known as an E-box. The basic region of the protein fits in the main groove of the DNA, and nine of the ten DNA-contacting residues are completely conserved in the different families of neural bHLH proteins. These direct contacts are responsible for the common ability of neural bHLH proteins to bind to the core E-box sequence, but are unlikely to account for the divergence in DNA-binding specificity and biological activities between different neural bHLH protein families.

Hes genes are repressor-type bHLH genes that are homologous to the *Drosophila* hairy and enhancer of split, and are one of the major target gene families activated by Notch signaling (Jarriault et al., 1995; Ohtsuka et al., 1999; Kageyama et al., 2008). Hes genes regulate neural stem cell self-renewal by repressing premature onset of the proneural bHLH genes, such as Ascl1 and Neurog2, which promote neuronal differentiation of neural stem cells (Kageyama et al., 2005; Nelson et al., 2007; Ohsawa and Kageyama, 2008).

One function of the proneural bHLH genes in vertebrates is to induce expression of Delta, thereby completing the molecular circuitry underlying the basis of lateral inhibition (Skeath and Carroll, 1994; Technau et al., 2006). Several DSL-ligands have been identified in vertebrates, including members of the Delta-like (Dll) and Serrate (also called Jagged) gene family (Shutter et al., 2000; Popovic et al., 2007). Whereas most

studies of vertebrate proneural bHLH genes have focused on their role in neural subtype specification, recent evidence has shown that Ascl1 and Neurog2 can directly regulate Dll1 expression in certain regions of the nervous system (Castro et al., 2006; Nelson and Reh, 2008).

Proneural bHLH factors act as transcriptional activators, and only a few, such as those belonging to the Olig family and bHLHb4/5, have been shown to act as repressors (Novitch et al., 2001; Bramblett et al., 2002; Xu et al., 2002). Many proneural bHLH genes seem to work in concert with one another: Ascl1, Neurog1 and Neurog2 are co-expressed in the dorsal telencephalon, and the three genes could together account for the generation of all progenitors of the cerebral cortex (Nieto et al., 2001). Although one study showed that Ascl1 is dispensable for the generation of neuronal progenitors in the sympathetic ganglia (Sommer et al., 1995), a large body of evidence suggests that co-expression of proneural bHLH genes is not redundant: mice that carry a mutation in Ascl1 have severe defects in neurogenesis in the ventral telencephalon and the olfactory sensory epithelium (Guillemot et al., 1993). Neurog1 or Neurog2 single mutant mice lack complementary sets of cranial sensory ganglia, and Neurog1/2 double knockout mice additionally lack spinal sensory ganglia and a large fraction of ventral spinal cord neurons (Ma et al., 1999; Fode et al., 2000). Ascl1 is the only known proneural gene to be expressed in the ventral telencephalon, and studies have shown that in Ascl1 mutants, progenitor populations in this region still persist and differentiate normally (Guillemot et al., 1993; Casarosa et al., 1999; Cau et al., 2002). However, there are highly region-specific requirements for Ascl1 for development of different GABAergic neuron subpopulations (Peltopuro et al., 2010). These studies seem to imply that in the CNS as

well as the PNS, other unidentified genes with proneural activity may exist.

While the proneural activity of a few genes (e.g. Ascl1) is well established, it is a separate question as to whether expression of these genes is sufficient to account for the selection of all neural progenitors. Genetic studies in *Drosophila* and other vertebrate models have provided evidence that a small number of proneural bHLH transcription factors are both necessary and sufficient in the context of the ectoderm to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation. In *Xenopus*, expression of Neurog1, Neurog2, or NeuroD is sufficient to promote the ectopic expression in ectodermal cells of markers characteristic of post-mitotic neurons (Lee et al., 1995; Olson et al., 1998; Ma et al., 1999). Gain-of-function studies have shown that Ascl1 is an instructive determinant of neuronal subtype identity: when ectopically expressed in the CNS, it has the ability to override endogenous differentiation programs, thereby re-specifying progenitor identity (Parras et al., 2002). Ascl1 is also thought to play a role in the specification of neuronal subtype identitity by inducing the expression of GABAergic differentiation markers in dorsal telencephalic neurons (Fode et al., 2000). These findings implicate bHLH proteins as key determinants in the competence of progenitor cells for neuronal differentiation. However, bHLH factors undergo complex temporal regulation during differentiation: some are transiently expressed in individual neural progenitors and are downregulated before progenitor cells exit the proliferative zone and begin to differentiate, while other bHLH factors persist or even increase (Gradwohl et al., 1996; Bhattacharya et al., 2004). Therefore, the ability of proneural genes to promote full neuronal differentiation must rely on the induction of downstream regulatory genes that implement neuronal differentiation programs (Bertrand

et al., 2002).

In addition to their roles in initiating neurogenic cell differentiation programs in neural precursors, a large number of bHLH proneural genes have been identified that also simultaneously promote cell cycle exit, leading to the possibility that bHLH factors are involved in coupling these two processes. For example, blocking Hes activity impedes the proliferation of neural stem cells and results in changes in two stages of differentiation: 1) the selection of neuronal instead of glial fate, and 2) the selection of a specific, GABAergic neuronal phenotype (Kabos et al., 2002). Since regulation of cell cycle entry and exit subsequently alters other transcriptional programs driving nervous system development, it becomes increasingly important to unveil the molecular mechanisms coupling cell cycle exit and neuronal differentiation.

Coupling cell cycle exit and neuronal differentiation

The eukaryotic cell cycle consists of four distinct phases: G1 phase (the first gap) before DNA synthesis occurs, S phase when DNA replication occurs, G2 phase (the second gap) after DNA synthesis, and the M (mitotic) phase when cell division occurs. A successful cell cycle is dependent on the precise adherence to order and termination of each phase. This strict regulation is guaranteed by control mechanisms that permit the transition to the next phase via distinct checkpoints only if certain critical events are fulfilled (Paulovitch et al., 1997).

Neuronal fates are often determined around their final cell cycle (Edlund and Jessell, 1999; Cremisi et al., 2003), and research has shown that these determination

events are linked to specific phases of the cell cycle. For example, in experiments where young cortical progenitor cells are transplanted into older animals, young cells change their fate in accordance with the older environment, but only if they are transplanted at G1 or S phase. Cells transplanted at M phase retain their early fates (McConnell and Kaznowski, 1991). Retinal cells have also been shown to lose their responsiveness to some extrinsic neuronal determinants as they enter M phase (Belliveau and Cepko, 1999).

The decision as to whether somatic cells continue to proliferate or become terminally differentiated neurons is dictated by extracellular and intracellular factors that act on the cell cycle machinery. Major players in this scenario are holoenzymes composed of regulatory (cyclin) and catalytic cyclin-dependent kinase (CDK) subunits (see Figure 1.3). CDKs are activated through a series of steps, beginning with the association with a cyclin subunit, followed by phosphorylation or dephosphorylation of specific amino acids. The G1/S transition is the key step for cell cycle progression and is controlled by CDK4 and CDK6, which act in mid-G1, and CDK2, which operates in the late G1 phase (Watanabe et al., 1999; Pucci et al., 2000). Neuronal differentiation is associated with a reduction in the overall amount of CDK activity during G1 phase. Consistent with this observation, the accumulation of CDK inhibitors (CKIs) has been observed in many differentiated cell types (Matsuoka et al., 1994; Parker et al., 1995; Rothschild et al., 2006; Buck et al., 2009).

CDK activity is suppressed via interactions with two major classes of inhibitor proteins: the Ink4 class of proteins that exhibit specificity for CDKs 4/6 and the Cip/Kip class that shows a broader spectrum of CDK inhibitor activity (Harper, 1997). Non-proliferating cells can often be re-activated via removal of cell-type specific CKIs, and

this reactivation takes place irrespective of added growth factors. In addition, non-proliferating cells still express functional, significant levels of pre-assembled cyclin/CDK complexes, making cell cycle arrest a state that must constantly be maintained by active expression of CKIs (Pajalunga et al., 2007). These findings suggest that CKIs not only modulate kinase activity, but also contribute to the decision to enter the cell cycle as much as cyclins themselves.

Studies have shown that high levels of the CKI $p27^{kip1}$ are characteristic of post-mitotic neurons in regions such as the cortical plate and pre-plate during development, as well as in neurons located in the basal telencephalon and the diencephalon (Lee et al., 1996). The high expression of $p27^{kip1}$ in these cells correlated with $p27^{kip1}$ binding to and inactivating CDK2. Evidence has also shown that the CKI $p21^{cip1}$ participates in the regulation of neural differentiation. *In vitro* studies using PC12 pheochromocytoma cells showed that NGF induced neuronal differentiation is accompanied by an increase in $p21^{cip1}$ protein levels (Erhardt and Pittman, 1998). In agreement with this observation, it was demonstrated that ectopic expression of $p21^{cip1}$ in this cell line mimicked the changes in PC12 cells induced by NGF treatment.

In P19 embryonic carcinoma cells, neuronal differentiation induced by ectopic expression of proneural bHLH factors was preceded by elevated expression of $p27^{kip1}$ and cell cycle withdrawal (Farah et al., 2000). Neuronal differentiation of P19 mouse embryonic carcinoma cells with retinoic acid requires the up-regulation of $p27^{kip1}$ (Sasaki et al., 2000). Gain- and loss-of-function studies in zebrafish have shown a fundamental role for NeuroD in the context of cell cycle regulation during retinal photoreceptor genesis: conditional expression of NeuroD caused cells to withdraw from the cell cycle,

which was accompanied by the upregulation of cell cycle inhibitor $p27^{kip1}$. In the absence of NeuroD, these cells fail to exit the cell cycle and also express more cell cycle progression factors, such as cyclin D1 (Ochocinska and Hitchcock, 2008). Together, these data emphasize the importance of CKI expression for neuronal differentiation and also demonstrate that bHLH proteins can link neuronal differentiation and neural precursor cell cycle exit.

As additional modulators of CDK activity, the growth-arrest and DNA-damage inducible protein 45 (Gadd45) proteins play an important role in neuronal differentiation. The Gadd45 gene family, composed of Gadd45α, Gadd45β, and Gadd45γ, encode small, evolutionarily conserved acidic proteins that are highly homologous to one another and primarily localized to the cell nucleus (Zhan et al., 1994; Takekawa and Saito, 1998). Evidence has shown that Gadd45 proteins play a pivotal role in normal cell cycle progression: Gadd45 proteins specifically interact with and inhibit the kinase activity of the CDK1/cyclin B1 complex, which is involved in progression of G2 to M phase (Liebermann and Hoffman, 1998; Zhan et al., 1999). The inhibition of kinase activity of CDK1/cyclin B1 by both Gadd45α and Gadd45β has been shown to involve physical dissociation of the complex, but inhibition of the same complex by Gadd45γ occurs in the absence of complex disruption (Vairapandi et al., 2002). Primary cells from Gadd45α deficient mice were found to exhibit genomic instability and abnormal mitotic morphology (Hollander et al., 1999), and research has also shown that all three Gadd45 proteins cooperate in activation of S and G2/M cell cycle checkpoints following exposure to genotoxic stress (Vairapandi et al., 2002).

Since the CDK1/cyclin B1 complex plays a key role in progression from G2 to M

phase of the cell cycle (Elledge, 1996; O'Connor, 1997), the ability of Gadd45 proteins to inhibit activity of this complex may explain the role of Gadd45 proteins in the activation of G2/M cell cycle arrest following DNA damage. However, how Gadd45 proteins are involved in the regulation of an S phase checkpoint remains to be determined. It is possible that this function is linked to the role Gadd45 proteins play in DNA repair (Smith et al., 2000). There is conflicting data on this topic, however, as Yang et al. (2000) observed that although Gadd45β and Gadd45γ interact with CDK1/cyclin B1, they do not inhibit the kinases' activity, as microinjection of the proteins into normal human fibroblasts failed to induce G2/M arrest. These discrepancies may arise from differences in methodologies and/or cellular systems used. It has yet to be determined to what extent Gadd45 proteins function in the multiplicity of G2 checkpoint controls that have evolved to protect the fidelity of DNA replication and mitosis.

The cAMP/PKA pathway and cell cycle regulation

The evolutionarily conserved cAMP-cAMP-dependent kinase (PKA) signaling pathway is another important regulator of the cell cycle (see Figure 1.4). Elevation of cAMP levels and subsequent activation of PKA results in the proliferation of certain cell types but can also induce cell-cycle arrest and apoptosis in others (Richards, 2001; Zhang and Insel, 2004). cAMP signaling pathways can inhibit progression through each phase of the cell cycle, the most well-characterized of these being cAMP-induced arrest in G1, which is mediated through modulation of cyclin D1 and cyclin D3 and the CDK inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Kato et al., 1994; van Oirschot et al., 2001). Studies of G2

arrest in meiosis I of *Xenopus* and mouse oocytes have shown that cAMP signaling maintains this arrest in a PKA-dependent fashion (Nebreda and Ferby, 2000; Han et al., 2005). PKA itself has been shown to be involved in many aspects of cell cycle regulation, including centrosome duplication, S phase progression, G2 arrest, mitotic spindle formation, exit from M phase, and cytokinesis (Matyakhina et al., 2002).

PKA is a holoenzyme and exists as an inactive tetramer comprised of two regulatory (R) subunits and two catalytic (C) subunits. The accumulation of cAMP in response to activation of guanine-nucleotide-binding G-protein-coupled receptors induces most cellular responses through PKA. Binding of cAMP to the R subunits of PKA induces conformational changes that cause their dissociation from the two C subunits, which are then active as kinases. The C subunits then diffuse throughout the cell and phosphorylate target molecules. Four R subunits (RIα, RIβ, RIIα, RIIβ) and three C subunits (Cα, Cβ, and Cγ) have been identified in mammals (Zheng et al., 1993; Skalhegg and Tasken, 1997; Akamine et al., 2003; Kim et al., 2007; We et al., 2007).

A significant body of research exists that explores the role of cAMP and PKA in maintaining cell cycle arrest, with classic work being done using *Xenopus* oocytes. *Xenopus* oocytes are arrested at the G2/M border of meiosis and upon treatment with progesterone, reinitiate meiosis, undergoing the process of maturation in which the immature oocyte is transformed into a fertilizable egg (Masui, 2001). Early studies suggested that high intracellular levels of cAMP were responsible for this maintenance of G2 arrest by sustaining high levels of PKA activity. Experiments specifically directed at the role of PKA in maturation showed that injection of the biochemically purified catalytic subunit inhibited progesterone-induced maturation, but only if the injections

were done within one hour of hormone treatment (Maller and Krebs, 1980) . This suggested that a high level of PKA activity affects primarily initial, early steps in maturation. Injection of the regulatory subunit of PKA or a heat-stable protein inhibitor of PKA (PKI) caused a protein synthesis-dependent release of the oocytes from G2 arrest without any hormonal treatment. PKA appears to play a similar role in maintaining somatic mammalian cells in an interphase state, since microinjection of PKI into mammalian fibroblasts resulted in mitotic induction (Lamb et al., 1991). Only recently have studies revisited the role of PKA in G2 arrest in the context of the identification and characterization of various molecular pathways involved in oocyte maturation and regulation of entry into M phase.

G2-arrested *Xenopus* oocytes contain pre-M-phase promoting factor (MPF), a complex of cyclin B and Cdc2 that is inactive due to inhibitory phosphorylations of Cdc2 on Thr^{14} and Tyr^{15}. Upon progesterone stimulation, there are two parallel pathways that lead to the activation of the cyclin B/Cdc2 complex and release of the oocyte from G2 arrest: in one pathway, Aurora-A kinase is activated and Mos protein is synthesized, activating the MAPK pathway that inhibits Myt1 (the kinase that maintains phosphorylation of Thr^{14} and Tyr^{15} of Cdc2). The other pathway leads to the activation of Cdc25C phosphatase by the upstream Polo kinase cascade. Activated Cdc25C phosphatase then dephosphorylates and activates Cdc2. The convergence of these two pathways induces an activation of cyclin B/Cdc2, driving the oocyte from G2 into M-phase (Duckworth et al., 2002; Schmitt and Nebreda, 2002). A functional link between PKA signals and the Cdc2 kinase signaling pathway has been established in mouse: PKA was shown to regulate the Wee1 kinase family, whose function is to inhibit cyclin

B/Cdc2 activity via phosphorylation of Cdc2 on Thr^{14} and Tyr^{15}, as well as binding to and sequestering the complex in the cytoplasm (Wells et al., 1999).

Phosphorylation is a prevalent mechanism by which transcription factors are regulated in response to cellular signals, and PKA-promoted protein phosphorylation is no exception. PKA exerts many of its transcriptional effects by the action of the cAMP-response element (CRE) binding protein (CREB), which binds as a dimer to a conserved CRE sequence, TGACGTCA (Mayr and Montminy, 2001). One of the major mechanisms by which CREB is activated is by phosphorylation at a key serine residue (Ser^{133}), which allows for interactions with transcriptional co-activators CREB-binding protein (CBP) and its paralogue p300 (Gonzalez and Montminy, 1989; Chrivia et al., 1993; Arias et al., 1994). Transcription of cellular genes typically peaks after 30 minutes of stimulation with cAMP, conciding with the time required for the levels of PKA catalytic subunit to become saturated in the nucleus. Under continuous stimulation, CREB activity attenuates over the next two to four hours, due to dephosphorylation by the serine/threonine phosphatases PP-1 and PP-2A (Hagiwara et al., 1992; Wadzinski et al., 1993). In addition to being an effector of PKA signaling, CREB also plays a critical role in activity-dependent gene regulation required for long-term synaptic plasticity and basic neuronal survival, and is a substrate for many additional protein kinases including Ca^{2+}/calmodulin-dependent (CaM) kinase and ribosomal S6 kinase (Silva et al., 1998; Bonni et al., 1999; Riccio et al., 1999; Barco et al., 2002; Lonze et al., 2002; Mantamadiotis et al., 2002).

As a cAMP-responsive activator, CREB plays many roles in neural cell function and is regulated through the action of diverse intracellular signaling cascades. Recent

findings show that CREB is constitutively activated in dividing immature neural cells, which are present in neurogenic regions of both embryonic and adult vertebrate brains (Nakagawa et al., 2002; Dworkin et al., 2007). CREB has been shown to be involved in growth-factor dependent survival of sympathetic and cerebellar neurons: NGF and BDNF have both been found to promote cell survival by stimulating expression of the anti-apoptotic gene *BCL2* through CREB (Bonni et al., 1999; Riccio et al., 1999). Transgenic models expressing dominant-negative forms of CREB have revealed additional roles for the CREB family of activators in the control of cell survival and proliferation. Several cell cycle regulators—such as cyclin D1 and cyclin A—contain functional CREs and appear to be regulated by CREB (Desdouets et al., 1995; Lee et al., 1999).

Since the cAMP-PKA signaling cascade is one of the most multifunctional systems described to date, and because one of its major effectors, CREB, lies at the hub of a diverse array of intracellular signaling pathways and is a transcriptional regulator of numerous functions in developing and adult neural cells, it is critical that cellular mechanisms exist within neurons that regulate their activity.

Regulation of PKA by protein kinase inhibitors

PKA activity is modified via two mechanisms: 1) inhibition of its kinase activity, and 2) the regulation of its intracellular localization. The R subunits function through both of the aforementioned mechanisms to modulate PKA activity: in the absence of cAMP, the R subunits associate with the C subunits, thereby inhibiting their activity (Corbin et al., 1988; Taylor et al., 1999). In addition, the R subunits also localize PKA to

the cytoplasm by binding to A-kinase anchoring proteins (AKAPs) that in turn anchor PKA to specific subcellular structures (Colledge and Scott, 1999; Michel and Scott, 2002).

In addition to the two R subunits, a second level of regulation of PKA activity occurs via the activity of protein kinase inhibitor (PKI) proteins. PKIs are heat-stable proteins, approximately 70-75 amino acids in length, and are high-affinity, specific inhibitors of PKA (Scott et al., 1985). PKI proteins regulate PKA activity by competitively binding to its free C subunits, thereby inhibiting phosphorylation of PKA substrates (Ashby and Walsh, 1972). The N-terminal region of PKIs contain the amino acid sequence R-R-N-A, which acts as a pseudosubstrate site, and is required for binding with high affinity to the C subunits of PKA. This affinity is matched only by the R subunits that comprise the PKA holoenzyme (Hofmann, 1980). However, PKIs lack the specific binding site for cAMP that is present on the R subunits of PKA. As a result, PKI-mediated inhibition of PKA occurs specifically in the presence of cAMP following the cAMP-mediated dissociation of the R and C subunits.

PKIs also play a role in regulating the intracellular localization of the C subunit of PKA. The free C subunit of PKA shuttles between the cytoplasm and nucleus, phosphorylating substrates in both cellular compartments, one of the most notable nuclear targets being CREB (Gonzalez and Montminy, 1989; Harootunian et al., 1993). PKI proteins contain a nuclear export signal that causes the ATP-dependent, rapid export of the PKI-bound C subunit out of the cell nucleus back to the cytoplasm (Fantozzi et al., 1994; Wen et al., 1994; Wiley et al., 1999). In the cytoplasm, when cAMP levels are reduced by phosphodiesterase activity, the C subunit is able to reassociate with the R

subunits to reform the PKA holoenzyme and restore cAMP regulation to the cell. By enhancing the rate of export of the C subunit from the nucleus, PKIs are thought to affect the kinetics and/or extent of PKA activity in the nucleus.

Three PKI protein isoforms—PKIα, PKIβ, and PKIγ—are produced from three different genes that have widespread but distinctive tissue distributions (Collins and Uhler, 1997; Zheng et al, 2000). PKIα was originally identified in rabbit skeletal muscle, and since then homologous proteins have been identified in mice and humans (Walsh et al., 1971; Olsen and Uhler, 1991). Sequence analysis shows that PKIα is composed of two distinct functional domains: the pseudosubstrate site is responsible for making PKIα the most potent inhibitor of the catalytic subunit of PKA, even amongst the other two isoforms found in mice (Gamm and Uhler, 1995; Collins and Uhler, 1997), and the leucine-rich nuclear export signal, which enables PKIα to transport the free C subunit out of the nucleus and back into the cell cytoplasm where it can reassociate with its R subunits and form the inactive holoenzyme. In mice, PKIα is the most abundantly expressed isoform, with high transcript expression levels in the cerebral cortex, cerebellum, and hippocampus (Van Patten et al., 1992; Seasholtz et al., 1995; de Lecea et al., 1998). Within these regions, PKIα is widely distributed, which further suggests that it plays a critical role in regulating PKA activity.

The PKIβ protein was first isolated from rat testis, but the cDNA was first cloned from mouse brain. Like the other two isoforms, homologous proteins were identified in mice and humans. In mice, PKIβ is highly expressed in certain brain regions such as cerebellum, pons, medulla, and hypothalamus (Van Patten et al, 1991; Seasholtz et al 1995). It is also expressed at lower levels in the cerebral cortex and hippocampus. In

humans, two PKIβ transcripts of 1.9 kb and 1.4 kb were detected, and PKIβ was identified as the predominant PKI isoform expressed in the brain (Zheng et al, 2000).

PKIγ was the most recently discovered isoform, and has expression in the brain in both mouse and humans, although research has not been done to determine specific regions of expression (Collins and Uhler, 1997; Zheng et al., 2000). There is high conservation between PKIγ and PKIα, mostly within the pseudosubstrate site and the nuclear export signal (Collins and Uhler, 1997). Despite this conservation, PKIγ is less potent than PKIα. One hypothesis for this difference in inhibition is the presence of a specific cysteine residue (Cys^{13}) at the N-terminal portion of PKIγ, which was postulated to interfere with α-helix and β-turn regions that play a role in the ability of PKIα to bind with high affinity to the C subunit of PKA (Knighton et al., 1991). However, modification of Cys^{13} failed to produce an effect on the inhibitory activity of PKIγ (Collins and Uhler, 1997).

Although all three PKI proteins inhibit PKA activity and can also facilitate nuclear export of the PKA C subunit, they may act by preferentially recognizing the various C subunit isoforms with different potencies. However, unlike the regulatory subunits of PKA, PKI inhibition of the catalytic subunit is not relieved by cAMP, and the physiological mechanism by which PKI dissociates from the C subunit has not been established. Initially, the function of PKI proteins was thought to be limited to the regulation of basal PKA activity based on the finding that the total amount of intracellular PKI in certain tissues would allow for the inhibition of about 20% of total cellular PKA (Walsh and Ashby, 1973). This would allow for a mechanism whereby basal levels of cAMP in a cell would not activate PKA. Only when intracellular cAMP levels rose above

a defined threshold would an increase of PKA activity occur.

However, more recent studies suggest that the endogenous tissue levels of PKI proteins were grossly underestimated due to the purification procedures' failure to detect the PKIγ isoform (Collins and Uhler, 1997). *In situ* hybridization studies show overlapping regions of expression between PKIα and PKIβ, suggesting that enough PKI may be present in these areas to inhibit the majority of PKA activity (Seasholtz et al., 1995). In PKIα null mice, the loss of PKI activity resulted in a significant reduction of basal PKA activity in skeletal muscle (Gangolli et al., 2000). PKIβ knockout mice exhibited a significant reduction of PKI activity in testis—where it is normally highly expressed in the adult mouse—but an additive effect was not seen when these mice were interbred with the PKIα knockout mice (Belyamani et al., 2001). The substantial amount of residual inhibitory activity in these knockout mice is presumably due to compensation by the PKIγ isoform, but to date no PKIγ knockout mice have been generated to test this hypothesis.

In vitro models of neuronal differentiation

To harness the potential of ES cells as a tool for scientific exploration and a source of possible cell replacement, it is essential to establish a consistent and rational approach for robust production of specialized neural cell types. New protocols will need to be devised—or existing ones modified—using integrative principles of developmental and stem cell biology (Anderson, 2001). The first hurdle that must be overcome with ES cells is directed differentiation towards a cell lineage of interest, such as those of

neuronal cells.

In the absence of feeder cells and anti-differentiation agents such as leukemia inhibitory factor (LIF), mouse ES cells spontaneously differentiate into embryoid bodies (EBs) when cultured in suspension. The EB structure recapitulates certain aspects of early embryogenesis (Doetschman et al., 1985), but exhibits stochastic differentiation into a variety of cell lineages. Spontaneous differentiation of EBs yields only a small fraction of neural lineage cells, and as such, EBs must be treated with morphogens or growth factors to achieve directed differentiation or selective expansion of a specific lineage of neuronal cells. The most commonly used approach for neuronal differentiation from mouse ES cells is their spontaneous aggregation into EBs and treatment of these aggregates with RA in the absence of LIF. Mouse ES cells treated with this protocol consistently yield a significant population of neuronal cells upon differentiation, predominantly consisting of glutamatergic and GABAergic neurons. These cells express voltage-gated ion channels and form functional synapses with neighboring neurons, and also generate action potentials that are functionally coupled by inhibitory and excitatory synapses as revealed by measurement of post-synaptic currents (Gajovic et al., 1998; Kawasaki et al., 2000; Okada et al., 2004).

While signaling through RA is critical during development (Maden, 2002), there is little evidence to suggest that RA in the aforementioned ES *in vitro* differentiation protocol acts to induce neural specification. Renoncourt et al. (1998) have shown that EBs treated with RA can differentiate into neuronal cell types characteristic of the ventral CNS, but within this mixed population of cells, rostral neural markers were absent, suggesting that RA may selectively promote the differentiation of caudal neuronal types.

Supporting this hypothesis, RA was found to be required for the differentiation of spinal motor neurons (Wichterle and Peljto, 2002). Therefore, although RA treatment of EBs results in robust, reproducible neuronal differentiation of ES cells, this protocol is severely limited in that the neurons generated are likely subgroups of cells representative of those in the caudal and ventral parts of the CNS, and even within these lineages, the cells are at a wide range of developmental stages.

Another commonly used method for promoting the proliferation of the neural precursor population is the use of FGF2, a survival and proliferation factor for early neural precursor cells (Okabe et al., 1996). ES cell aggregates cultured in suspension and then plated on adherent substrates in the presence of FGF2 result in the majority of the cells undergoing apoptosis, but a small population of surviving neuronal precursors continue to proliferate. With continued selection and expansion, nestin-positive neural precursor cells become highly enriched, and withdrawal of FGF2 results in spontaneous differentiation of neurons and glia (Okabe et al., 1996). The cells generated under this protocol also fulfill the criteria of functional, post-mitotic neurons, as they exhibited both excitatory and inhibitory synaptic connections, and have the capability of being induced into dopaminergic neurons (Lee et al., 2000). While the FGF2 protocol provides a distinct advantage over the RA protocol in that the neural precursor cells are better developmentally synchronized, the overall efficiency of neural induction during the early stages of differentiation is very low.

A major difficulty with observing the molecular pathway that a given cell population has executed *in vivo* is the complexity of dissecting the signaling cues that a cell has perceived from its environment. *In vitro* work attempts to simplify the context

within which a cell finds itself, and cellular context can further be reduced with the use of a clonal cell line. Clonal cell lines have been used extensively in research as they provide a homogenous population of cells that can be grown indefinitely *in vitro* and with relative ease. Furthermore, they are extremely useful when taking into consideration the translation from mouse to human systems. From the standpoint of fundamental biology, this transition is generally regarded as a straightforward step since the principles gleaned from lower vertebrates are likely to prevail in primates. However, because cell lineage development depends on the interplay between extrinsic signals and cell intrinsic programs, it makes the understanding of the biological clock of cell lineage development even more key to directing human ES cells to a particular phenotypic fate. The method described in this dissertation offers a protocol for dissecting mechanisms underlying early neural development as well as for developing cells for potential application in neurological conditions.

Like neural stem cells, ES cells and EC cells retain the ability to differentiate into neurons both *in vivo* and *in vitro* (Schuldiner et al., 2001; Wei et al., 2002; Hornstein and Benvenisty, 2004). ES cells are undifferentiated, pluripotent cells derived from the inner cell mass of blastocyst embryos. Like ES cells, EC cells are pluripotent, but are also transformed (McBurney, 1993). While many EC cell lines have been characterized with respect to neuronal differentiation, the P19 mouse EC cell line has proved to be a particularly tractable system for studying neuronal and glial differentiation because of the ease with which this cell line is cultured without the need for feeder cell layers. Many of the individual gene expression changes characterized during neural stem and progenitor cell differentiation are recapitulated in P19 cells (Jonk et al., 1994; Blelloch et al., 2004;

Hatada et al., 2008).

P19 cells are pluripotent, have a normal complement of chromosomes, and have the capacity to differentiate into derivatives of all three germ layers depending on the type of chemical inducers and culture conditions used (McBurney, 1983). For example, after treatment with high concentrations of RA and aggregation, they are easily differentiated into neurons, glia, and fibroblast-like cells (Jones-Villeneuve et al., 1982). These cell types appear with high temporal fidelity: the fibroblast-like cells emerge first, followed by the neuronal and glial cells. A microarray hybridization analysis of gene expression has been described using P19 cells induced to differentiate with RA, and demonstrated that over 200 known neuronal- and glial-specific genes were induced by RA differentiation (Wei et al., 2002; Teramoto et al., 2005). These results suggest that RA-induced P19 neuronal differentiation recapitulates at least part of the transcriptional network involved in the development of the nervous system.

As previously discussed, RA is involved in the development of vertebrate nervous system *in vivo*, playing a role in the stimulation of axon outgrowth, the migration of neural crest cells, and the specification of rostro-caudal positioning in the developing CNS among other processes (Maden and Holder, 1992). Studies of RA-induced P19 neuronal differentiation led to the initial discovery of a number of genes that were subsequently shown to be important for neural development *in vivo* (Bain et al., 1994). However, the majority of these studies focused on the roles of individual intrinsic or extrinsic factors, and as such, little is known about the transcriptional and signaling networks and how their coordinated interactions influence cell fate and the terminal differentiated phenotypes of the resulting cells. In order to reveal active processes at the

molecular level and to dissect key components of molecular pathways, differential gene expression studies provide a foundation for the elucidation of dynamic molecular mechanisms. The advent of DNA microarray technology allows for the high throughput examination of thousands of genes simultaneously (Schena et al., 1998).

Dissection of the transcriptional networks that control the cell fate determination during P19 neural differentiation under the regulation of RA signaling determined genome-wide expression patterns of terminally differentiated neuron populations using a microarray containing 9000 cDNA clones (Wei et al., 2002). Of the 9000 cDNA clones, 910 were preferentially expressed in neurons. In developing embryos, neurite formation requires extensive cytoskeleton remodeling. Microtubules provide structural support and act as substrates for the fast axonal transport of vesicles (Valtorta and Leoni, 1999). Genes encoding for a number of microtubule-related proteins—such as β-III-tubulin and microtubule-associated protein 2 (Map2)—are preferentially expressed during P19 neuronal differentiation. In addition to the microtubule network, there is a rapid rearrangement of the actin cytoskeleton during neurite outgrowth (Valtorta and Leoni, 1999). Correspondingly, proteins that have important roles in regulating the actin system, such as drebrin (Shirao, 1995), profilin 2 (Schluter et al., 1997) and rhoB (Tapon and Hall, 1997) are also upregulated in P19 cells.

Compared to other models of neuronal differentiation—such as the PC12 pheochromocytoma cells, SHSY-5Y neuroblastoma cells, and the Neuro2A neuroblastoma cells—P19 cells represent a much earlier embryonic state as determined by the expression of embryonic marker proteins (Marikawa et al., 2009). In addition, P19 cells have advantages over human teratocarcinoma-derived NT2 cells, which differentiate

more slowly in response to RA treatment, are karyotypically hypotriploid, and have not been tested in embryos for pluripotency (Bain et al., 1994).

P19 cells have been extensively used to study neuronal differentiation not only because of their ease of handling, but also because they can be easily transfected both transiently and stably (Heicklen-Klein et al., 2000; Wakabayashi et al., 2000; Liu et al., 2004). Transfection of several proteins including Ascl1, Neurogenin2, and Math1 has been shown to be sufficient to induce the differentiation of neurons even in the absence of RA treatment (Farah et al., 2000). The neurons produced by the transfection of Ascl1 express many of the neuronal proteins that have been previously characterized for RA-induced differentiation, including neuronal-specific β-III-tubulin, neurofilament proteins, synapsin I, and glutamic acid decarboxylase (Farah et al., 2000). Transfection of P19 cells also resulted in cells that possessed electrophysiological properties of neurons (Farah et al., 2000). Remarkably, the expression of glial-specific proteins such as glial fibrillary acidic protein (GFAP) was not found in the Ascl1-transfected cells as compared to RA-induced differentiation. These and other studies suggest that a significant number of the intrinsic and extrinsic cues responsible for differentiation of neurons *in vivo* are recapitulated in the P19 cell cultures *in vitro*.

Aims

In summary, neuronal differentiation encompasses an elaborate developmental program controlled by both intrinsic and extrinsic signaling programs. Previous research

provides a sound basis for the conclusion that bHLH transcription factors are critical regulators for both the initiation of neuronal differentiation and the specification of neurons into distinct regional subtypes. However, the precise molecular mechanisms for the basis of proneural bHLH action and downstream target effectors remain to be determined. Since the regulatory network that controls nervous system development and function is too complex to be studied as a whole, in the discourse to follow, particular emphasis is placed on identifying novel neuroregulatory genes that mediate Ascl1-induced neuronal differentiation. The continued elucidation of the molecular mechanisms that drive neurogenesis is important for applications of neural replacement therapy, which requires the direction of cells to specific neural subtype identities.

This thesis describes the development and characterization of a novel model system of P19 EC cells with doxycycline-inducible expression of Ascl1 in order to elucidate targets of its regulatory network. Microarray hybridization technology was employed to elucidate potential transcriptional targets during neurogenesis and from the resulting data identified a potential effector of Ascl1 (PKIβ) and a novel transcriptional target of Ascl1 (Gadd45γ). The studies described in this dissertation provide a platform for the future study of transcriptional regulation of bHLH proteins in the development and function of the nervous system.

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Figure 1.1. Patterning of the neural tube generates unique domains for neural progenitors. **A.** Schematic diagram of the embryonic neural tube showing patterning along the neural axis. The neural tube is highly regionalized by gradients of morphogens that create anteroposterior (AP) and dorsoventral (DV) axes. In vertebrates, the DV patterning of the developing neural tube is achieved by counteracting activities of morphogenic signaling gradients set up by sonic hedgehog (Shh) in the ventral floor plate and notochord, and bone morphogenic proteins (BMPs) in the dorsal roof plate. High levels of BMP signaling sets up dorsal patterns of transcription factor expression (e.g. Math1, Neurog1/2, and Ascl1). Shh signaling is thought to regulate the initial expression of transcription factors in the ventral neural tube (e.g. Nkx2.2, Olig2, Pax6 and Irx3). This double gradient activates distinct combinations of transcription factor expression at different dorsal/ventral levels of the central nervous system, which confers unique identities to cells based on their position in the gradient. Along the AP axis, Wnt signaling controls anterior–posterior axis formation and neural patterning, and later in development Shh is involved as an axon guidance molecule, impacting the positioning of axons following its morphogenic effects on neuron formation in the DV axis. **B.** Summary of the major signaling pathways involved in early vertebrate development. The Shh signaling pathway involves two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc binds Shh, whereas Smo acts as a signal transducer. In the absence of ligand, Ptc interacts with and inhibits Smo. This inhibition activates a transcriptional repressor (e.g. Gli in vertebrates). In the presence of ligand, the interaction of Ptc and Smo is altered and Smo is no longer inhibited. Gli proteins may then enter the nucleus and function as transcriptional activators. In BMP signaling, binding of a BMP dimer to its type II receptor recruits type I receptors. The proximity of the receptors allows the type II receptor to phosphorylate the type I receptor. Signal transduction through BMPs results in mobilization of members of the Smad family, which ultimately translocate to the nucleus and function as transcriptional activators. The canonical Wnt signaling pathway progresses via the binding of Wnts to receptors on other cells (e.g. Frizzled), which then, by a chain of cytoplasmic signaling events, removes β-catenin from a degradation pathway and promotes its import into the nucleus, where it is recruited to the TCF DNA-binding factors and modifies patterns of gene expression. Retinoic acid (RA) signaling is mediated by RA binding to retinoic acid receptors (RARs), which form heterodimers with retinoid X receptors (RXRs). This complex in turn binds to retinoic acid response elements (RAREs) in the regulatory regions of target genes (i.e. HOX genes).

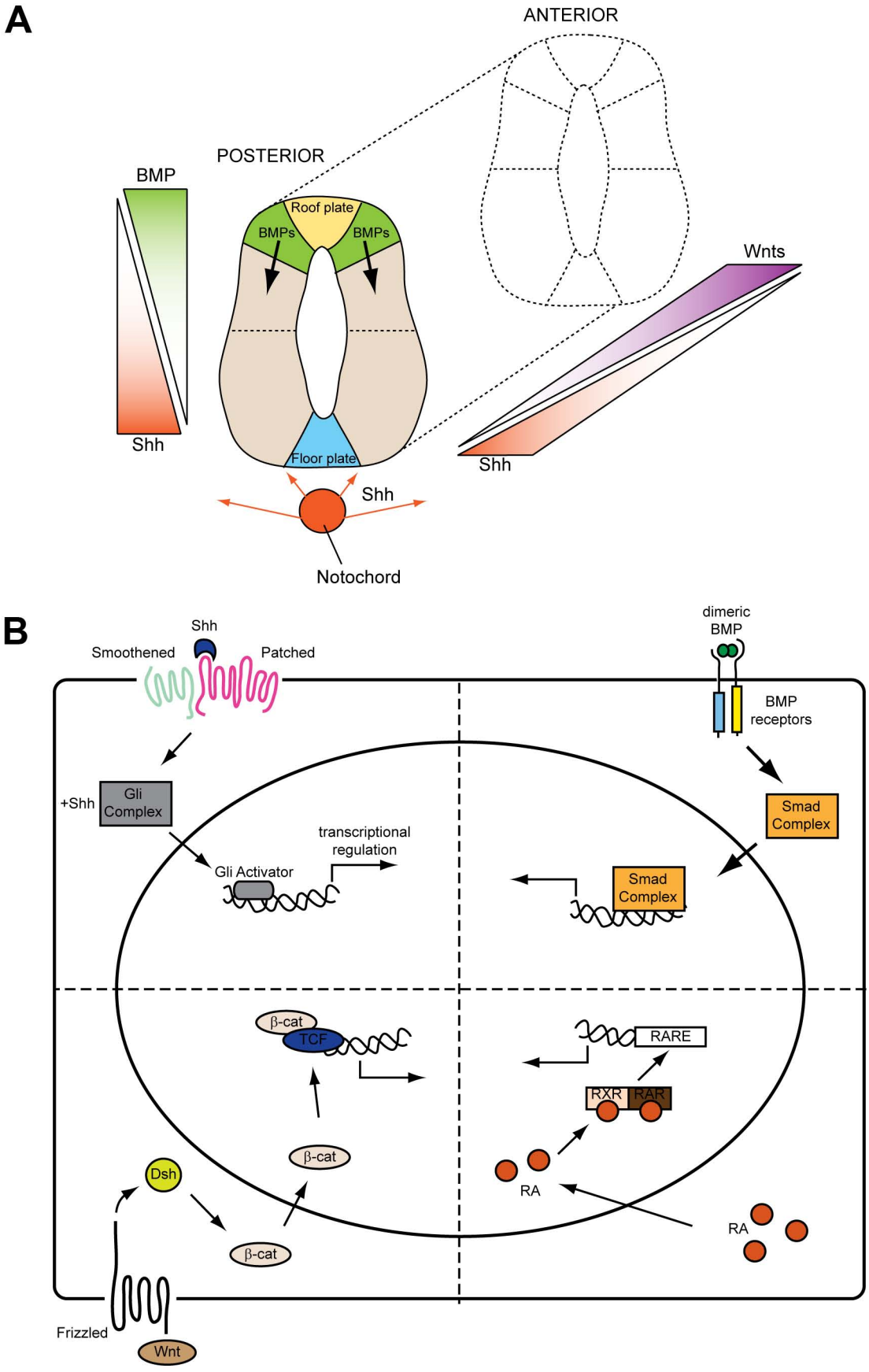
A
ANTERIOR
POSTERIOR
BMP
Shh
Roof plate
BMPs
BMPs
Floor plate
Wnts
Shh
Shh
Notochord
B
Shh
Smoothened
Patched
dimeric
BMP
BMP
receptors
+Shh
Gli
Complex
Smad
Complex
transcriptional
regulation
Gli Activator
Smad
Complex
β-cat
TCF
RARE
RXR
RAR
β-cat
Dsh
RA
RA
β-cat
Frizzled
Wnt

Figure 1.2. Structure and properties of neural bHLH proteins. A. Phylogenetic tree of bHLH factors involved in cortical development. Shown are a small set of bHLH factors that have been shown to have important roles in cell fate decisions during corticogenesis (adapted from Ross et al., 2003). **B.** Schematic representation of the structure of a bHLH protein. bHLH proteins are characterized by two α-helices connected by a loop. In general, transcription factors including this domain are dimeric, each with one helix containing basic amino acid residues that facilitate DNA binding. bHLH proteins typically bind to a consensus sequence called an E-box (CANNTG) to initiate transcriptional regulation.

A

bHLH Factor	Drosophila Homologue	Function	DNA Element
NeuroD, Neurog, Olig, Ascl, E proteins	Atonal	Transcriptional activators	E box
	Tap/Biparous		
	Oli		
	Achaete-Scute		
	Daughterless		
Id	EMC	Sequester E proteins	None
Hes	Hairy/(E/Spl)	Transcriptional repressor	N Box

B

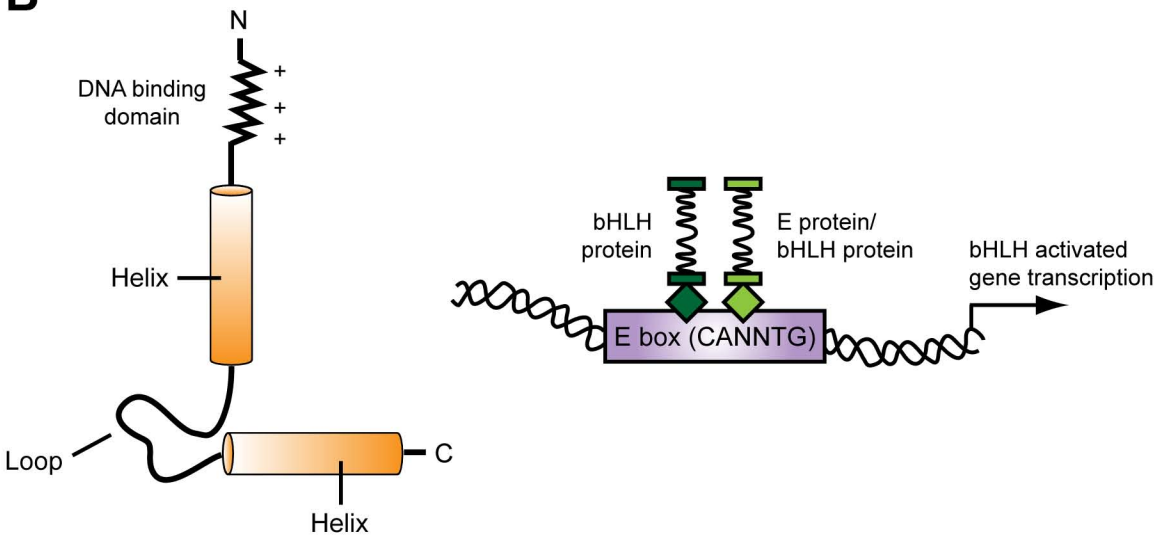

Figure 1.3. The cell cycle and its regulation by CDKs. In a simplified representation, a typical somatic cell cycle can be divided into four sequential phases: G1, S, G2, and M. DNA replication is carried out during S (synthesis) phase, and chromosome segregation occurs during a massive reorganization to cellular architecture at the M (mitosis) phase. Two "gap phases" separate these major cell cycle events: G1, which occurs between M and S phase, and G2, which occurs between S phase and M phase. During development of the central nervous system, cells can exit the cell cycle and enter a quiescent state known as G0 where they may ultimately terminally differentiate into neurons and glia. Cyclin dependent kinases (CDKs) control progression through each of these phases of the cell cycle. Cyclin-CDK complexes and their approximate times of activity during the cell cycle are shown. For clarity, extended cyclin families are indicated only by their class name (e.g. cyclin D rather than cyclin D1, D2, D3).

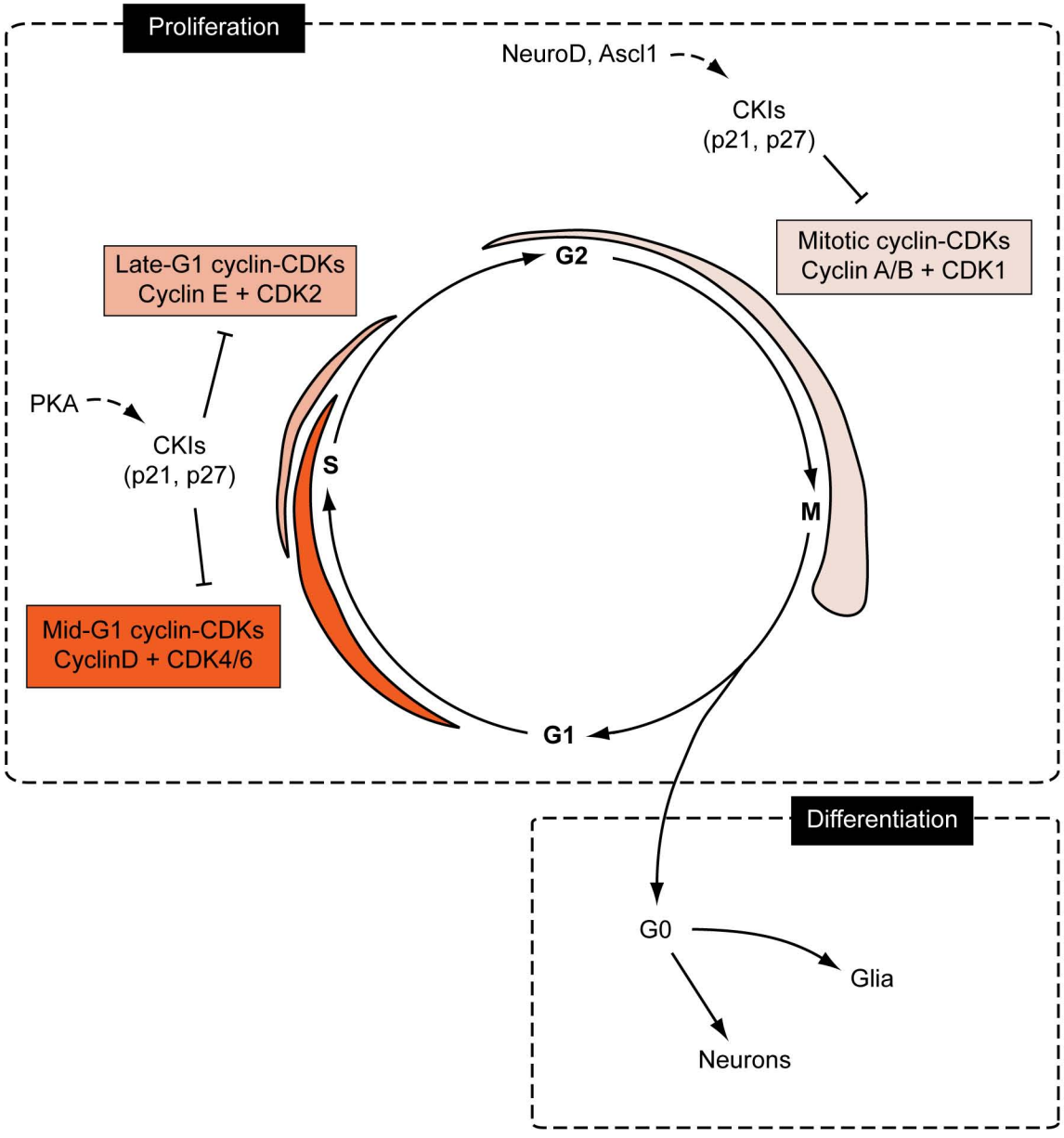
Proliferation
NeuroD, Ascl1
CKIs
(p21, p27)
Mitotic cyclin-CDKs
Cyclin A/B + CDK1
Late-G1 cyclin-CDKs
Cyclin E + CDK2
G2
PKA
CKIs
(p21, p27)
S
M
Mid-G1 cyclin-CDKs
CyclinD + CDK4/6
G1
Differentiation
G0
Glia
Neurons

Figure 1.4. Representative pathway for the activation of the cAMP-dependent protein kinase, PKA. Ligand binding activates a G protein-coupled receptor, which then undergoes a conformation change resulting in dissociation of the α-subunit (Gαs). Gαs activates adenylate cyclase (AC), which catalyzes the conversion of ATP to cyclic-AMP (cAMP). In the absence of cAMP, PKA exists as an inactive, tetrameric holoenzyme composed of two regulatory (R) and two catalytic subunits (C). The R subunits not only inhibits PKA activity, but also participate in regulating the intracellular localization of PKA by binding to A-kinase anchoring proteins (AKAPs) that in turn anchor the PKA holoenzyme to specific cellular structures (e.g. adenylate cyclase). In the presence of cAMP, each R subunit binds to two molecules of cAMP at separate allosteric binding sites, causing the dissociation of the C subunits. Once released, the free C subunits can enter the cell nucleus via diffusion where they phosphorylate specific serine and threonine residues in PKA substrates (such as the transcription factor CREB), which allows it to bind to target CRE sequences and activate gene transcription. Protein kinase inhibitors (PKIs) inhibit the activity of PKA by binding to the free C subunit via the pseudosubstrate site and inhibiting the phosphorylation of PKA substrates. In addition, binding of PKI to the C subunit exposes the nuclear export signal (NES) of PKI and allows it to transport the C subunit out of the nucleus and into the cytoplasm in an ATP-dependent manner.

Ligand
AC
Gαs
GPCR
cAMP
cAMP
ATP
AKAP
AKAP
C
C
R
R
Inactive PKA
free catalytic
subunits
P
CREB
CRE
CREB-activated
gene transcription
PKI
PKI
NES
NUCLEUS
PKI
CYTOPLASM

CHAPTER II

AN ENDOGENOUS INHIBITOR OF cAMP-DEPENDENT PROTEIN KINASE IS NECESSARY FOR ASCL1-INDUCED NEURONAL DIFFERENTIATION

Summary

The cAMP-dependent second messenger pathway plays a critical role in the developing nervous system. In particular, cAMP-dependent protein kinase (PKA) has been implicated in mediating the effects of sonic hedgehog and bone morphogenetic proteins during development. Both retinoic acid treatment and transfection studies of P19 embryonic carcinoma cells overexpressing proneural basic helix-loop-helix (bHLH) transcription factors, such as Ascl1, have been used to study neuronal differentiation *in vitro*. In our studies, microarray hybridization analysis showed that following overexpression of Ascl1, P19 cells undergo a transient increase in the expression of the endogenous PKA protein kinase inhibitor (PKI)β. This induction of PKIβ gene expression was confirmed by quantitative RT-PCR and was accompanied by a significant increase in PKIβ protein levels. shRNA constructs targeting PKIβ were effective in reducing levels of both PKIβ mRNA and protein and prevented the neuronal differentiation of P19 cells. We were able to partially rescue this obstruction by overexpressing PKIβ protein and found that the rescue was dependent on the inhibition of PKA. Our results define a requirement for PKIβ and its association with PKA during

neuronal differentiation of P19 cells.

Introduction

Pluripotent mammalian neural stem cells give rise to a variety of neuronal and glial cell types. This differentiation involves the dynamic interplay of extrinsic, environmental signals, cell-cell interactions, and intrinsic transcriptional regulatory events. The bone morphogenic proteins (BMPs) interact with complementary regional signals such as fibroblast growth factors (FGFs), and sonic hedgehog (Shh) to regulate earlier stages of neural stem cell expansion, self-renewal, lineage restriction, and incipient lineage commitment. The ability of these trophic signals to act within specific neurodevelopmental niches depends on the composite interactions of cell-cell contact-associated signals, such as the Notch signaling pathway, and transcriptional modulatory events, such as those mediated by members of the basic helix-loop-helix (bHLH) transcription factors (reviewed in Takahashi and Liu, 2006). bHLH factors regulate the fate of neural progenitor cells by exercising control over proliferation, initiation of differentiation, neurite outgrowth, and synaptogenesis (Sun et al., 2001; Nguyen and Woo, 2003). Gain- and loss-of-function studies have shown that precise temporal and spatial expression of bHLH transcription factors is critical for proper development of the nervous system (Casarosa et al., 1999).

One major effector of Notch signaling is repression by the bHLH genes *Hes1* and *Hes5* (Jarriault et al., 1995; Kageyama, 1995; Hsieh et al., 1997). *Hes1* and *Hes5* themselves encode for transcriptional repressor proteins that function to antagonize

proneural bHLH genes such as mammalian achaete-schute homolog 1 (*Ascl1* or *Mash1*). Ascl1 is essential for the survival of neural progenitor cells, and plays a central role in generating neuronal diversity by regulating subtype specification as well as differentiation (Bertrand et al., 2002). Ascl1 is one of the earliest markers expressed in neural progenitor cells (Parras et al., 2004), and in the embryonic ventral telencephalon, is essential for the production of neuronal precursor cells (Casarosa et al., 1999; Nieto et al., 2001). In the dorsal telencephalon, Ascl1, in concert with other proneural bHLH proteins from the Neurogenin family, promotes the neuronal commitment of multipotent progenitors while inhibiting their astrocytic differentiation (Nieto et al., 2001). When Ascl1 is expressed in differentiating neurons, it can heterodimerize with Id proteins (a subfamily of bHLH proteins that negatively regulate Ascl1), or it can heterodimerize with a family of ubiquitously expressed bHLH factors known as E-proteins to activate gene expression by binding to the E-box DNA sequence (Johnson et al., 1992).

Previous research utilizing P19 embryonic carcinoma cells has shown that these cells function as pluripotent stem cells. Once induced to differentiate into neurons by retinoic acid and aggregation, they exhibit biochemical and developmental processes similar to those that occur in early embryogenesis. Furthermore, they share several properties in common with embryonic stem cells isolated from mice and humans (Thomson and Marshall, 1998). Transient transfection of NeuroD2, Ascl1, Neurog1 or related proneural bHLH proteins, along with their putative dimerization partner E12, showed that these key transcription factors are sufficient to convert uncommitted P19 cells into differentiated neurons (Farah et al., 2000). The overall effects of Ascl1 expression in P19 cells are similar to those observed *in vivo* (Johnson et al., 1992): the

differentiation of these transfected cells is preceded by elevated expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ and cell cycle withdrawal. Furthermore, these differentiated neurons exhibit electrophysiological properties of neurons (Farah et al., 2000). However, little is known about the signaling cascades triggered downstream of Ascl1 that are involved in the differentiation and eventual function of these cells.

As a downstream effector of the sonic hedgehog and BMP pathways, cAMP-dependent protein kinase (PKA) is an essential integrator of signaling pathways (Tiecke et al., 2007; Ohta et al., 2008; Ghayor et al., 2009; Pan et al., 2009). During development, the cAMP/PKA pathway is critically involved in regulation of gene expression, cell growth, and cell differentiation. PKA exists as a tetrameric holoenzyme composed of two catalytic subunits and two regulatory subunits. Two forms of the catalytic subunits have been identified in mammalian tissues with the amino acid sequences of these isoenzymes, Cα and Cβ, differing by only 7% (Lee et al., 1983; Uhler et al., 1986; Hedin et al., 1987). Cα appears to be the major form, and is expressed constitutively in most cells, while the expression of Cβ is more tissue specific (Uhler et al., 1986; Hedin et al., 1987).

The regulatory subunits of PKA are divided into two categories: type I and type II. Functionally, these can be distinguished based on their potential for autophosphorylation. The type II holoenzymes contain an autophosphorylation site (Hofmann et al., 1975; Rosen and Erlichman, 1975), whereas the type I subunits are not autophosphorylated, but have a high affinity binding site for cAMP (Hofmann et al., 1975). Several isoforms of each regulatory subunit have been identified: RIα and RIIα are expressed in most cells (Lee et al., 1983; Scott et al., 1987), while the expression of RIβ and RIIβ are more specific, the latter being expressed primarily in neuronal tissues, testis,

and adrenal cells (Stein et al., 1987; Oyen et al., 1988; Cadd and McKnight, 1989). The regulatory subunits are modular, highly dynamic proteins that have multiple functions: in the presence of cAMP, the regulatory subunits each bind to two molecules of cAMP, which results in their dissociation from the catalytic subunits of PKA. These free catalytic subunits then go on to phosphorylate specific serine or threonine residues on PKA substrates, eliciting changes in their biological function (Corbin et al., 1988; Taylor et al., 1990). In addition, the regulatory subunits also serve to target the PKA holoenzyme to the A-kinase anchoring proteins (AKAPs) within the cell via binding to a stable dimerization/docking domain at the amino terminus of the subunit (Banky et al., 1998; Newlon et al., 1999).

A major PKA substrate that plays a role in the cell-intrinsic regulation of neurogenesis is the cAMP-response element binding (CREB) transcription factor (Nakagawa et al., 2002; Giachino et al., 2005). CREB functions as an integrator of numerous intracellular signals and has a clear role in neuronal development: CREB is activated in differentiating neurons, and deletion of CREB in early postnatal animals decreases the survival of neuronal precursor cells (Giachino et al., 2005). In addition, immature neurons contain the phosphorylated form of CREB, and pharmacological activation of PKA/CREB signaling enhances neuronal proliferation (Nakagawa et al., 2002). Since PKA has a wide distribution throughout the nervous system—and because PKA-mediated phosphorylation of neuronal targets via transcription factors like CREB plays a major role in the regulation of neuronal differentiation—it becomes critical that mechanisms exist to tightly regulate its activity.

Protein kinase inhibitor (PKI) proteins are important physiological regulators of

PKA that regulate its activity via two mechanisms: 1) competitive inhibition with the catalytic subunit of PKA, and 2) compartmentalization by binding to the catalytic subunit in the nucleus and translocating it to the cytoplasm where it can reform the inactive PKA complex with the regulatory subunits. Three isoforms of PKIs have been characterized in mammals and show conserved tissue-specific expression (Collins and Uhler, 1997; Zheng et al., 2000). In this study, we characterized PKA activation in P19 cells and demonstrated induction of all three isoforms of PKI during Ascl1-induced P19 differentiation. The magnitude of induction varied by isoform, and each PKI transcript also exhibited a distinct temporal pattern of expression. RNAi knockdown of each isoform showed that PKIβ—the most highly induced isoform in our model system—and its inhibition of PKA activity is necessary for Ascl1-induced neuronal differentiation in P19 cells.

Materials and Methods

Materials

The following primary antibodies were used in these experiments: CREB, phosphorylated CREB, GAPDH, polyclonal Map2 (Cell Signaling Technology), Flag, monoclonal Map2 (Sigma-Aldrich), and Ascl1 (BD Pharmingen). In addition, a polyclonal antibody was raised against peptides for PKIβ (64-KDQGQPKTPLNEGK-78) and synthesized from Invitrogen. Secondary horseradish peroxidase-conjugated antibodies were obtained from Cell Signaling Technology. A secondary Alexa Fluor conjugated antibody (goat anti-mouse Alexa Fluor 546) was purchased from Invitrogen.

Lentiviral shRNA vectors were obtained from Open Biosystems.

Cell culture, transfection, and treatment

P19 cells were cultured in Minimal Essential Medium Alpha (MEMα; Gibco) supplemented with 7.5% calf serum (CS; HyClone), 2.5% fetal bovine serum (FBS; HyClone), and 1% penicillin/streptomycin (Gibco). HEK-293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS (HyClone). Cells were kept at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. Cells were maintained below 80% confluence and passaged by dissociating them into single cells using TrypLE Express (Gibco). Cells were transfected using the TransIT-LT1 transfection reagent (Mirus) following the manufacturer's instructions. When necessary, the appropriate parental expression plasmid DNA was added to maintain a constant total amount of DNA. US2-Neo and US2-Cα DNAs were constructed from previously described plasmids (Huggenvik et al., 1991). US2-CαK72M encodes for a protein that renders PKA catalytically inactive by mutating a lysine residue near the N-terminus of the kinase in the protein kinase subdomain II to a methionine. This residue has frequently been mutated to eliminate the catalytic activity of protein kinases (Zoller et al., 1981; Huggenvick et al., 1991). 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate (8-CPT-cAMP; Sigma-Aldrich) was dissolved in DMSO to a concentration of 20 mM. 8-CPT-cAMP was pre-diluted in serum-free media to a working concentration of 200 μM and added to cells that had been serum-starved for a minimum of 2 hours prior to treatment.

Differentiation of P19 cells

Tissue cultures plates were laminin coated using the procedure described in Huang et al., (2010). P19 cells were seeded at a density of 10^5 cells/ml and allowed to recover for 24 hours prior to transfection. After 12 hours of transfection, cells were treated with 7.5 μg/ml puromycin (InvivoGen). 24 hours after transfection, the media was changed to stop puromycin selection. On day four, the media was changed to Neurobasal media (Gibco) supplemented with B27 (Gibco) and GlutaMAX (Invitrogen). Media was changed every 24 hours thereafter.

Construction of PKIβ expression vectors

The sequence resulting in the 78 amino acid isoform of PKIβ was PCR amplified from a PKIβ 7.1 plasmid (previously described in Scarpetta and Uhler, 1993) using the primer pair shown in Table 2.1. The resulting PCR fragment was subcloned into the pGEM-T Easy vector system (Promega). The DNA was *EcoRI/XbaI* digested and then subcloned into *EcoRI/XbaI* digested US2 vector downstream of the ubiquitin promoter. This plasmid was further modified such that base pair complementation was not possible with the shRNA we found to be most effective at knocking down PKIβ expression, but the final amino acid sequence of the protein remained the same. Silent mutations within the shRNA target sequence were introduced via PCR using the primer pairs found in Table 2.1. Briefly, the first round of PCR generated a 5' mutant fragment and a 3' mutant fragment that had 24 overlapping nucleotides. These were then used as templates for the second round of PCR using the outer primer pairs indicated in Table 2.1. The amplified

fragment was *EcoRI/XbaI* digested and subcloned into *EcoRI/XbaI* digested US2 to create the US2-PKIβ expression vector.

In order to create the null mutant pUS2- PKIβ DNA construct, four amino acid residues (Phe^{18}, Arg^{23}, Arg^{26}, Arg^{27}) were mutated to alanine residues using the mutagenic oligonucleotide primers shown in Table 2.1. Mutations were introduced via PCR as described for the US2-PKIβ expression vector. The full-length mutated PCR product was *EcoRI/XbaI* digested and subcloned into *EcoRI/XbaI* digested US2 to create the US2-PKIβnull expression vector. All of the PKIβ constructs were sequenced to ensure that only the intended mutations were introduced. All oligonucleotides were synthesized by Invitrogen.

RNA isolation and RT-PCR analysis

Total RNA was extracted from P19 cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Single stranded cDNA was synthesized from 2 μg of total RNA using SuperScript II Reverse Transcriptase and random hexamers (Invitrogen). Gene expression was evaluated by quantitative real-time PCR (RT-PCR) using the SYBR Green PCR Master Mix (Applied Biosystems) and the MyiQ single-color real-time PCR detection system (Bio-Rad) according to manufacturer's instructions. The specificity of the PCR amplification procedures was checked with a heat dissociation protocol (from 72°C to 98°C) after the final cycle of the PCR. Each reaction was done in triplicate. Expression levels were calculated using the delta-delta CT method, with GAPDH as the normalization control. The primer sequences used to amplify target genes can be found in Table 2.1. A paired student's *t*-test was performed to compare the two

groups, with data presented as means ± standard deviation with the significance level set at $p < 0.05$.

Dual luciferase reporter assay

Dual luciferase assays were performed with the Dual Luciferase Reporter Assay kit (Promega) following the recommended protocols. Samples were read on a FLUOstar OPTIMA microplate reader (BMG Labtech). To correct for differences in transfection efficiencies, firefly luciferase activity was normalized to that of renilla luciferase (US2-RL). The US2-RL plasmid construction has been previously described (Huang et al., 2010). Experiments were repeated a minimum of three times, and results were expressed as mean ± standard deviation. The statistical significance of transactivation data was determined using a student's paired *t*-test with the significance level set at $p < 0.05$.

Catalytic activity assays

The PepTag assays were performed according to the manufacturer's instructions. This assay utilizes the Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) peptide substrate tagged with a fluorescent dye. Upon phosphorylation, the net charge of this peptide changes from +1 to -1, which subsequently alters its migration when run on an agarose gel. Briefly, lysed cell extract expressing PKIβ or PKIβnull proteins was incubated with the tagged Kemptide substrate and activator buffers at 30°C, and the reaction was run on a 1% agarose gel. Active protein was detected by its substrate (Kemptide) migrating towards the anode. Quantitative assay of kinase activity was based on density measurements of the bands using ImageJ software (http://rsb.info.nih.gov/ij) from three

independent experiments. The statistical significance of differences in kinase activity was determined using a student's paired *t*-test with the significance level set at $p < 0.05$.

SDS-PAGE and western blot analysis

Cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Hyclone) and lysed in buffer containing 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 10 mM sodium fluoride, complete EDTA-free protease inhibitors (Roche), and 1 mM PMSF. Lysates were sonicated, and protein concentrations were determined by the bicinchonic acid protein assay (Bio-Rad). Equal amounts of total protein were denatured at 95°C in the presence of SDS, DTT, and β-mercaptoethanol. Samples were resolved on 15% Tris-HCl gels and transferred onto a 0.2 μm nitrocellulose membrane (Whatman). Membranes were blocked for 2 h in PBS supplemented with 5% non-fat dried milk, 2% polyvinylpyrrolidone (PVP-40), and 0.1% Triton X-100 and subsequently incubated in primary antibody diluted in PBS supplemented with 0.5% bovine serum albumin and 0.1% Triton X-100 overnight at 4°C. Membranes were washed three times for 10 min with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20), and then incubated with a 1:2,000 dilution of HRP-conjugated secondary antibody in TBST supplemented with 5% non-fat dried milk. Following the final set of three 10 min washes with TBST, the blots were developed using Lumi-Light Western Blotting Substrate (Roche) according to the manufacturer's instructions. Quantitative assay of antigen expression was based on density measurements of protein bands using ImageJ software.

Rapid amplification of cDNA ends (RACE)

The 5'-cDNA ends were obtained with the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc). For 5'-RACE, 1 μg total RNA from the 36 hour time point (see Figure 2.4) was reverse transcribed with the 5'-RACE CDS Primer and SMART II A Oligonucleotide (provided). Three gene-specific primers for PKIβ were designed based on the sequence reported in the NCBI database and can be found in Table 2.1. 5'-RACE PCR was performed with either PKIβ 3.1, 3.2, or 3.3 along with Universal Primer A Mix according to the SMART™ RACE cDNA Amplification Kit user manual. Negative controls containing only the UPM or only gene-specific primers were also performed. The amplified cDNA products were isolated, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Immunocytochemistry

Cells were washed once in DPBS and then fixed in 4% formaldehyde in PBS for 20 min. Cells were washed three times in PBS, and then blocked for 1 h in PBS supplemented with 2% goat serum and 0.1% Triton X-100. Cells were probed with primary antibodies diluted in blocking solution for 2 h at 23°C. After washing in PBS cells were incubated with AlexaFluor conjugated secondary antibodies for 1 h at 23°C, followed by three PBS washes. For nuclear counterstaining, the cells were incubated in 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) for 10 min before being washed twice in PBS and visualized. To collect still images we used an inverted Olympus IX70 fluorescence microscope using an Illix CCD imaging system and Micro Computer Image Device software (Imaging Research Inc.). The percent of cells

differentiated under each condition was calculated using the ratio of differentiated cells to the total number of cells (visualized by DAPI staining). Cells were qualified as differentiated if the processes were three times the length of the cell body. The statistical significance between groups was determined using a student's paired *t*-test with the significance level set at $p < 0.05$.

Results

Characterization of the PKA-CREB pathway in undifferentiated P19 cells

Previous studies to assess cAMP-inducible transcription with the F9 embryonic carcinoma cells showed they were refractory to cAMP, and only become cAMP-responsive following RA-induced differentiation (Strickland and Mahdavi, 1978; Strickland et al., 1980). Evidence suggested that CREB played a pivotal role in determining cAMP inducibility of genes in F9 cells: expression of exogenous CREB allowed undifferentiated F9 cells to activate the somatostatin promoter in response to PKA, and was dependent on both the somtatostatin cAMP response element (CRE) and the PKA phosphoacceptor site of CREB (Gonzalez and Montminy, 1989; Montminy et al., 1990). The lower levels of PKA activity in undifferentiated F9 cells were not due to the absence of known positive-acting factors such as the catalytic subunit of PKA or CREB (Plet et al., 1982). This suggests the presence of negative regulatory factors that repress cAMP induction in at least some embryonic carcinoma cell lines.

P19 embryonic carcinoma cells have been widely used to study neuronal differentiation. To determine whether P19 cells exhibit characteristics similar to F9 cells

in the activity level of the PKA-CREB pathway, we used a membrane-permeable analog of cAMP (8-CPT-cAMP) to carry out a PKA activation time course in P19 cells. Cells were treated for varying lengths of time with cAMP and then subjected to western blot analysis. Similar levels of total CREB and PKA catalytic subunit (Cα) protein were observed at all time points (Figure 2.1A). Basal levels of pCREB were close to undetectable, but levels increased after very short exposure to cAMP (15 min) and remained elevated until 2 h, after which levels declined. The kinetics of CREB phosphorylation is typically transient in nature, peaking at approximately 30 minutes post-stimulation and subsequently diminishing to basal levels after 3-4 hours following dephosphorylation of Ser^{133} by the protein phosphatases PP-1 and/or PP-2A (Hagiwara et al., 1992; Wadzinski et al., 1993).

We also examined the ability of P19 cells to respond to exogenous PKA. Functional PKA signaling in HEKT cells has been extensively studied, and as such we utilized this cell line as a positive control for activity (Roche et al., 1996; Chow and Wang, 1998; Papadopoulou et al., 2004). P19 and HEKT cells were transfected with exogenous, wild-type Cα and with a mutant form of Cα (K72M), which eliminates the catalytic activity of the kinase (Brown et al., 1990; Huggenvik et al., 1991). As expected, western blot analysis again shows comparable levels of CREB between HEKT and P19 cells, and higher basal levels of pCREB in HEKT cells transfected with the empty parental vector (Figure 2.1B). In both cell lines, the levels of pCREB protein increases when cells are transfected with exogenous Cα. This increase is PKA activity-dependent, since no increase in pCREB was seen in cells transfected with the inactive Cα protein (K72M). An antibody against the catalytic subunit of PKA detected both the exogenous

Cα and CαK72M, the latter of which has previously been reported to migrate faster than its wild-type counterpart, most likely due to a lack of autophosphorylation (Iyer et al., 2005).

Finally, we characterized the transcriptional response of P19 cells to both cAMP treatment and exogenous Cα by utilizing a CRE-containing reporter vector composed of the human chorionic gonadotropin (HCG) promoter driving expression of firefly luciferase. The promoter of the HCG gene has been extensively used for reporter analysis, and the proximal 180 bp of the promoter contains two adjoining CREs that mediate basal and cAMP-stimulated transcription (Delegeane et al., 1987; Jameson et al., 1989; Mellon et al., 1989; Pittman et al., 1994). Since the major transcription factor that binds to these CREs is CREB, and because CREB is a substrate for phosphorylation by PKA, this CRE-luciferase reporter is an effective means of quantitating PKA activity (Mayr and Montminy, 2001).

P19 cells exhibited a 14-fold increase in CRE-luciferase activity in the presence of exogenous Cα, and a 17-fold increase in response to cAMP treatment (Figure 2.1C). HEKT cells exhibited a 38-fold increase in CRE-luciferase activity in the presence of exogenous Cα, and a 23-fold increase in response to cAMP treatment. This is in agreement with the results shown in Figure 2.1A, suggesting that PKA-regulated transcription via CREB is functional in P19 cells. In both cell lines, transfection with CαK72M did not significantly induce CRE-luciferase activity. Together, these data show that P19 cells are a tractable system for studying cAMP-mediated effects, and suggest that PKA-CREB phosphorylation in P19 cells is similar to many other cell types but distinct from that reported for F9 embryonic carcinoma cells.

Characterization of the PKA-CREB pathway in differentiating P19 cells

Transfection of proneural bHLH proteins such as Ascl1 has previously been shown to be sufficient to convert P19 cells into a relatively homogenous population of electrophysiologically differentiated neurons (Farah et al., 2000; Vojtek et al., 2003; Huang et al., 2010). P19 cells were transiently transfected with either an empty plasmid expression vector (pUS2) or an expression vector for Ascl1 (pUS2-Ascl1). After 120 hours following transfection, a significant percentage of cells had adopted a neuronal morphology with round cell bodies and one or more long processes (Figure 2.2A). Neuron-specific class III β-tubulin (recognized by the TuJ1 monoclonal antibody) is widely accepted as a neuronal marker, and immunocytochemistry showed a high percentage (> 30%) of cells that had TuJ1-immunoreactive processes 5 days after transfection with Ascl1. In contrast, cells transfected with only pUS2 maintained the morphology of undifferentiated P19 cells.

To study changes in PKA activity as P19 cells differentiate into neurons, we again treated cells with cAMP, and employed an expression vector encoding the vasodilator-stimulated phosphoprotein (VASP) with a Flag epitope tag at the N-terminus of the protein. VASP harbors three phosphorylation sites: serine 157 (S157), serine 239 (S239), and threonine 278 (T278) (Butt et al., 1994; Gertler et al., 1996; Lambrechts et al., 2000; Blume et al., 2007). The first and second sites in VASP are phosphorylated by PKA both *in vitro* and *in vivo*, and as such we chose this protein as a measure of PKA activity. Furthermore, phosphorylation on S157 causes a shift in mobility from 46 to 50 kDa that is easily resolved on a SDS-PAGE gel (Reinhard et al., 1992; Butt et al., 1994;

Haffner et al., 1995; Collins and Uhler, 1999).

In undifferentiated P19 cells, the majority of VASP is in its unphosphorylated form (Figure 2.2B). A slight increase in phosphorylated VASP is seen 24 h after transfection with Ascl1. After 48 h of transfection with Ascl1, a significant decrease in the ratio of phosphorylated:unphosphorylated VASP was observed, and by 72 h VASP existed entirely in its lower molecular weight, unphosphorylated form. Levels of phosphorylated CREB mirrored those of the VASP protein: an increase in pCREB was observed 24 hours after transfection with Ascl1, but pCREB was largely absent at 48 and 72 h. Levels of total CREB protein were comparable between samples. This decline is not due to a reduction in PKA protein, as levels of catalytic subunit remain comparable across all time points. Treatment of undifferentiated P19 cells with cAMP caused an increase in pCREB levels, as expected based on our results shown in Figure 2.1. Because both VASP and CREB are direct phosphorylation targets of PKA, these results suggest the presence of a negative regulatory factor that is inhibiting the activity of PKA at the later time points. However, this inhibition appears to be unaffected by treatment with cAMP since treatment of differentiating cells with cAMP resulted in high levels of phosphorylation of both VASP and CREB at all time points.

Regulation of PKA during neuronal differentiation

Preliminary microarray hybridization studies of genes induced after transient transfection of P19 cells with Ascl1 showed a significant, but transient, increase in expression of all three PKI isoforms (data not shown, personal communication, Dr. David L. Turner, University of Michigan). Since the PKI proteins are important physiological

regulators of PKA-mediated phosphorylation events, we pursued the possibility that PKIs could be responsible for the reduction in PKA activity observed at later time points during the Ascl1-induced differentiation process. To confirm the microarray findings, we verified this change in gene expression using RT-PCR (Figure 2.3A). In concordance with the microarray data, PKIα and PKIγ were induced after forced expression of Ascl1. Maximal (18-fold) induction of PKIα was seen at 120 h, while maximal (6-fold) PKIγ induction was observed at 36 and 48 h. To determine whether either of these isoforms is required for neuronal differentiation, we employed shRNA vectors targeting the specific PKI isoforms. Five different shRNA constructs, each targeting a different region of the transcript, were tested for PKIα and PKIγ (Figure 2.3B). Whereas a number of these shRNAs were found to significantly reduce mRNA transcript levels, we were unable to detect a phenotypic effect on neuronal differentiation since these cells differentiated normally (Figure 2.3C).

PKIβ was the most highly induced isoform in our microarray hybridization analyses, and quantitative RT-PCR confirmed an increase in the relative expression of PKIβ transcript from 1.6 at 0 h to 4,000 at 36 h, resulting in a 2,500-fold increase after the overexpression of Ascl1 (Figure 2.4A). This increase in PKIβ transcript level closely matches the time at which a decrease in levels of pVASP and pCREB is observed (see Figure 2.2B). Western blot analysis using a PKIβ antibody shows that the expression of the PKIβ protein also transiently increases during the Ascl1-induced differentiation process. The PKIβ protein is first detectable at 36 h, which corresponds to the peak mRNA expression in the RT-PCR results, and then declines. The PKIβ immunoreactivity on the Western blots appears at the expected molecular weight of 15.5 kDa (Scarpetta

and Uhler, 1993). The peak induction of PKIβ protein comes after that of the Ascl1 protein, with Ascl1 expression peaking at 24 h and becoming undetectable by 120 h. This expression pattern is in accordance with previous data showing that Ascl1 is transiently expressed in proliferating neural precursors; the protein appears before overt neuronal differentiation and disappears as markers of the mature neuronal phenotype are expressed (Lo et al., 1991; Casarosa et al., 1999). The relative intensity of PKIβ or Ascl1 protein to GAPDH protein is shown underneath each blot. These combined results show that both PKIβ mRNA and protein levels increase during Ascl1-induced neuronal differentiation of P19 cells

PKIβ is necessary for Ascl1-induced neuronal differentiation

Once the expression pattern for PKIβ mRNA and protein levels was defined, we used specific shRNA vectors to knockdown the expression of PKIβ to determine whether its expression is required for the neuronal differentiation of P19 cells. Five different shRNAs were assayed for efficacy of knockdown of the PKIβ gene at 36 hours, the time point where mRNA expression peaks (Figure 2.5A). Compared to the positive control (PC; cells transfected with Ascl1), all five shRNAs significantly reduced PKIβ mRNA transcript levels ($p < 0.05$). However, shRNA1 did not decrease PKIβ expression as much as shRNA4 (which showed the greatest fold-reduction in PKIβ expression). To determine whether knocking down PKIβ results in changes in neuronal differentiation, we co-transfected P19 cells with Ascl1 and either the shRNA1 or shRNA4 constructs, and then used immunocytochemistry to assay for differences in neuronal differentiation 120 hours post-transfection (see Figure 2.2A).

Cells transfected with shRNA1 showed fewer TuJ1-positive cells than the positive control (Figure 2.5B), but the difference in percentage of cells differentiated was not found to be statistically significant (Figure 2.5C). Transfection with shRNA4, however, significantly ($p < 0.01$) reduced the percentage of differentiated cells. Western blot analysis of cell lysates using an antibody against microtubule-associated protein 2 (Map2; another commonly used marker of neuronal differentiation) supported our immunocytochemistry findings (Figure 2.5D): as expected, a substantial increase in Map2 expression is observed in cells transfected with Ascl1. A slight decrease in Map2 expression is observed in cells co-transfected with shRNA1, while a dramatic decrease is seen in cells co-transfected with shRNA4. The levels of Map2 protein in cells transfected with shRNA4 are comparable to those of the negative control. These results show that not only is the shRNA4 construct the most effective at knocking down PKIβ mRNA and protein expression, but it also perturbs Ascl1-induced neuronal differentiation in P19 cells.

Characterization of PKIβ in P19 cells

Since the PKIβ transcript is known to undergo significant alternative splicing (Scarpetta and Uhler, 1993; Kumar and Walsh, 2002), RACE amplification was used to characterize the PKIβ transcript in differentiating P19 cells. Using three different reverse primers (see Table 2.1) one major transcript was successfully amplified (Figure 2.6A) which was subsequently isolated, cloned, and sequenced. A representative sequence from one amplified DNA clone is shown in Figure 2.6B with the starting sequence of each exon underlined. The predicted exon organization of the mouse PKIβ gene includes

exons 1, 5, 6, 7, 9 and 10. RACE amplification results show that in P19 cells, exon 7 is absent (Figure 2.6C). Open boxes represent non-coding regions, while the closed box represents the coding region. Exon 7 does not contain any elements crucial to the inherent activity of PKI, but it does include the region that makes it a highly potent inhibitor of cGMP-dependent protein kinase (PKG) (Kumar and Walsh, 2002).

Rescue of PKIβ during neuronal differentiation

If the changes in neuronal differentiation observed in Figure 2.5 are dependent on PKIβ protein expression, the effect should be rescued by introducing exogenous PKIβ protein. However, since the shRNA4 construct targets a sequence within the PKIβ coding region (see Figure 2.6C), we took advantage of the redundancy of the genetic code and created a PKIβ coding variant where the nucleotide sequence was altered to impede binding of shRNA4, but still produced the wild-type protein (Figure 2.7A). PKIs are competitive inhibitors of the catalytic subunit of PKA, and contain an autoinhibitor sequence Arg-Arg-Asn-Ala that serves to prevent phosphorylation. Studies on the PKIα isoform shows that the autoinhibitor sequence is important for inhibition, as substitutions of Arg^{18} and Arg^{19} significantly reduced PKI potency (Scott et al., 1986). Other extra-autoinhibitory sequence residues in PKIα are also important for the high potency inhibition of PKA: two residues outside the pseudosubstrate sequence that contribute significantly to PKI interactions with the catalytic subunit of PKA are Arg^{15} and Phe^{10} (Glass et al., 1989; Baude et al., 1994). The substitution of both residues leads to dramatic decreases in the efficacy of PKIα. The amino acids important for full inhibitory potency are conserved between PKIα and PKIβ, so we examined whether the residues

important in PKIα function are also critical for PKIβ inhibition of PKA. We introduced mutations in the corresponding four conserved amino acids (Phe^{18}, Arg^{23}, Arg^{26}, and Arg^{27}) in the PKIβ coding variant sequence (Figure 2.7A). All four residues were mutated to alanines. A PKA kinase activity assay was used to determine whether these proteins were functionally active (PKIβ) or inactive (PKIβnull). Based on densitometry and the coupled kinase assay, exogenous PKIβ reduced kinase activity 7-fold, while the PKIβnull mutant failed to inhibit the activity of PKA (Figure 2.7B).

Transfection of P19 cells with the exogenous wild-type PKIβ construct partially restores the ability of P19 cells to differentiate in response to Ascl1 in the presence of shRNA4: immunohistochemistry shows increased number of Map2-positive cells with extended neurites (Figure 2.8A). When P19 cells were co-transfected with the functionally null PKIβ construct, we did not see a rescue effect as evidenced by the lack of Map2-positive projections. Quantitation of the percentage of cells differentiated under each condition showed that 68% of the cells differentiated 120 h post-transfection with Ascl1 (Figure 2.8B). Co-transfection with shRNA4 reduced the percentage of differentiated cells to 1.6% of the total population ($p < 0.01$). When exogenous PKIβ was introduced, differentiation was restored with 53% of the cells expressing Map2. This rescue requires the residues previously shown to be important for PKIα inhibition of PKA, because the null PKIβ mutant did not rescue the differentiation, as evidenced by significantly fewer differentiated cells compared to the positive control ($p < 0.01$). Western blot analysis also showed increased Map2 protein levels in cells co-transfected with the exogenous PKIβ (Figure 2.8B). However, when cells were co-transfected with the null PKIβ expression vector, no significant difference in Map2 protein levels was

observed between this condition and cells transfected with shRNA4. Together, these data suggest that mutated residues critical for PKIβ inhibition of PKA are also critical for PKIβ-mediated neuronal differentiation downstream of Ascl1.

Discussion

Examining the PKA-CREB signaling pathway in P19 cells showed that 8-CPT-cAMP was capable of activating the PKA pathway, as evidenced by increased levels of pCREB and pVASP. Exogenous Cα produced similar levels of activation (Figure 2.1). In response to Ascl1-induced neuronal differentiation, P19 cells exhibited a sharp decrease in PKA activity, but are still responsive to cAMP treatment (Figure 2.2). While a large body of research has defined the CREB protein family as the principle mediators of positive changes in gene expression in response to cAMP following phosphorylation by PKA, recent observations of cAMP-mediated induction of specific genes occurring via PKA-independent mechanisms have challenged this dogma of the PKA-CREB pathway. For example, a family of CREB co-activators—the transducers of regulated CREB activity (TORCs)—can bind to and activate CREB independently of PKA-mediated phosphorylation, and have furthermore been shown to translocate from the cytoplasm to the nucleus in response to cAMP elevation (Bittinger et al., 2004; Screaton et al., 2004). To date, several cAMP-responsive neuronal genes with diverse functions have been shown to be regulated in a PKA-independent manner. Examples include glial fibrillary acidic protein (Anciaux et al., 1997), neuronal nitric oxide synthase (Boissel et al., 2004), and cyclin D1 (Datta et al., 2005). Given the diverse range of important biological

responses regulated by cAMP that cannot be explained by PKA, it is possible that an unknown pathway is responsible for the increase in pCREB and pVASP levels seen in response to cAMP stimulation.

Since PKIs are major inhibitory regulators of PKA—and because microarray hybridization studies detected transient increases in PKI expression during neuronal differentiation—we hypothesized that PKIs could be responsible for the observed inhibition of PKA activity during Ascl1-mediated differentiation. The PKIα and PKIγ isoforms were induced in response to Ascl1, but to a lesser extent than PKIβ. Furthermore, shRNA-mediated knockdown of PKIα and PKIγ did not produce significant changes in the extent of neuronal differentiation (Figure 2.3-5). However, we found that progression of neuronal differentiation in P19 cells is dependent on PKIβ, and that an alternative splice variant of PKIβ exists in P19 cells (Figure 2.6). The effects produced by knocking down PKIβ mRNA and protein expression are rescued via transient transfection with exogenous PKIβ protein, and this rescue is dependent on four amino acid residues critical for binding to the catalytic subunit of PKA (Figure 2.7-8). These results suggest that binding to and inhibition of the catalytic subunit of PKA is required during the Ascl1-induced neuronal differentiation of P19 cells.

The balance of proliferation with cell cycle withdrawal is fundamental to the normal generation of the wide array of distinct cell types that comprise the mature vertebrate CNS, and both bHLH transcription factors and the cAMP-PKA pathway are good candidates for mechanisms of cell cycle control. bHLH transcription factors are known to play a role in the cell cycle exit that precedes differentiation: transfection of proneural bHLH proteins promote cell cycle withdrawal driven by increased $p21^{cip1}$

expression in HeLa cells, and $p27^{kip1}$ expression in P19 cells (Mutoh et al., 1998; Farah et al., 2000). However, the direct activities of bHLH factors alone are not likely to be sufficient for precise cell cycle exit in proliferating neural progenitors at early neurogenic stages, suggesting the necessity of another regulatory mechanism that links directly to the cell cycle machinery and functions to precisely control cell cycle withdrawal.

cAMP and PKA levels are known to fluctuate throughout the cell cycle in mammalian cells and mouse embryos, and both are critical in phenotypic specification and transition in the adult and developing nervous system (Matyakhina et al., 2002; Chen et al., 2005; Shin et al., 2009). The involvement of PKA in the maintenance of meiotic arrest is widely accepted: classical studies using *Xenopus* oocytes have shown that injection of PKI or the regulatory subunits of PKA causes resumption of meiosis, while forced expression of the catalytic subunit of PKA prevents it (Maller and Krebs, 1977; Huchon et al., 1981; Bornslaeger et al., 1986). Studies across species, however, have shown that the role of PKA in cell cycle regulation varies depending on the system used: activation of PKA results in the proliferation of certain cell types, but induces cell cycle arrest and apoptosis in others (Desdouets et al., 1995; Roger et al., 1995; Thompson et al., 1999; Yan et al., 2000). These discrepancies likely stem from the fact that PKA substrates that are phosphorylated/dephosphorylated during regulation of the cell cycle are still being elucidated. Despite these incongruities, it is clear that tight regulation of PKA activation is extremely important for proper cell cycle exit, an event that is necessary for progenitor cells to begin differentiating into neurons.

The composition and specific biochemical properties of PKA holoenzymes partly accounts for differential cellular responses to discrete extracellular signals. The

characteristics of PKA are largely determined by the structure and properties of its regulatory subunits, since the catalytic subunit isoforms show common kinetic features and substrate specificity (Edelman et al., 1987; Taylor et al., 1992). Four regulatory (R) subunits have been identified—RIα, RIβ, RIIα, and RIIβ—that are differentially distributed in mammalian tissues, and exhibit distinct regulation and biochemical properties. The binding affinity of cAMP for the RIIβ isoform *in vivo* is much lower relative to RIα and RIIα (Edelman et al., 1987; Taylor et al., 1992), suggesting that regulatory subunit isoforms can decode cAMP signals that differ in duration and intensity. For example, neurons and endocrine cells express predominantly RIIβ, and are adapted to persistent high concentrations of cAMP (Stein et al., 1987).

In addition to regulatory subunits, the specificity of PKI for the catalytic subunits of PKA makes them very good candidates for modulating the activity of PKA. In addition to reducing PKA activity by competitively binding to the catalytic subunits of PKA, PKI proteins also contribute to reducing PKA activity by trafficking the catalytic subunit between subcellular locations (Fantozzi et al., 1994). Once bound to PKA, it is generally accepted that PKIs undergo a conformational change that exposes a leucine-rich nuclear export signal, which shuttles the catalytic subunit back to the cytoplasm where it can reform an inactive tetramer with the regulatory subunits. However, in a study examining the effects of PKI on the subcellular localization of the catalytic subunit of PKA, Wiley et al. (1999) found that while PKI was capable of redistributing nuclear catalytic subunit to the cytoplasm and blocking subsequent gene induction, increasing concentrations of PKI resulted in saturation of the export process and the reappearance of the catalytic subunit of PKA in the nucleus. These data suggested that free PKI may

interact with components of the export machinery itself in the absence of catalytic subunit. Therefore, overexpression of PKI may interfere with the nuclear export machinery and decrease its ability to transport the PKI/catalytic subunit complex out of the nucleus. This contrasts with previous reports suggesting that the nuclear export signal on PKI is masked and only becomes available when the catalytic subunit binds to PKI (Wen and Taylor, 1994; Wen et al., 1994; Wen et al., 1995).

By facilitating the nuclear export of PKA, PKI proteins can affect the kinetics and extent of PKA activity in the nucleus and rapidly reset the PKA system for subsequent gene induction responses necessary for neuronal differentiation. Additionally, the isoform-specific expression of PKIβ during Ascl1-induced neuronal differentiation may provide for a distinct threshold of activation for PKA. Because PKIβ is such a potent, specific inhibitor of PKA, changes in its expression could profoundly affect the duration over which the PKA activation threshold is breached. Furthermore, depending on which isoforms of PKI are expressed in cells, the physiological consequences concomitant upon modulating PKA activity could be very different. These distinctions are determined by the regulatory properties of the PKI isoforms and like PKA, their activities are likely to be influenced by their position within the cell.

Three PKI protein isoforms—PKIα, PKIβ, and PKIγ—are produced from three different genes that have widespread but distinctive tissue distributions (Collins and Uhler, 1997; Zheng et al., 2000). Mice deficient in PKIα exhibited defects in skeletal muscle, but showed no defect in development or fertility (Gangolli et al., 2000). PKIβ-deficient mice exhibited a partial loss of PKI activity in testis, but remained fertile with normal testis development and function (Gangolli et al., 2001). Remarkably, PKIα/β

double-knockout mice were also viable and fertile with no obvious physiological defects, presumably due to compensation by PKIγ (Belyamani et al., 2001).

Studies indicate that multiple forms of PKIβ exist, related by covalent modification and alternate translational initiation (Van Patten et al., 1991; Van Patten et al., 1997; Zheng et al., 2000; Kumar and Walsh, 2002). PKIβ was first isolated from rat testis as a 70 amino acid protein, but the genomic sequence suggested that an alternate form might exist, arising as a consequence of alternate translational initiation. This species, now termed PKIβ78, is equipotent with PKIβ70, and also occurs *in vivo*. Six additional species of PKIβ are also evident in tissues: two of these represent the phospho forms of PKIβ78 and PKIβ70, while the other four represent phospho and dephospho forms of two higher molecular mass PKIβ species. These latter forms are currently termed PKIβ109 and PKIβY, and their molecular identities have yet to be fully deciphered (Kumar et al., 1997). Our data indicate that the form expressed in P19 cells corresponds to the 78 amino acid isoform of PKIβ, whose predicted molecular weight is at 15.5 kDa and is expressed in the brain (Kumar et al., 1997). Furthermore, the gene organization of PKIβ elucidated from our RACE studies indicates that PKIβ78 is a specific inhibitor of PKA. Other isoforms of PKIβ exist that are dual-specificity inhibitors of both PKA and PKG, but the sequences required for PKG inhibition are located in exon 7, a region that is absent in the cDNA of PKIβ in P19 cells. A homologous gene to mouse PKIβ has been identified in humans (*PKIB*). Human *PKIB* shares a 70% homology to mouse PKIβ, most notably within the sequences for the pseudosubstrate site and nuclear export signal. In humans, PKIB is the predominant isoform expressed in the brain, and the *PKIB* cDNA encodes for a peptide of 78 amino

acids (Zheng et al., 2000). Because of the sequence homology between human and mouse PKIβ and similar regions of tissue expression pattern, it seems plausible that the critical role of PKIβ in neuronal differentiation of murine cells *in vitro* may carry over to *in vitro* differentiation of human progenitor cells.

Both PKA and the PKI proteins are widely distributed throughout the body, and several tissues express multiple isoforms of PKI, each of which has a distinct inhibitory potency for PKA. The overlapping expression of these proteins suggests that isoforms of PKI may have undiscovered roles that are necessary for normal function in a variety of tissues, including the brain. Our research utilizes a novel cell line model for studying the physiological roles of PKIs in neuronal differentiation. Our findings provide novel insight about the roles of PKIβ in the complex molecular network that regulates neurogenesis, showing an essential role for inhibition of PKA by PKIβ in the Ascl1-induced neuronal differentiation of P19 cells.

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Figure 2.1. Activity of the cAMP/PKA signaling pathway in P19 cells. A. P19 cells were treated with 200 μM 8-CPT-cAMP for the indicated lengths of time. Untreated cells (0 min) were also included as negative controls. Western blotting shows an induction of pCREB in response to cAMP treatment in P19 cells. **B.** P19 and HEKT cells were transfected with the indicated DNAs for 24 hours. Western blot analysis showed an increase in pCREB levels in both P19 and HEKT cells that was abolished upon mutation of a critical lysine residue. **C.** Transcriptional activity of a CRE-luciferase reporter in response to Cα and cAMP. In both cell lines, co-transfection with Cα or cAMP treatment yielded an increase in the relative luciferase activity of the CRE-luciferase reporter.

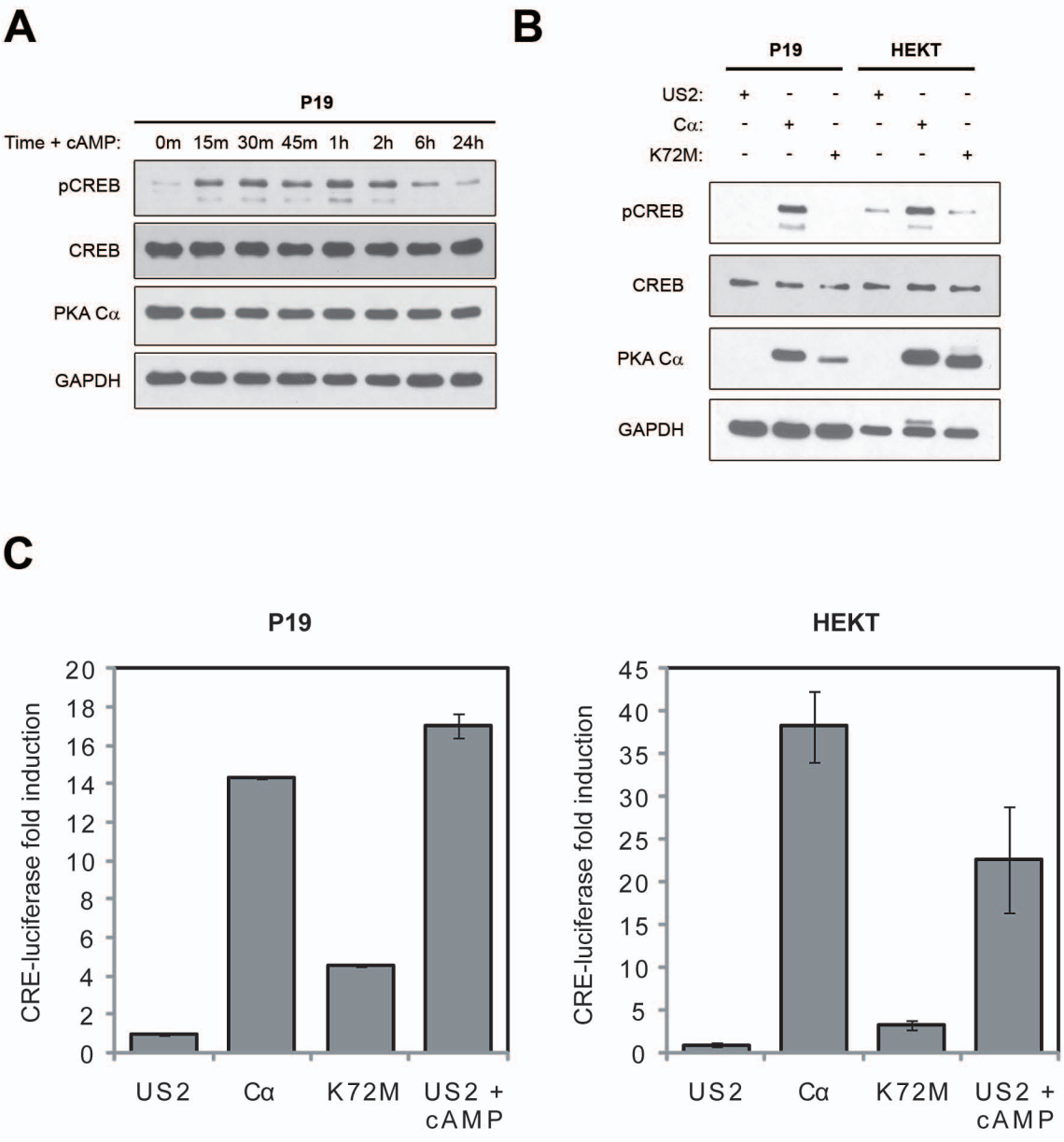

A
P19
Time + cAMP:
0m 15m 30m 45m 1h 2h 6h 24h
pCREB
CREB
PKA Cα
GAPDH
B
P19
HEKT
US2:
Cα:
K72M:
pCREB
CREB
PKA Cα
GAPDH
C
P19
HEKT
CRE-luciferase fold induction
US2
Cα
K72M
US2 + cAMP

Figure 2.2. PKA activity changes during neuronal differentiation of P19 cells. A. TuJ1 staining (red) of P19 cells transiently transfected with US2 or US2-Ascl1 120 h post-transfection. Nuclei were visualized with DAPI and appear blue. In the absence of Ascl1, no TuJ1-positive processes were observed. In the presence of Ascl1, TuJ1-positive cells were evident that had a distinct neuronal morphology. Scale bar = 100 μm. **B.** Western blot for changes in PKA activity in response to Ascl1-induced neuronal differentiation revealed a significant decrease in PKA activity at 48 h as evidenced by a decrease in pVASP and pCREB expression. However, cells remained responsive to cAMP treatment as illustrated by subsequent increases in VASP and CREB phosphorylation.

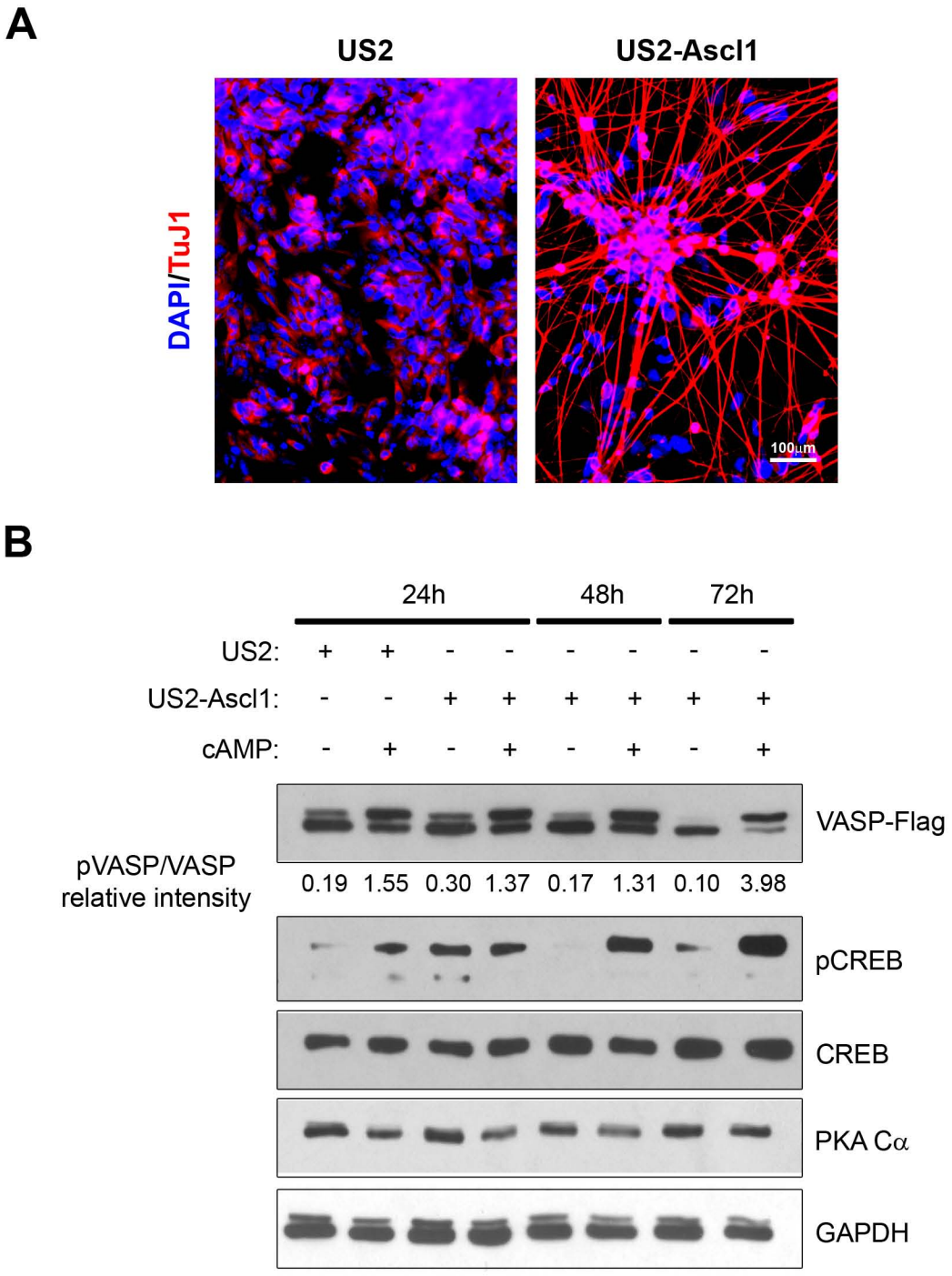

A
US2
US2-Ascl1
DAPI/TuJ1
100μm
B
24h
48h
72h
US2: + + - - - - - -
US2-Ascl1: - - + + + + + +
cAMP: - + - + - + - +
pVASP/VASP
relative intensity
0.19 1.55 0.30 1.37 0.17 1.31 0.10 3.98
VASP-Flag
pCREB
CREB
PKA Cα
GAPDH

Figure 2.3. PKIα and PKIγ are not required for Ascl1-induced neuronal differentiation. A. RT-PCR analysis of PKIα and PKIγ gene expression over a time course of Ascl1-induced neuronal differentiation. P19 cells undergo a transient increase in PKIα and PKIγ mRNA expression. Results are shown as the mean ± S.D. normalized to GAPDH levels. **B.** RT-PCR analysis of shRNA constructs for efficacy of knockdown for each gene. Negative controls (NC) are cells transfected with the empty US2 vector. Positive controls (PC) are cells transfected with Ascl1. *$p < 0.05$. **C.** Representative images showing that P19 cells differentiate normally even when PKIα or PKIγ are knocked down. TuJ1 staining is shown in red, and DAPI-stained nuclei appear blue. Scale bar = 100 μm.

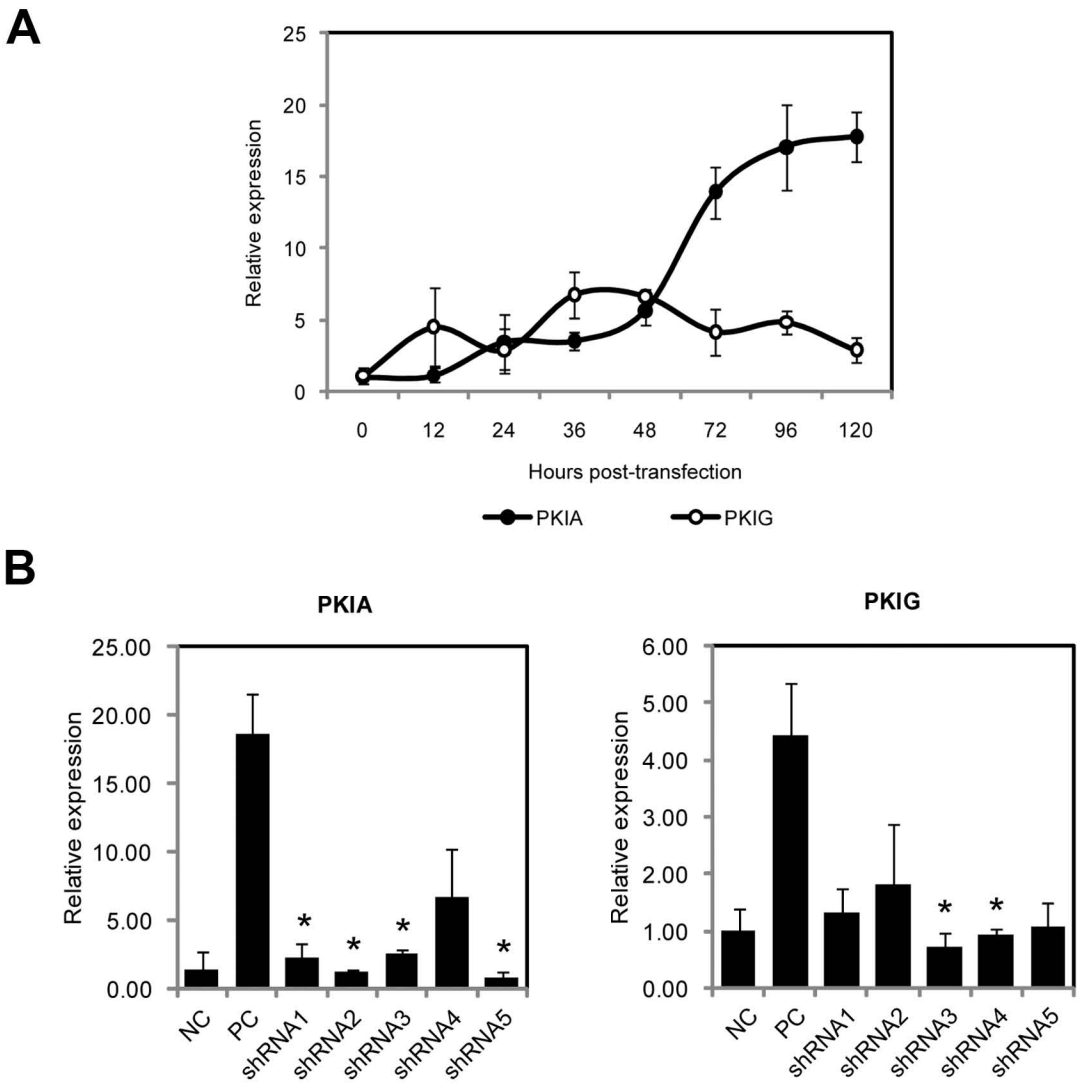
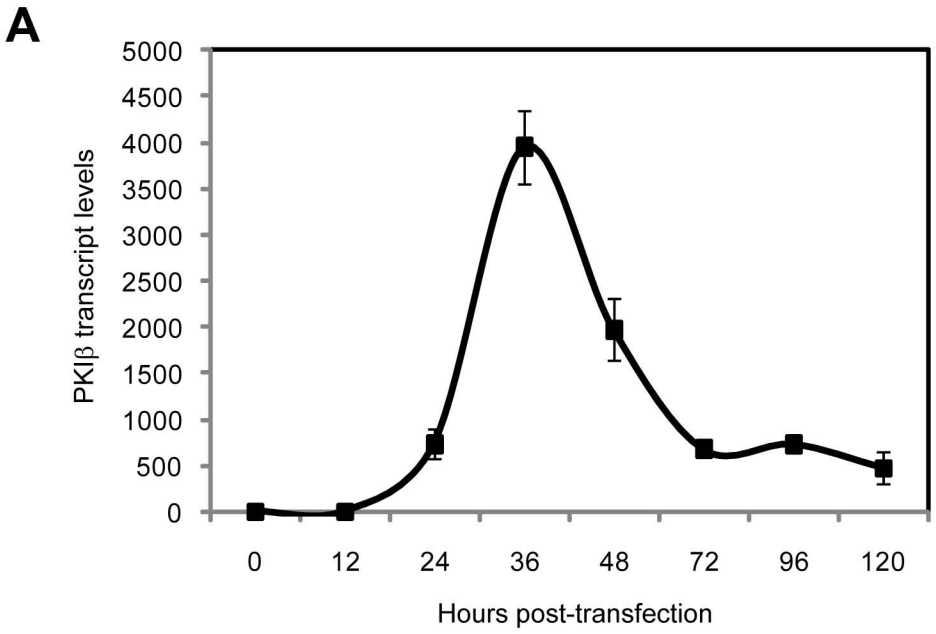

A
Relative expression
25
20
15
10
5
0
0
12
24
36
48
72
96
120
Hours post-transfection
PKIA
PKIG
B
PKIA
PKIG
Relative expression
25.00
20.00
15.00
10.00
5.00
0.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
NC
PC
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
C
US2
Ascl1
+PKIA shRNA
+PKIG shRNA
DAPI/TuJ1
DAPI/TuJ1
100μm

Figure 2.4. PKIβ expression during Ascl1-induced differentiation. A. Cells induced to differentiate via transient transfection of Ascl1 show a 2500-fold increase in *PKIβ* expression at the peak 36 h time point relative to the 0 h time point. PKIβ mRNA levels are given as the mean ± S.D. normalized to GAPDH levels. **B.** Western blotting for expression of PKIβ under the same conditions in (A). Induced PKIβ protein was observed between 36 and 72 h post-transfection, and occurred after the induction of Ascl1 expression. Densitometric analysis was performed to quantify and compare protein levels with GAPDH controls, and relative intensity values are shown underneath each corresponding blot.


A
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
PKIβ transcript levels
0
12
24
36
48
72
96
120
Hours post-transfection

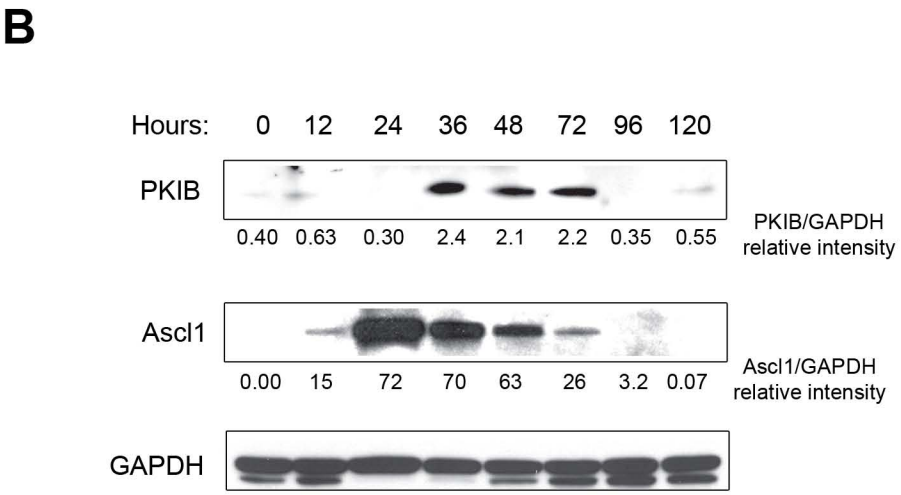

B
Hours: 0 12 24 36 48 72 96 120
PKIB
0.40 0.63 0.30 2.4 2.1 2.2 0.35 0.55
PKIB/GAPDH
relative intensity
Ascl1
0.00 15 72 70 63 26 3.2 0.07
Ascl1/GAPDH
relative intensity
GAPDH

Figure 2.5. PKIβ is necessary for Ascl1-induced P19 neuronal differentiation. A. RT-PCR analysis of the efficacy of shRNAs targeted to the PKIβ gene. Negative controls (NC) are cells transfected with the empty US2 vector. Positive controls (PC) are cells transfected with Ascl1. Out of five different shRNA vectors tested, shRNA1 was the least effective, while shRNA4 was the most effective. Results are shown as the mean ± S.D. normalized to GAPDH levels. *$p < 0.05$, **$p < 0.01$. **B.** Immunostaining for expression of TuJ1 (red) shows that co-transfection with PKIβ shRNA4 results in fewer TuJ1-positive cells than either the positive control (Ascl1) or the cells co-transfected with shRNA1. Nuclei were visualized with DAPI staining and appear blue. Scale bar = 100 μm. **C.** Quantitation of the percentage of cells differentiated in (B). Co-transfection with shRNA4 resulted in a significant reduction in the percentage of TuJ1-positive cells. Percentages are expressed as the mean ± S.D. * $p < 0.01$. **D.** Western blotting using antibodies against PKIβ and Map2 show that shRNA4 is the most effective at reducing both protein levels.

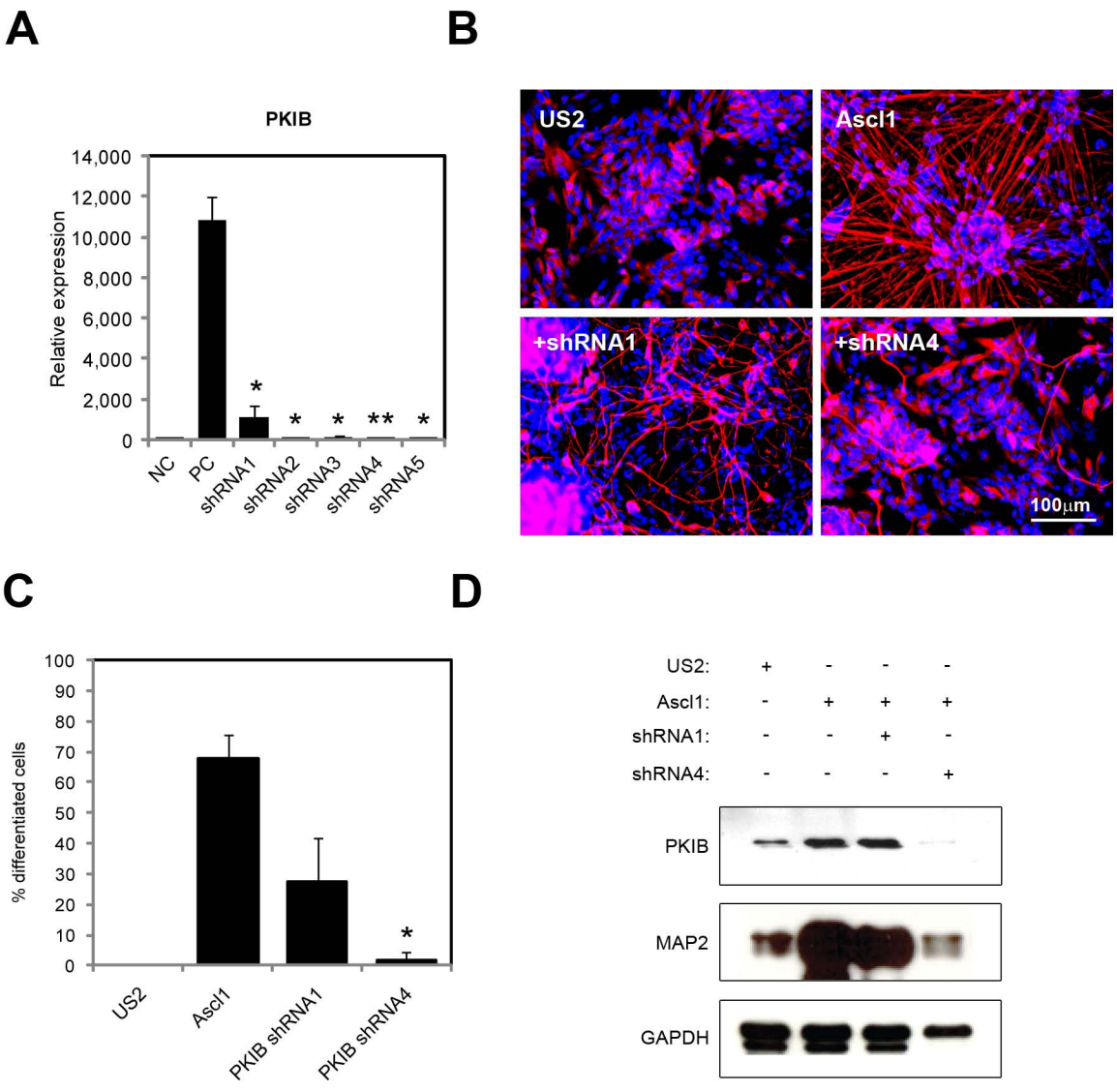

A
B
C
D
PKIB
Relative expression
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
NC
PC
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
US2
Ascl1
+shRNA1
+shRNA4
100μm
% differentiated cells
100
90
80
70
60
50
40
30
20
10
0
US2
Ascl1
PKIB shRNA1
PKIB shRNA4
US2: + - - -
Ascl1: - + + +
shRNA1: - - + -
shRNA4: - - - +
PKIB
MAP2
GAPDH

Figure 2.6. Organization of the PKIβ gene in P19 cells. A. PKIβ gene organization was determined using the Smart™ Race cDNA amplification kit, and a representative gel of the amplified products is shown. **B.** Representative PKIβ nucleotide sequence from an isolated cDNA clone. The beginning of each exon of the PKIβ gene is underlined. **C.** Schematic of PKIβ exon organization based on 5' RACE sequencing. Open boxes represent non-coding regions, and the closed box represents the coding region. Exon sizes are indicated above the exon boxes. Target sequences for two representative PKIβ shRNA constructs are underlined.

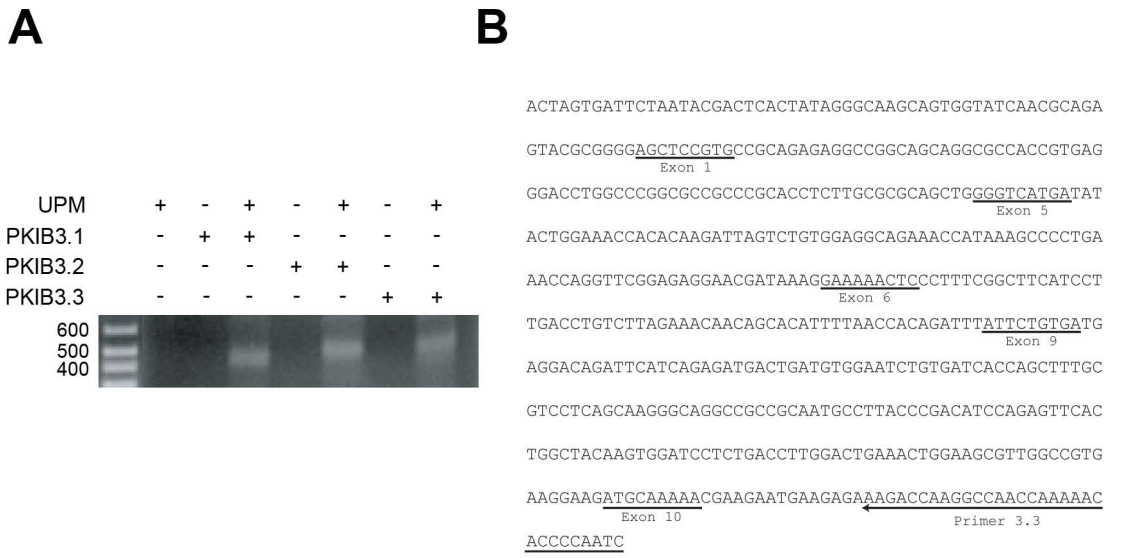
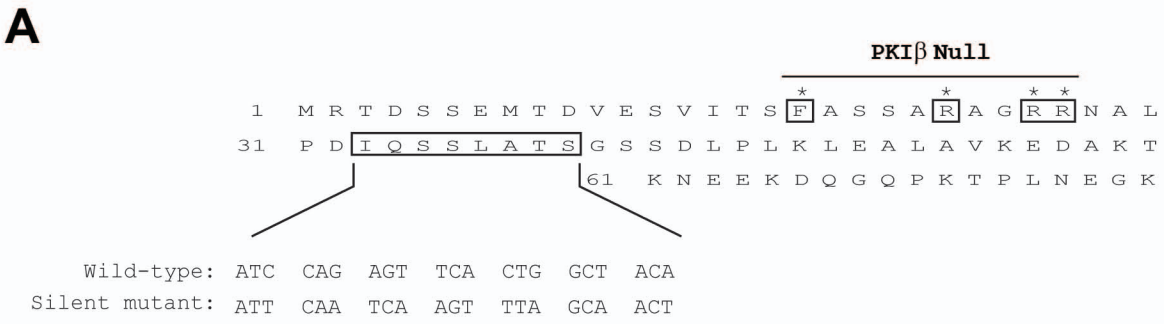

A
B
UPM + - + - + - +
PKIB3.1 - + + - - - -
PKIB3.2 - - - + + - -
PKIB3.3 - - - - - + +
600
500
400
ACTAGTGATTCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGA
GTACGCGGGGAGCTCCGTGCCGCAGAGAGGCCGGCAGCAGGCGCCACCGTGAG
Exon 1
GGACCTGGCCCGGCGCCGCCCGCACCTCTTGCGCGCAGCTGGGGTCATGATAT
Exon 5
ACTGGAAACCACACAAGATTAGTCTGTGGAGGCAGAAACCATAAAGCCCCTGA
AACCAGGTTCGGAGAGGAACGATAAAGGAAAAACTCCCTTTCGGCTTCATCCT
Exon 6
TGACCTGTCTTAGAAACAACAGCACATTTTAACCACAGATTTATTCTGTGATG
Exon 9
AGGACAGATTCATCAGAGATGACTGATGTGGAATCTGTGATCACCAGCTTTGC
GTCCTCAGCAAGGGCAGGCCGCCGCAATGCCTTACCCGACATCCAGAGTTCAC
TGGCTACAAGTGGATCCTCTGACCTTGGACTGAAACTGGAAGCGTTGGCCGTG
AAGGAAGATGCAAAAACGAAGAATGAAGAGAAAGACCAAGGCCAACCAAAAAC
Exon 10
Primer 3.3
ACCCCAATC
C
83
91
67
176
4822
EX1
EX5
EX6
EX9
EX10
attctgtgatgaggacagattcatcagagatgactgatgtggaat
ctgtgatcaccagctttgcgtcctcagcaagggcaggccgccgca
atgccttacccgacatccagagttcactggctacaagtggatcct
shRNA4
ctgatcttccactgaaactggaagcgttggccgtgaaggaagatg
caaaaacgaagaatgaagagaaagaccaaggccaaccaaaa
tctgaaataggctgggtataaagggaggatacaat
shRNA1

Figure 2.7. Inhibitory activity of exogenous PKIβ expression vectors. A. Amino acid sequence of murine PKIβ. The numbering of the sequence begins with the known initiator methionine, and is placed on the left of the diagram. Amino acid residues known to be important in high affinity binding of PKIα for the C subunit of PKA are indicated with an asterisk on the top line (Phe^{18}, $Arg^{23, 26, 27}$). All four of these residues were mutated to alanines to determine their importance for PKIβ function. **B.** PKA enzyme activity, as determined by kinase assays using a fluorescent PKA substrate peptide (f-kemptide), is inhibited by exogenous PKIβ. A representative UV-illuminated agarose gel of the products of kinase reactions run with f-kemptide and transfected cell homogenates is shown. PKA activity phosphorylates kemptide, which changes its net charge from +1 to -1. This allows the phosphorylated and nonphosphorylated forms of the substrate to be rapidly separated on an agarose gel. Densitometric analysis quantitated a 7-fold reduction of PKA activity in the presence of exogenous PKIβ. This inhibition is dependent on residues critical to binding of PKA, as the PKIβ Null protein failed to inhibit PKA activity. $*p < 0.05$.


A
PKIβ Null
1 M R T D S S E M T D V E S V I T S F A S S A R A G R R N A L
31 P D I Q S S L A T S G S S D L P L K L E A L A V K E D A K T
61 K N E E K D Q G Q P K T P L N E G K
Wild-type: ATC CAG AGT TCA CTG GCT ACA
Silent mutant: ATT CAA TCA AGT TTA GCA ACT

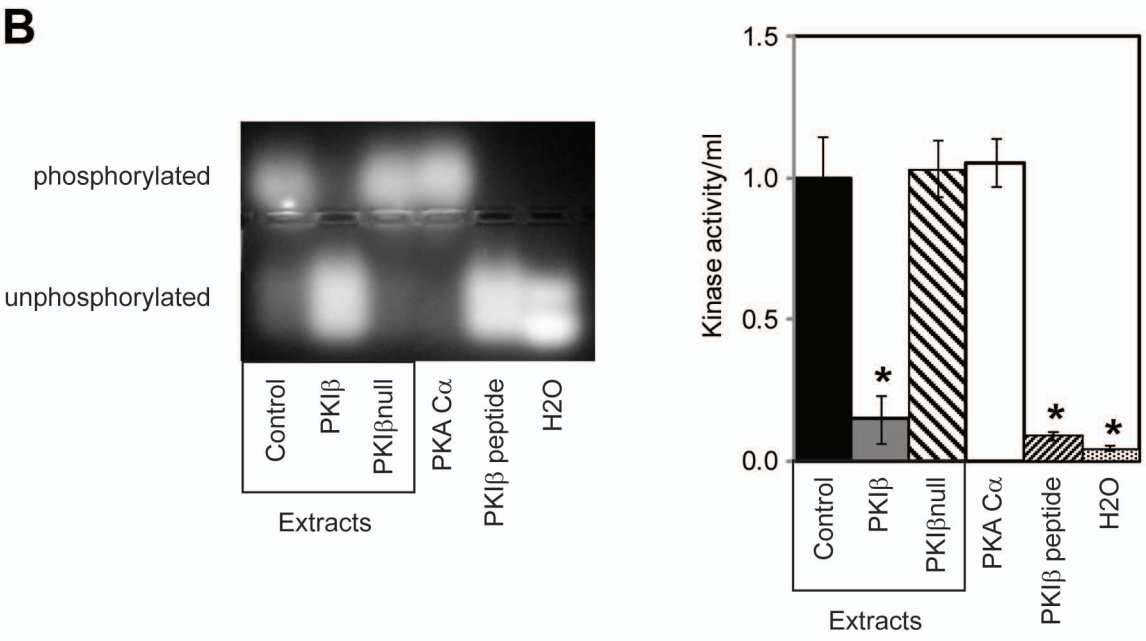

B
phosphorylated
unphosphorylated
Control
PKIβ
PKIβnull
PKA Cα
PKIβ peptide
H2O
Extracts
Kinase activity/ml
1.5
1.0
0.5
0.0
*
*
*
Control
PKIβ
PKIβnull
PKA Cα
PKIβ peptide
H2O
Extracts

Figure 2.8. Exogenous PKIβ rescues neuronal differentiation, and is dependent binding to PKA. A. As previously shown (see Figure 2.5B), transient co-transfection of P19 cells with shRNA4 resulted in fewer cells differentiating. Immunostaining for Map2 (red) shows that introduction of exogenous PKIβ rescued the phenotype, producing more Map2-positive cells that adopt a neuronal morphology. A functionally null PKIβ did not rescue the phenotype, as evidenced by decreased Map2-immunoreactivity and a lack of Map2-positive processes. Nuclei were visualized with DAPI staining and appear blue. Scale bar = 100 μm. **B.** Quantitation of the percentage of cells differentiated from (A). To be considered differentiated, cells had to be Map2-positive and also have processes three times the length of the cell body. Using these parameters, cells from three independent fields per condition were counted and expressed as the mean ± S.D. *$p < 0.05$. Western blotting for Map2 protein showed changes in expression that supported the immunostaining results shown in (A).

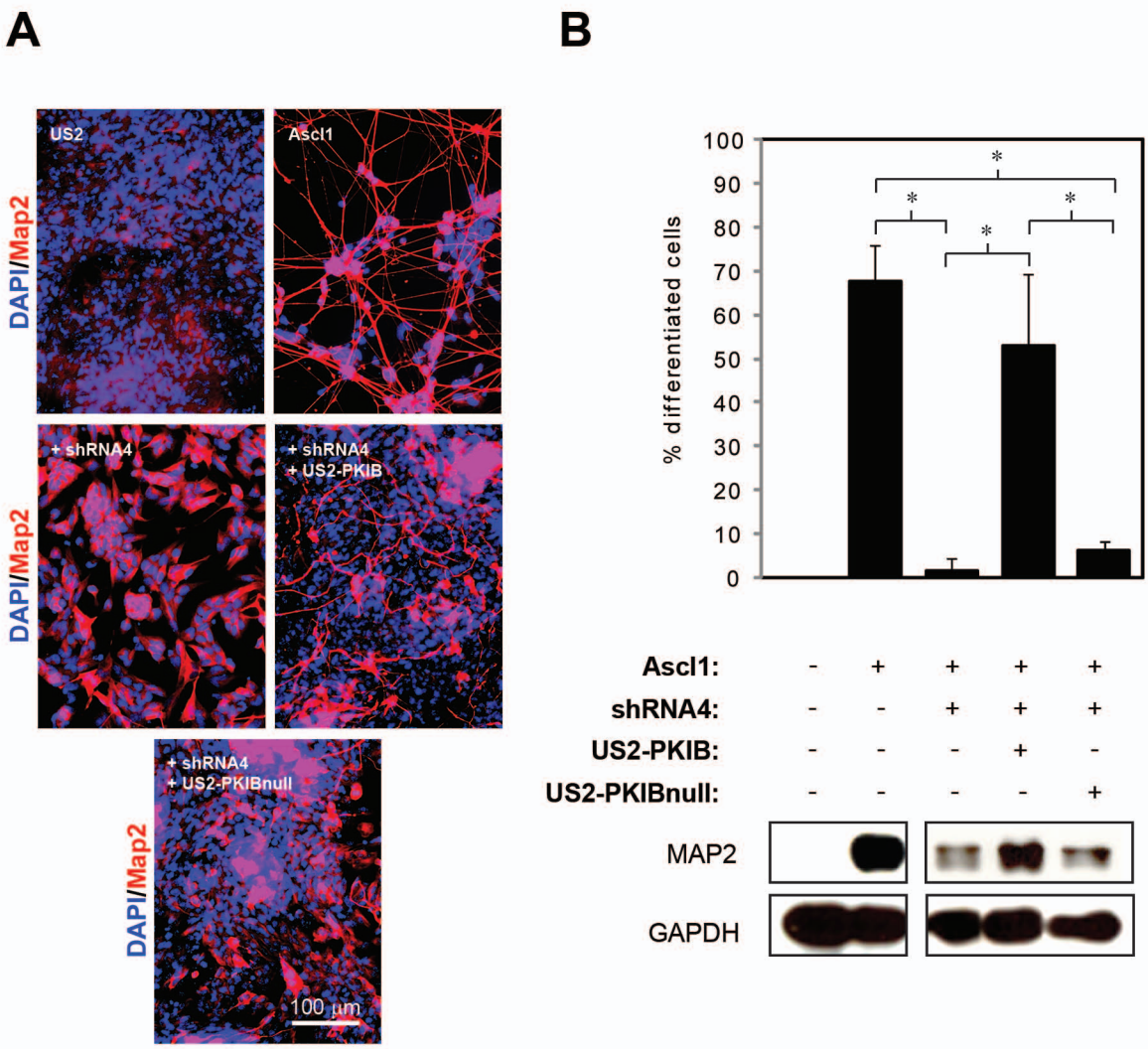

A
B
US2
Ascl1
+ shRNA4
+ shRNA4
+ US2-PKIB
+ shRNA4
+ US2-PKIBnull
DAPI/Map2
DAPI/Map2
DAPI/Map2
100 µm
% differentiated cells
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
Ascl1: - + + + +
shRNA4: - - + + +
US2-PKIB: - - - + -
US2-PKIBnull: - - - - +
MAP2
GAPDH

Table 2.1. Oligonucleotides used in the experimental methods

Section	Primer Name	Sequence
PKIβ 7.1 amplification		
	PKIβ78 5'	GGGGAATTCCCACCATGAGGACAGATTCATCAGAGATGACTGATG
	PKIβ78 3'	GGGTCTAGATTCATTTTCCTTCATTTAGGGGTGTTTTTG
US2-PKIβ cloning		
	PKIβmut 5'out	ATTTAGGTGACACTATAGAATA
	PKIβmut 3'in	TGAAGTTGCTAAACTTGATTGAATGTCGGGTAAGGCATTGCG
	PKIβmut 5'in	ATTCAATCAAGTTTAGCAACTTCAGGATCCTCTGATCTTCCACT
	PKIβmut3'out	TAATACGACTCACTATAGGG
US2-PKIβnull cloning		
	PKIβnull 5'out	GTACGGTGGGAGGCCTATATAAG
	PKIβnull 3'in	ATTGGCGGCGCCTGCGGCTGCTGAGGACGCGGCGCTGGTGATCACAGATTC
	PKIβnull 5'in	GAATCTGTGATCACCAGCGCCGCGTCCTCAGCAGCCGCAGGCGCCGCCAAT
	PKIβnull 3'out	GATCCTCTAGAATCACTTGTCATCG
RT-PCR Analysis		
	GAPDH 5'	AGGTCGGTGTGAACGGATTTG
	GAPDH 3'	TGTAGACCATGTAGTTGAGGTCA
	PKIα 5'	CGAGCCCAGAGCCCAGCACAC
	PKIα 3'	GCCATTTTCTTCCCCTCCGTTTCA
	PKIβ 5'	CGCCGCCCGCATCTCTT
	PKIβ 3'	ACAAAAACCAACCGGAACCAGAAA
	PKIγ 5'	AAACTAGGCCTGAGGAGCGATGAG
	PKIγ 3'	CCATCCGAGAGGTACAGCAGGTC
5' RACE		
	PKIβ 3.1	GCATCTTCCTTCACGGCCAACGCTTCC
	PKIβ 3.2	GGGGGTGTTTTTGGTTGGCCTTGGTCTTT
	PKIβ 3.3	GGGGTGTTTTTGGTTGGCCTTGGTCT

CHAPTER III[1]

DIRECT TRANSCRIPTIONAL INDUCTION OF GADD45γ BY ASCL1 DURING NEURONAL DIFFERENTIATION

Summary

The basic helix-loop-helix transcription factor Ascl1 plays a critical role in the intrinsic genetic program responsible for neuronal differentiation. Here, we describe a novel model system of P19 embryonic carcinoma cells with doxycycline-inducible expression of Ascl1. Microarray hybridization and real-time PCR showed increased gene expression of many neuronal markers in a time- and concentration-dependent manner. Interestingly, the gene encoding the cell cycle regulator Gadd45γ was increased earliest and to the greatest extent following Ascl1 induction. Here, we provide the first evidence identifying Gadd45γ as a direct transcriptional target of Ascl1. Transactivation and chromatin immunoprecipitation assays identified two E-box consensus sites within the Gadd45γ promoter necessary for Ascl1 regulation, and demonstrated that Ascl1 is bound to this region within the Gadd45γ promoter. Furthermore, we found that overexpression

[1] This chapter has been published: Huang, H. S., Kubish, G. M., Redmond, T. M., Turner, D. L., Thompson, R. C., Murphy, G. G., and Uhler, M. D. (2010) *Mol Cell Neurosci* **44**(3), 282-296. HSH performed research for Figures 3.3C, 3.5C, 3.7, 3.8, and 3.10. GMK performed research for Figures 3.2, 3.3A-B, 3.4, 3.5A, and 3.6. RCT performed the microarray hybridization studies for Tables 3.1-3.3. GGM performed the electrophysiological studies for Figure 3.5B. MDU performed research for Figures 3.1, 3.5C, and 3.7.

of Gadd45γ itself is sufficient to initiate some aspects of neuronal differentiation independent of Ascl1.

Introduction

Transcription factors of the basic helix-loop-helix (bHLH) class play important roles in many aspects of neuronal development. The importance of bHLH genes for neurogenesis was first appreciated in *Drosophila melanogaster*, where it was shown that genes belonging to the achaete-scute complex are required for the development of some neurons in the peripheral and central nervous system (PNS and CNS; Romani et al., 1989; González et al., 1989). Genetic studies in *Drosophila* and *Xenopus* have also shown that bHLH proteins are both necessary and sufficient to commit ectodermal progenitors to a neuronal-specific fate, and that this activity involves the Notch signaling pathway (Turner and Weintraub, 1994; Artavanis-Tsakonas et al., 1999). The proneural function of bHLH genes appears to have been evolutionarily conserved: homologues of achaete-scute genes have been identified in a variety of vertebrate species, and these genes regulate the development of specific classes of neurons (Johnson et al., 1990; Guillemot et al., 1993). For example, mammalian achaete-scute homolog 1 (Ascl1) is expressed in subsets of proliferating precursor cells in the PNS and CNS of the mouse embryo, and loss-of-function studies have shown that Ascl1 is required for the development of autonomic neurons and olfactory receptor neurons (Guillemot et al., 1993). The neurogenic effects of bHLH proteins—such as Ascl1—make them useful in strategies to yield neuron-enriched grafts. Recently, transduction of Ascl1 into donor neuronal progenitor cells before transplantation dramatically enhanced neuronal yield and donor cell survival, both *in vitro* and *in vivo* (Yi et al., 2008).

The function of the vertebrate CNS is dependent on the generation of neuronal progenitor cells at the proper developmental time, making the balance between proliferation and cell cycle withdrawal fundamental to the formation of the mature vertebrate CNS. Proneural bHLH proteins promote cell cycle arrest, presumably through activation of cyclin-dependent kinase inhibitors (Farah et al., 2000). Despite the importance of neurogenic bHLH families in neuronal development, primary target genes and transcriptional programs directly regulated by neurogenic bHLH proteins have yet to be systematically defined.

P19 cells are pluripotent embryonic carcinoma (EC) cells that differentiate into cell types of all three germ layers (McBurney et al., 1982), and are a commonly used model to study neuronal differentiation *in vitro*. Treatment of P19 cells with retinoic acid followed by aggregation results in neuronal and glial differentiation (Bain et al., 1996). Many bHLH genes are induced in this method of differentiation, including Ascl1, and its pattern of expression closely matches those observed *in vivo* (Johnson et al., 1992). More recently, transient transfection of neural bHLH proteins such as Ascl1 was shown to be sufficient to convert P19 cells into a relatively homogeneous population of electrophysiologically differentiated neurons (Farah et al., 2000). These findings suggest that undifferentiated P19 cells express the genes necessary to support the initiation of neuronal differentiation in response to neurogenic bHLH transcription factors. One limitation to the current studies of Ascl1-induced neuronal differentiation is their reliance on transient transfection, which results in difficulty controlling Ascl1 expression temporally or quantitatively. Furthermore, the levels of transfected DNA are heterogeneous at a cellular level. To circumvent these problems, we developed an

inducible P19 cell line in which the expression of the Ascl1 gene was under the control of the tetracycline transcriptional repressor (Gossen and Bujard, 1992).

In our studies, we used microarray hybridization analysis combined with tetracycline-regulated Ascl1-expressing cell lines to delineate the transcriptional consequences of Ascl1 induction. We showed that doxycycline induction of Ascl1 in P19 cells caused expression of neuronal marker proteins, including cytoskeletal and synaptic proteins, in a time- and dose-dependent manner and generated neurons that were polarized and electrically excitable. Microarray analysis of genes induced over the time course of differentiation showed changes in several genes not previously characterized as Ascl1 responsive in P19 cells. One highly induced gene, growth-arrest and DNA-damage inducible protein 45 gamma (Gadd45γ), was of particular interest because of its role in cell cycle regulation (Smith et al., 1994; Wang et al., 1999; Zhan et al., 1999; Yang et al., 2000). Using reporter constructs of the human Gadd45γ gene that contained four evolutionarily conserved E-box consensus sites adjacent to the Gadd45γ promoter, we showed transactivation of Gadd45γ with Ascl1 in P19 cells. Additionally, chromatin immunoprecipitation (ChIP) assays showed that Ascl1 associates with the Gadd45γ promoter in living P19 cells, supporting our data that Gadd45γ is a direct transcriptional target of Ascl1. Finally, using a Gadd45γ-inducible P19 cell line, we found that overexpression of Gadd45γ recapitulated a subset of Ascl1-mediated gene regulatory events.

Materials and Methods

Materials

The following primary antibodies were used in the experiments: TetR, Tau (Chemicon), GAPDH, Map2 (Cell Signaling Technology), Ascl1 (BD Pharmingen), TuJ1 (Covance), Gap43 (Sigma-Aldrich), Isl1 (DSHB University of Iowa), Synaptophysin (Syp; BD Biosciences), and Gadd45γ (Sigma-Aldrich). Secondary horseradish peroxidase-conjugated antibodies were obtained from Cell Signaling Technology. Alexa Fluor conjugated antibodies (goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 546, and goat anti-rabbit Alexa Fluor 488) were all purchased from Invitrogen. Five different shRNAs were assayed for efficacy of knocking down the mouse Gadd45γ gene and were obtained from Open Biosystems.

Cell culture, transfection, and treatment

P19 EC cells were cultured in Minimal Essential Medium Alpha (MEMα; Gibco) supplemented with 7.5% calf serum (CS; HyClone), 2.5% fetal bovine serum (FBS; HyClone), and 1% penicillin/streptomycin (Gibco). The Ascl1-inducible P19 cell line (P19T1A2) was maintained in the same media as P19 EC cells, with the addition of G418 (200 μg/ml, HyClone) and hygromycin (100 μg/ml, Invitrogen). HEK-293T cells were grown in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS. Cells were kept at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. Cells were maintained below 80% confluence and passaged by dissociating them into single cells using TrypLE Express (Gibco). Cells were transfected using the TransIT-LT1 transfection reagent (Mirus) following the manufacturer's instructions. When necessary, the appropriate parental expression plasmid DNA was added to maintain a constant total amount of DNA.

Construction of the Dox-controlled Ascl1 expression system

Initially, the pCMV-TetOnAdv or pUS2-TetOn plasmids were used to generate Doxycycline (Dox, a tetracycline derivative) inducible P19 cells. However, these attempts were largely unsuccessful. Therefore, we constructed the pUS2-TetOnAdv plasmid by subcloning the 1325 bp SalI/EcoRI fragment of pUS2 into XhoI/EcoRI digested pTetOnAdv (Clontech). Using the TransIT-LT1 reagent, P19 cells were transfected with the pUS2-TetOnAdv vector together with a 10-fold lower amount of the 2.2 kb BamHI fragment of pCMV-Neo containing the neomycin phosphotransferase gene under the control of the SV40 promoter. Stable clones were selected with 200 μg/ml of G418. G418-resistant colonies were screened by transient co-transfection with the pTRE-Luciferase vector (pTRE-Luc, Clontech), which encodes for the firefly luciferase protein under control of the TRE promoter, and RL-SV40 (Promega), which encodes for renilla luciferase protein and served as a control for transfection efficiency. A Dual Luciferase Reporter Assay (Promega) was used to identify clones exhibiting low background and high luciferase activity upon addition of Dox. The cell line used in further experiments was designated P19T1.

The pTRE-Ascl1 expression vector was constructed by subcloning the 700 bp EcoRI/XbaI fragment encoding Ascl1 from pCS2-Ascl1, and ligating this fragment with EcoRI/XbaI digested pTRE-tight (Clontech). P19T1 cells were co-transfected with pTRE-Ascl1 together with pTK-Hyg (Clontech), and cultured in the presence of 100 μg/ml hygromycin for selection. Hygromycin-resistant colonies were screened for the expression of neuron-specific class III β-tubulin (TuJ1) and Ascl1 upon addition of Dox by immunocytochemistry and western blot analysis.

Construction of the Dox-controlled Gadd45γ expression system

The pTRE-Gadd45γ-IRES2-EGFP vector was constructed by first inserting the 1.4 kb NheI/NotI fragment of pIRES2-EGFP (Clontech) into NheI/NotI digested pTRE-tight (Clontech) to generate the plasmid pTRE-tight-IRES2-EGFP. pUS2-Ascl1 was then digested with EcoRI and SnaBI. The resulting 760 bp fragment was ligated into EcoRI/SmaI digested pTRE-tight-IRES2-EGFP to generate pTRE-Ascl1-IRES2-EGFP. Finally, the human Gadd45γ coding region was PCR amplified from pCMV6-XL5-hGadd45γ (Origene) using the primer pair shown in Table 3.4. The resulting 500 bp amplification product was purified, digested with EcoRI/XbaI, and ligated to EcoRI/XbaI digested pTRE-Ascl1-IRES2-EGFP, thereby replacing the Ascl1 coding sequence with the Gadd45γ coding sequence. P19T3 cells were transfected with 26 μg of pTRE-Gadd45γ-IRES2-EGFP and 1 μg of pUS2-puro. Puromycin resistant clones were identified by enhanced green fluorescent protein (EGFP) fluorescence after 24 h of doxycycline treatment and expanded. The clone P19T3GIE2 was chosen for detailed characterization based on the high induction of EGFP fluorescence.

Differentiation of P19T1A2 cells

For differentiation of P19T1A2 cells, tissue culture plates were laminin coated using a procedure adapted from Ray et al. (1995). Briefly, plates were coated in a solution of 5 μg/ml laminin (Invitrogen) diluted in phosphate buffered saline (PBS, HyClone). The plates were sealed in plastic bags and kept in an incubator overnight (37°C, 5% CO_2). After aspirating off the laminin solution, the plates were washed twice with PBS before seeding the P19T1A2 cells at a density of 5.0 x10^5 cells/ml. For the first

four days of differentiation, cells were maintained in MEMα supplemented with 7.5% CS, 2.5% FBS, 1% penicillin/streptomycin, 200 μg/ml G418, 100 μg/ml hygromycin, and 0.5 μg/ml doxycycline. On day four, the media was changed to Neurobasal media (Gibco) supplemented with 1% penicillin/streptomycin, B27 (Gibco), GlutaMAX (Invitrogen), G418 (200 μg/ml), hygromycin (100 μg/ml), and doxycycline (0.5 μg/ml).

Construction of reporter plasmids

The pEL2 reporter vector containing the EGFP coding region fused to the firefly luciferase coding region was constructed to monitor reporter expression in living cells (EGFP) as well as to quantitate reporter expression by enzymatic assay (firefly luciferase). pEL2 was constructed by ligating the PCR-amplified coding region of firefly luciferase generated using pGL3basic (Promega) as template and the primer pairs shown in Table 3.4. The resulting PCR fragment was Acc65I/NotI digested prior to subcloning into BsrBI/NotI digested pEGFP-1 (Clontech).

Oligonucleotides used in generating the following reporter plasmids can be found in Table 3.4. The 1222 bp promoter sequence for the human Gadd45γ gene was PCR amplified from human genomic DNA (Clontech), and subcloned into pEL2 (1222-EL2). The AVID alignment program implemented in VISTA was used to compare conserved regions between human and mouse, and four E-box (CANNTG) sequences were identified clustered in a highly conserved region of the promoter of the human Gadd45γ gene. PCR procedures were used to generate five stepwise deletion constructs (938-, 665-, 281-, 194-, and 188-EL2) of the full length Gadd45γ promoter (1222-EL2). The amplified PCR fragments were subcloned into the pGEM-T-Easy vector system

(Promega). The DNA was HindIII/BamHI digested and then subcloned into HindIII/BamHI digested pEL2. A 281-EL2 construct harboring substitutions to the two proximal E-box consensus sites was constructed by oligo-directed mutagenesis and PCR. The E-box consensus at -281/-275 was mutated from CACGTG to GAATTC, and the consensus at -194/-188 was mutated from CAGCTG to ACGCGT. Constructs containing the two mutated sites were generated in separate rounds of PCR. The PCR amplicon containing the distal E-box mutation was EcoRI/MluI digested. The amplicon containing the proximal E-box mutation was MluI/BamHI digested. These fragments were then ligated into EcoRI/BamHI digested pEL2. All of the Gadd45γ deletion subclones were sequenced to ensure that only the intended deletions were introduced. All oligonucleotides were synthesized by Invitrogen.

Electrophysiological recordings

All recordings were carried out at room temperature using an external solution that contained (in mM) 132 NaCl, 5.3 KCl, 1.3 NaH_2PO_4, 1.7 $MgSO_4$, 5.4 $CaCl_2$, 12 Hepes, 6.3 glucose, pH, 7.4. Whole-cell recordings on P19T1A2 cells treated with Dox for six days were made using a Dagan 3900A amplifier in bridge mode. Neurons were visualized with an Olympus BX51WI upright microscope equipped with differential interference contrast optics. Patch-pipettes made from Clark Borosilicate Standard Wall glass (Warner Instruments) and pulled using a P-97 Flaming-Brown pipette puller (Sutter Instruments) with resistances of 9-11 MΩ were used and filled with the following internal solution (in mM): KCl 140, NaCl 5, $MgCl_2$ 1, Na_2EGTA 10, Hepes 10, pH 7.4. Seal resistances of >2 GΩ were achieved prior to rupturing into whole-cell mode. Action

potentials were elicited by delivering a 5 ms current step of increasing amplitude (0.01 nA steps).

Microarray analysis

Total RNA (200 ng) was amplified and labeled using the Illumina Total Prep RNA Amplification Kit (Ambion). Labeled cRNA (1.5 μg) was hybridized at 55°C for 22 h to Sentrix-6 Mouse V1.0 BeadChip microarrays (Illumina). Microarrays were washed and scanned for data collection as directed by the manufacturer. Microarray data were analyzed using BeadStudio software (Illumina). Differential gene expression was determined using quantile normalization and the Illumina Custom error model. mRNAs for analysis were selected based on mRNAs detected in at least one condition with $p < 0.01$. For differential expression analyses, a cutoff of $p < 0.01$ was used. All analyses used a subset of Illumina probes that matched sequences in the Refseq database and mapped to the mouse genome at a single location (Pinglang Wang and Fan Meng, University of Michigan, personal communication).

RNA isolation and RT-PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Single stranded cDNA was synthesized from 2 μg of total RNA using SuperScript II Reverse Transcriptase and random hexamers (Invitrogen). Gene expression was evaluated by real-time quantitative PCR (RT-PCR) using the SYBR Green PCR Master Mix (Applied Biosystems), and the MyiQ single-color real-time PCR detection system (Bio-Rad) according to manufacturer's instructions. The specificity of the PCR amplification procedures was checked with a heat dissociation

protocol (from 72°C to 98°C) after the final cycle of the PCR. Each reaction was done in triplicate. Expression levels were calculated using the delta-delta CT method, with GAPDH as the normalization control. The primer pairs used to amplify target genes are shown in Table 3.4.

Dual luciferase reporter assay

Dual luciferase assays were performed using the Dual Luciferase Reporter Assay (Promega) following recommended protocols. Samples were read on a FLUOstar OPTIMA microplate reader (BMG Labtech). To correct for differences in transfection efficiencies, firefly luciferase activity (pEL2) was normalized to that of renilla luciferase (pUS2-RL). The pUS2-RL plasmid was constructed by subcloning the 1.3 kb BglII/XbaI fragment of pUS2 containing the Ubc promoter into BglII/NheI digested pRL-SV40. Experiments were repeated a minimum of three times and results were expressed as mean ± standard deviation. The statistical significance of Ascl1 transactivation data was determined by employing a student's paired t-test ($p < 0.01$).

Chromatin immunoprecipitation assay

P19T1A2 cells were treated with 5 μg/ml Dox for 24 h as described above. An antibody against Ascl1 was used for immunoprecipitation (BD Pharmingen) and the chromatin immunoprecipitation (ChIP) assay was performed as described by the manufacturer (Cell Signaling). The immunoprecipitates were subjected to RT-PCR using primers specific to the Gadd45γ promoter. The resulting amplified fragment contained both the E3 and E4 E-boxes of the Gadd45γ promoter. The ChIP amplifications were performed using SYBR Green PCR Master Mix (Applied Biosystems) in quadruplicate.

The reaction conditions were as follows: 95°C for 7 min, followed by 60 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 20 sec. Threshold cycle numbers (CT) were determined with the MyiQ single-color real-time PCR detection system (Bio-Rad). The DNA levels from the ChIP RT-PCR assay were calculated using the delta-delta CT method, with primers for the ribosomal protein L30 (RPL30) as the normalization control. PCR primer sets for the ChIP assays are shown in Table 3.4.

SDS-PAGE and western blot analysis

Cells were washed twice with Dulbecco's PBS (DPBS; Hyclone) and lysed in buffer containing 10 mM $NaH_2PO_4 \bullet H_2O$, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 10 mM sodium fluoride, complete EDTA-free protease inhibitors (Roche), and 1 mM PMSF. Lysates were sonicated, and protein concentrations were determined by the bicinchonic acid protein assay (Bio-Rad). Equal amounts of total protein were denatured at 95°C in the presence of SDS and β-mercaptoethanol. Samples were resolved on linear gradient Tris-HCl gels (Bio-Rad) and transferred onto 0.2 μm polyvinylidene difluoride membranes. Detection was carried out using Lumi-Light Western Blotting Substrate (Roche) according to the manufacturer's instructions.

For resolving Gadd45γ protein in P19T1A2 cells, samples were transferred to 0.2-μm nitrocellulose membranes (BA-83, Whatman). Membranes were blocked for 4 h in PBS supplemented with 5% non-fat dried milk, 2% polyvinylpyrrolidone (PVP-40), and 0.1% Triton X-100 and subsequently incubated with a 1:200 dilution of anti-Gadd45γ in PBS supplemented with 0.5% bovine serum albumin and 0.1% Triton X-100 overnight at 4°C. Membranes were washed three times for 10 min with TBST (50 mm Tris, pH 7.5,

150 mm NaCl, and 0.05% Tween 20), and then incubated with a 1:2,000 dilution of goat anti-mouse-HRP (Cell Signaling Technology) in TBST supplemented with 5% non-fat dried milk as the secondary antibody for 2 h. Following the final set of three 10 min washes with TBST, the blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) according to the manufacturer's instructions.

Recombinant His-tagged Gadd45γ protein was purified from *E. coli* essentially as described (Collins and Uhler, 1999), and the purified Gadd45γ protein had an apparent molecular weight of 17 kDa on SDS-PAGE. Quantitative assay of antigen expression was based on density measurements of protein bands using ImageJ software (http://rsb.info.nih.gov/ij).

Immunocytochemistry

Cells were washed twice with DPBS, and then fixed in 4% formaldehyde solution for 10 minutes. Cells were washed twice in PBS, and then blocked for one hour in PBS supplemented with 2% goat serum and 0.1% Triton X-100. Cells were probed with primary antibodies diluted in blocking solution for two hours at 23°C. After washing in PBS, cells were incubated with AlexaFluor conjugated secondary antibodies for one hour at 23°C, followed by three PBS washes. For nuclear counterstaining, the cells were incubated in 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) for 10 minutes before being washed twice in PBS and visualized. To collect still images, we used an inverted Olympus IX70 fluorescence microscope using an Illix CCD imaging system and Micro Computer Image Device software (Imaging Research Inc.). Confocal images were obtained using an inverted Olympus FV1000 laser scanning confocal

microscope. Prior to image collection, the acquisition parameters for each channel were optimized to ensure a dynamic signal range and to ensure no signal bleed through between detection channels.

Results

Generation of rtTA-expressing clones derived from P19 cells

A total of 156 putative rtTA-stable clones were screened by transfection with the reporter plasmid pTRE-Luc and grown with or without doxycycline (Dox) in the medium. The majority of the clones showed no regulation of firefly luciferase activity (e.g. P19T4; Figure 3.1A). Six clones showed high constitutive firefly luciferase activity in the absence of Dox (e.g. P19T5), and six clones showed high firefly luciferase activity only in the presence of Dox (e.g. P19T3, P19T1, and P19T6). Clones P19T1, P19T3, and P19T6 showed the highest induction of luciferase activity, exhibiting 212-, 263-, and 532-fold increases, respectively, in the presence of Dox. Upon passage, the induction by Dox in the P19T3 and P19T6 cells progressively diminished. However, the P19T1 cells showed consistent induction over 20-30 passages. After extended passages, induction of pTRE-Luc activity was roughly correlated with the amount of rtTA protein expressed in the cells. Figure 3.1B shows that clone P19T1, which had the highest sustained induction of luciferase activity in the presence of Dox, also expressed the most rtTA protein. We selected the P19T1 clone for generating secondary transfectants in subsequent experiments.

Generation of a stably transfected cell line showing Dox-responsive Ascl1 expression

P19T1 clones stably co-transfected with plasmids pTRE-Ascl1 and pTK-hygro were generated as described in Materials and Methods. Of the 206 hygromycin-resistant clones isolated, six showed a significant reduction of growth in the presence of Dox. Microtubule-associated protein 2 (Map2) and neuron-specific class III β-tubulin (TuJ1) are widely accepted as neuronal marker proteins, and are induced in P19 cells transiently transfected with Ascl1 (Farah et al., 2000). Immunocytochemistry was performed with Map2 and TuJ1 antibodies. Three pTRE-Ascl1 transfected clones—P19T1A2, P19T1A3, and P19T1A12—produced a high percentage of cells (>30%) that were Map2- and TuJ1-immunoreactive in the presence of Dox, although their levels of Ascl1 expression varied (data not shown). This induction of Map2 and TuJ1 immunoreactivity was never observed following treatment of parental P19 or P19T1 cells with Dox (data not shown). We chose the P19T1A2 clone for subsequent experiments because of the low level of spontaneous differentiation and the high level of differentiation in the presence of Dox.

Optimizing growth conditions for Ascl1-induced neuronal differentiation in P19 cells

Neuronal differentiation and survival *in vivo* and *in vitro* depends on a variety of factors. Pure neuronal cultures require specific growth factors for optimal survival and neurite production. In addition to these soluble factors, the culture substrate is essential for neuronal adhesion and influences the number, shape, and growth rate of neurites (Rogers et al., 1983). Experiments were performed to define the relative importance of these various influences and optimize growth conditions for our model of neuronal differentiation.

Substrata commonly used for neuronal cell culture include polymers of basic amino acids such as poly-D-lysine and polyornithine, and extracellular matrix constituents such as collagen, fibronectin, and laminin (Carbonetto et al., 1983; Lochter and Schachner, 1993). Three substrata were evaluated: poly-D-lysine, poly-ornithine, and laminin. Neurite outgrowth was consistently enhanced in the presence of laminin (data not shown). P19T1A2 cells were seeded onto laminin-coated tissue culture plates and treated with or without Dox using four different growth conditions: 1. MEMα (7.5% CS, 2.5% FBS; Figure 3.2A, E), 2. MEMα (1% FBS; Figure 3.2B, F), 3. MEMα (7.5% CS, 2.5% FBS) for the first three days of differentiation, followed by a change to Neurobasal media (B27, GlutaMAX) for the remaining duration of the differentiation protocol (Figure 3.2C, G), and 4. OPTI-MEM (1% FBS; Figure 3.2D, H).

At ten days and for all media conditions, more P19 cells adopted a neuronal morphology and expressed the appropriate neuronal-specific markers—such as Map2—in the presence of Dox than in the absence of Dox (Figure 3.2E-H). However, the Map2-positive cells cultured in OPTI-MEM (Condition 4) appeared less differentiated than in other conditions as evidenced by a lack of neurites (Figure 3.2H). Cells grown under reduced (1% FBS) serum conditions also expressed significant Map2 in the absence of Dox, although the expression was not found in long neurites (Figure 3.2B). This observation is consistent with previous reports suggesting that cultivating EC cells in a low serum environment can cause them to spontaneously differentiate into neurons (Pachernik et al., 2005).

Changing culture media from MEMα to Neurobasal media on the fourth day following Dox treatment (Condition 3) was chosen as the optimal growing condition

because it resulted in the highest percentage of Map2-positive cells in the presence of Dox (Figure 3.2G), while few cells (< 0.1%) expressed Map2 in the absence of Dox (Figure 3.2C). Compared to other commonly used media (e.g. MEMα and DMEM), Neurobasal media has been shown to select against the proliferation of glia and increase neuronal viability (Brewer et al., 1993). Supporting these data, cells differentiated using Neurobasal media (Condition 3) also expressed the highest levels of Map2 protein when quantitated via western blot analysis (Figure 3.2I). Because equal amounts of protein are loaded on the western blot, contributions of undifferentiated cells (seen as blue nuclei in Figures 3.2E, 3.2F and 3.2H) to the total protein significantly dilute the Map2 signal seen on the western blot. Similar immunocytochemistry and western blot experiments with two additional P19 clones showing dox-inducible expression of Ascl1 (designated P19T1A3 and P19T1A12) also demonstrated that Condition 3 was optimal for neuronal differentiation (data not shown).

Under the growth conditions delineated above, the time course of differentiation with the P19T1A2 cells was evaluated. Map2-positive cells first appeared three days after induction of Ascl1. Their total number increased progressively during the time course of differentiation, and the cells adopted a neuronal morphology (Figure 3.3A). Ascl1 protein was detected as early as one day after Dox treatment, remained elevated until three days, and then declined over the remaining four days of differentiation (Figure 3.3B). We attributed the transient down-regulation of Map2 expression at day four to neuronal atrophy prior to the Neurobasal media change that offers the best trophic support (see Figure 3.2C). Neuronal atrophy was further confirmed by our examination of a second major neuronal marker, β-III-tubulin, whose expression was also transiently reduced prior

to the switch to Neurobasal media (data not shown). Supporting these results, we examined the occurrence of apoptosis in P19T1A2 cells and observed expression of cleaved PARP—an apoptotic marker—beginning at four days of differentiation (Figure 3.3C).

P19T1A2 cells respond to Dox in a dose-dependent manner

P19T1A2 cells were exposed to increasing concentrations of Dox (0, 1, 3, 10, 30, 100, 300, and 1000 ng/ml) in culture medium. Immunocytochemistry of P19T1A2 cells treated with varying concentrations of Dox for 24 h showed that individual Ascl1-positive cells became evident at a minimal concentration of 30 ng/ml of Dox, and that the percentage of Ascl1-positive cells increased with higher concentrations of Dox (Figure 3.4A). Ascl1 protein expression by western blot analysis was detectable in cells treated with as low as 3 ng/ml Dox, and was fully induced at 100 ng/ml Dox (Figure 3.4C). When P19T1A2 cells were treated with these same Dox concentrations for eight days, TuJ1 immunoreactivity also increased in a dose-dependent manner (Figure 3.4B). The shortest time to result in maximal TuJ1 staining was determined to be eight days (data not shown). Additionally, cells exposed to higher concentrations of Dox adopted a neuronal morphology and had an overall reduction in the density of the cells. The most striking effect was seen at Dox concentrations of 100 ng/ml or more where clustering of cell bodies and fasciculation of the neurite-like processes was observed. Western blot analysis verified the dose-dependent increase in β-III-tubulin protein expression (Figure 3.4D).

Identification of gene expression changes during Ascl1-induced neuronal differentiation

In order to determine the transcriptional profiles of P19T1A2 cells undergoing neuronal differentiation *in vitro*, we utilized microarray hybridization to characterize genes that were differentially expressed in P19T1A2 cells following Ascl1 induction. The abundance of mRNAs for over 270 known genes was induced four-fold or greater after eight days of differentiation, and the abundance of mRNA for over 80 genes was reduced by four-fold or greater (data not shown). The identities, associated functions, and mRNA fold changes of some key genes are provided in Table 3.1. Multiple values for fold changes represent data generated from distinct probes within the microarray. Many embryonic stem cell markers such as *Pou5F1* (also known as *Oct 3/4*) were observed to decrease in the microarray hybridization. While several of the genes have previously been shown to be Ascl1 regulated, many have not previously been reported to be Ascl1 responsive (e.g. *Npy*, *Fgf5*, and *Igf2*).

To confirm differential expression of selected upregulated genes from the microarray results shown in Table 3.1, western blot analysis was carried out on lysates collected from P19T1A2 cells treated with or without Dox (Figure 3.5A). All four of the protein products of selected genes found to be upregulated in the microarray—Gap43, Isl1, Synaptophysin, and Tau—were also upregulated in the presence of Dox. Each of these genes have previously been reported to play a role in neuronal differentiation and development (Mahalik et al., 1992; Jancsik et al., 1996; Jurata et al., 1996; Daly et al., 2000) and these results suggest that Ascl1 induction resulted in at least some transcriptional changes associated with neuronal differentiation.

A hallmark of neurons is their ability to propagate electrical signals, so we carried out studies to determine whether P19T1A2 cells have the electrophysiological properties

of neurons. Recordings were made from a total of eight cells that had been treated with Dox for six days and had an average resting membrane potential of -39.1 ± 0.6 mV. Five of the eight cells exhibited suprathreshold action potential-like waveforms. A representative action potential-like waveform is presented in Figure 3.5B. Another characteristic of differentiating neurons is their asymmetric development of processes into distinct axons and dendrites. The polarization of axons and dendrites underlies the ability of neurons to integrate and transmit information in the brain. These two types of processes differ from one another in morphology (Goslin and Banker, 1989; Craig and Banker, 1994), capacity for protein synthesis (Miyashiro et al., 1994), and in the molecular constituents of their cytoskeletons and plasma membranes (Barnes and Polleux, 2009). Using immunocytochemistry, we distinguished neurites expressing Map2, a marker of dendrites (Garner et al., 1988), from those that expressed the axonal marker neurofilament-L (NF-L; Szaro and Gainer, 1988; Figure 3.5C). The neurites that expressed Map2 had a shorter, tapered morphology characteristic of dendrites, and varicosities could be seen along the length of certain projections (Figure 3.5C', arrowheads). The neurites that expressed NF-L were more elongated and slender, characteristic of axonal projections. These electrophysiological and immunocytochemical findings suggest that Dox-treated P19T1A2 cells share at least some aspects of neuronal development in common with primary neurons.

Identification of early changes in gene expression following Ascl1 induction

Having confirmed a subset of the microarray results and characterized neuronal differentiation of P19T1A2 cells, we sought to examine the earliest changes in gene expression following Ascl1 induction. P19T1A2 cells were cultured in the presence or

absence of 0.5 μg/ml Dox for 0, 3, 6, 9, 12, 15, 18, 21, and 24 h. RNA and whole cell extracts were then isolated for microarray analysis, RT-PCR, and western blot analysis. Western blotting showed that Ascl1 protein was detectable as early as 3 h after treatment with Dox, and levels remained elevated throughout the 24 h time course (Figure 3.6).

Microarray hybridization results indicated that the mRNA abundance for 28 known genes was induced greater than four-fold after 24 h of Dox treatment. Furthermore, the mRNA abundance for 14 other genes was found to be reduced by greater than four-fold over the same time course (data not shown). Selected genes that were induced or repressed strongly at early time points are shown in Table 3.2. After background subtraction, the mRNA for the Gadd45γ gene was found to show the greatest fold induction (33-fold) after 24 h of Dox treatment.

Validation of the microarray expression data for Gadd45γ was carried out using RT-PCR. Multiple oligonucleotide primer pairs were evaluated for detection of Gadd45γ mRNA (data not shown), and data generated from a representative set are shown. The induction patterns for Gadd45γ mRNA expression were in concordance with the microarray data: elevated expression levels of Gadd45γ mRNA were first detected 12 h after the addition of Dox, and expression continued to increase, reaching a 38-fold induction at 24 h (Figure 3.7A). Upon longer treatment with Dox, we continued to see elevated levels of Gadd45γ mRNA (Figure 3.7B). Using identical Dox exposure as for the microarray and RT-PCR experiments, western blot analysis was performed to confirm that the increase in Gadd45γ mRNA expression also resulted in increased levels of Gadd45γ protein (Figure 3.7C).

Ascl1 regulates transcription of Gadd45γ and employs two proximal E-box consensus sites

bHLH transcription factors such as Ascl1 usually function as transcriptional activators by binding to specific E-box motifs (CANNTG). As described in the Experimental Methods, we identified evolutionarily conserved sequences adjacent to the human and mouse Gadd45γ promoters. We further identified four E-box sequences clustered within these conserved sequences. The promoter region of the human Gadd45γ gene was subcloned into the promoter-less pEL2 reporter vector (1222-EL2), and its transcriptional properties were assayed in wild-type P19 cells via transient transfection in the presence or absence of Ascl1 (Figure 3.8A). A statistically significant, 12-fold increase in the activity of the 1222-EL2 reporter was observed in the presence of Ascl1, consistent with our microarray and RT-PCR results showing induction of Gadd45γ transcription by Ascl1 (see Table 3.2 and Figure 3.7). All of the 5' Gadd45γ truncated constructs except 194- and 188-EL2 were able to drive transcription of the EL2 reporter in P19 cells, suggesting that the 281 bp fragment contains the core promoter elements sufficient to drive transcription in response to Ascl1 (Figure 3.8B). As described in the Experimental Methods, we introduced 4 bp substitution mutations in the two E-box consensus sites designated E3 and E4 and tested their effect on promoter activity. Figure 3.8B also shows the relative reduction in promoter activity of the mutated (281-EL2ΔEbox) versus wild-type (281-EL2) reporter. Mutations in the two E-box sites significantly reduced promoter activity by 4.2-fold, suggesting that the ability of Ascl1 to activate transcription of Gadd45γ was dependent on the presence of these two E-boxes in the promoter.

Recent research has shown cooperative activity between Ascl1 and the POU proteins Brn1 and Brn2 in mediating expression of certain genes critical for neurogenesis (Castro et al., 2006). However, the octameric motif recognized by the Brn transcription factors was not seen in the regions surrounding the essential E-boxes in the Gadd45γ promoter. Furthermore, VISTA analysis did not show any other highly conserved sequences in the Gadd45γ promoter region (data not shown). Therefore, the reduction of reporter activity driven by the Gadd45γ promoter was specifically due to loss of Ascl1 interaction with the core promoter region, and not due to loss of Ascl1 interaction with other DNA-binding cofactors such as the Brns.

Other bHLH factors are known to function together to regulate development of the nervous system (Bertrand et al., 2002). Ascl1 and the Neurogenin family constitute the main proneural proteins in mammals, and research has shown that they can cooperatively regulate neural progenitor cell cycle exit, the specification of neuronal subtype identities, and neuronal migration (Bertrand et al., 2002; Hand et al., 2005; Ge et al., 2006). Recently, the integrated activity of Ascl1 and Neurogenin-2 (Neurog2) with specific E-boxes was shown to temporally regulate Dll3 levels during neural tube development (Henke et al., 2009). To determine whether Neurog2 can also regulate the Gadd45γ promoter, the full-length 1222-EL2 reporter was transiently transfected into P19 cells in the presence or absence of Neurog2 (Figure 3.8C). A significant, 5.2-fold increase was observed in the presence of Neurog2, consistent with preliminary microarray data showing induction of Gadd45γ transcription by Neurog2, but overall to a lesser extent than the induction by Ascl1 (data not shown).

Ascl1 binds directly to the Gadd45γ promoter

To determine whether Ascl1 could bind directly to the Gadd45γ promoter in P19 cells, we performed chromatin immunoprecipitation (ChIP) experiments. Chromatin was immunoprecipitated with an antibody specific to Ascl1 from formaldehyde cross-linked P19T1A2 cells treated with or without Dox for 24 h. To determine whether Ascl1 localized to the Gadd45γ promoter, quantitative RT-PCR amplification was performed using primers encompassing the proximal E-box sequences (E3/E4). Figure 3.9A shows representative ChIP-PCR samples that were stopped in the linear amplification range, run on an agarose gel, and visualized with ethidium bromide. Chromatin immunoprecipitated with Ascl1 antibody from P19T1A2 cells treated with Dox showed significant enrichment (10-fold) for the Gadd45γ promoter sequence containing E-boxes E3 and E4. Negative controls with primers specific to the RPL30 gene had no significant enrichment. In Figure 3.9B, the ChIP-PCR amplification products were quantified and normalized to the input of each sample. Taken together, these results demonstrate that Ascl1 directly binds to the Gadd45γ promoter in differentiating P19T1A2 cells.

Gadd45γ is sufficient to induce a neuronal-like phenotype in P19 cells

In order to more closely examine the transcriptional events initiated by expression of Gadd45γ, a stable P19 cell line (P19T3GIE2) in which expression of both Gadd45γ and EGFP was under control of the TRE promoter was generated (see Experimental Methods). One day after induction of Gadd45γ with Dox, no TuJ1-immunoreactive cells were seen (Figure 3.10A). By eight days, however, a fraction of P19T3GIE2 cells adopted a neuronal morphology and expressed TuJ1 (Figure 3.10B). Preliminary microarray hybridization analyses showed a number of commonly induced or repressed

genes between P19T1A2 and P19T3GIE2 cells. The identities, associated functions, and mRNA fold changes of select genes are provided in Table 3.3.

Western blot analysis at various times following Dox treatment confirmed that P19T3GIE2 cells showed induction of some proteins that are characteristic of a P19T1A2 differentiation program such as β-III-tubulin and Gap43 (Figure 3.10C). β-III-tubulin protein was detected as early as one day post-Dox treatment, with a gradual increase up to eight days. Gap43 protein was detected two days post-Dox treatment, increased at four days, and then declined. Map2 protein expression was not detected at any time point by western blot or immunocytochemistry following Dox treatment of P19T3GIE2 cells (data not shown). Therefore, it appears that while Gadd45γ is sufficient to induce some proteins also induced by Ascl1 (such as β-III-tubulin and Gap43), it is not sufficient to induce others (such as Map2).

In order to validate the induction of β-III-tubulin and Gap43 by Gadd45γ as being Dox-dependent, we conducted western blot analysis on lysates from cells treated with and without Dox at 24, 48, and 120 h (Figure 3.10D). A clear induction of β-III-tubulin protein was seen in P19T3GIE2 cells treated with Dox at all time points shown. Western blot analysis also showed an increase in Gap43 protein at 48 and 120 h. The clear difference in β-III-tubulin expression at earlier time points between cells treated with or without Dox combined with the induction of Gap43 only in Dox-treated cells strongly demonstrates that overexpression of Gadd45γ is sufficient to induce a subset of Ascl1 transcriptional responses in P19 cells. Microarray hybridization analyses showed that Gadd45γ induction did not induce expression of bHLH proteins including Ascl1, Neurog2, and NeuroD2 (data not shown). Western blot analysis confirmed that Ascl1

protein is not induced in P19T3GIE2 cells (Figure 3.10E). Together, these data show that Ascl1 is not required for the Gadd45γ-induced transcription of *β-III-tubulin* or *Gap43*.

Discussion

Despite the importance of neurogenic bHLH families during neuronal development, knowledge of their physiological target genes is still incomplete. Ascl1 is one of the earliest markers expressed in neural progenitor cells and is essential for their survival and differentiation (Parras et al., 2004). Recently, Ascl1 was found to be the only gene within a pool of 19 candidate genes that was sufficient to induce neuron-like cells in mouse fibroblasts (Vierbuchen et al., 2010). Therefore, delineation of the gene regulatory networks controlled by Ascl1 is critical to understanding the transcriptional interactions that control neuronal differentiation. A major challenge to elucidating the Ascl1-induced genetic cascades is the cellular complexity of the developing embryo, as well as the limited number of defined cells that can be obtained from each embryo. These shortcomings may be circumvented by the use of *in vitro* models, such as the P19T1A2 cells characterized here. The P19T1A2 cells demonstrated tight regulation of Ascl1, and varying the levels of Ascl1 expression resulted in varying extents of neuronal differentiation and neurons that were electrically excitable (see Figure 3.5). The P19T1A2 cells can therefore provide a continuous source for generating a large number of stage-specific cells, which facilitates many types of analyses, including large-scale genomic profiling via microarray analysis.

Achieving stable expression of transfected genes in P19 cells has historically been more problematic than other continuous cell lines (McBurney, 1993). Clonal populations

of transformed P19 cells often segregate into non-expressing variants that can rapidly become predominant in the population. At least partially, successfully generating the P19T1A2 cell line was due to use of the human ubiquitin C promoter, first exon, first intron and partial second exon, which resulted in high, sustainable levels of rtTA expression in the P19 cells. This high efficiency of the ubiquitin C promoter in P19 cells has been reported previously (Yu et al., 2005).

To gain insight into the role of Ascl1 in neuronal development, we characterized downstream transcriptional targets of Ascl1. Using microarray hybridization assays of P19T1A2 cells induced to differentiate in the presence of Dox, we identified several genes showing differential expression that are also known to be important for neurite outgrowth, axon guidance, and differentiation. For example, FGF5 is frequently expressed in embryonic tissues and has recently been described as an embryonic stem cell marker (Pelton et al., 2002). We observed a 9-fold decrease in its mRNA expression after eight days of neuronal differentiation (see Table 3.1). In concordance with these data, FGF5 mRNA expression is inhibited during retinoic acid-induced neuronal differentiation (Martinez-Ceballos et al., 2005), and its expression also decreases upon specification of embryonic stem cells to a neuroectodermal fate (Shimozaki et al., 2003). These induced neuronal cells displayed functional neuronal properties such trains of action potentials and synapse formation, as well as polarization into dendritic and axonal domains. The present work also identified a number of genes that were not previously known candidates for regulating neuronal differentiation. For example, microarray hybridization showed that Gadd45γ was the earliest and most highly induced gene, a finding confirmed by RT-PCR and western blot analysis.

Differentiation of neuronal precursors is characterized by a loss of multipotency and cell-cycle exit. Previous studies have shown that simultaneous with the differentiation program induced by proneural bHLH proteins, an anti-proliferative response is also induced through the upregulation of cell cycle inhibitors such as the cyclin kinase inhibitor $p27^{kip1}$ (Farah et al., 2000). Several studies have shown that the Gadd45 proteins also regulate the cell cycle via interactions with PCNA (Smith et al., 1994), cyclin-dependent kinase inhibitor p21 (Yang et al., 2000), and Cdc2 to inhibit Cdc2-cyclin B1 kinase activity (Zhan et al., 1999). In addition, Gadd45 proteins activate the p38/Jun N-terminal kinase pathway by binding to MTK1/MEKK4 in response to environmental stress (Takekawa and Saito, 1998). Induction of Gadd45 genes in cell culture was shown to stop the cell cycle in G1 phase (Zhang et al., 2001), which is compatible with cell cycle exit—a requirement for terminal neuronal differentiation. Gadd45γ was identified in the medaka *Oryzias latipes* as a gene differentially expressed in the regions in which cells stop dividing and begin differentiating, e.g. the optic tectum, and the hypothalamic and telencephalic ventricles (Candal et al., 2004). It is therefore plausible that the upregulation of Gadd45γ observed in response to Ascl1 is important for the cell cycle withdrawal that precedes neuronal differentiation.

Novel roles for Gadd45 are also becoming apparent: a recent study identified Gadd45 as one factor in a system of proteins involved in the demethylation process in zebrafish embryos (Rai et al., 2008), and Gadd45β was found to be required for activity-induced DNA demethylation of specific promoters and expression of corresponding genes critical for adult neurogenesis (Ma et al., 2009). Since upregulation of Gadd45 proteins affects cell cycle regulation, cell survival, and cell death—all of which are

important processes during neuronal development—and because Gadd45γ is one of the earliest and most highly expressed genes in our model system of neuronal differentiation, it will be important in future studies to determine which of these multiple roles Gadd45γ fulfills in the gene regulatory network that guides Ascl1-induced neuronal differentiation.

In preliminary studies, we were unable to determine whether Gadd45γ was required for Ascl1-induced differentiation, because five shRNA constructs targeted against the mouse Gadd45γ gene were ineffective in sufficiently reducing Gadd45γ mRNA or protein levels in P19T1A2 cells. Despite this, our findings that Ascl1 overexpression drives P19 cells towards exit from the cell cycle and generation of neurons *in vitro*, and that Gadd45γ is strongly induced soon after Ascl1 induction suggests that Gadd45γ couples cell cycle exit to neuronal differentiation.

Research has shown that regulation of histone acetylation/deacetylation levels is essential for murine Gadd45γ promoter control, and that functional Oct and NF-Y elements are essential for basal expression of the promoter (Campanero et al., 2008). Gadd45γ has also previously been implicated as a transcriptional target of bHLH proteins. In *Xenopus*, injection of Ngnr1 or NeuroD promoted ectopic expression of Gadd45γ (de la Calle-Mustienes et al., 2002). Furthermore, Gadd45γ was identified as a direct NeuroD responsive target gene, with conserved induction in mammalian cells (Seo et al., 2007). Microarray studies from gain- and loss-of-function analyses in developing mouse dorsal or ventral telencephalon also identified Gadd45γ as an Ascl1 target gene, with expression predominantly in the subventricular zone (Gohlke et al., 2008). However, these previous studies did not identify promoter elements required for Gadd45γ transcriptional regulation by Ascl1 and did not characterize the Gadd45γ protein.

Our results provide the first evidence for a direct regulation of the Gadd45γ gene by Ascl1. We demonstrated the importance of a 281 bp region of the Gadd45γ promoter for Ascl1 induction: when E-boxes within this region were destroyed, the promoter displayed almost complete loss of Ascl1-induced activity (see Figure 3.8). Furthermore, ChIP-PCR analysis showed that Ascl1 binds directly to the Gadd45γ promoter in differentiating P19T1A2 cells (see Figure 3.9). The overlap between the Ascl1 and Gadd45γ gene regulatory networks suggests that Gadd45γ acts downstream of Ascl1 *in vitro* (see Figure 3.10 and Table 3.3). However, we have also identified genes belonging to the Ascl1 gene regulatory network, such as Map2 and Synaptophysin, whose expression appears not to be affected by over-expression of Gadd45γ (data not shown). These data suggest that Ascl1 requires the induction of other genes in addition to Gadd45γ in order to generate a more complete neuronal differentiation program. Recent research has shown that Ascl1 alone is sufficient to induce some neuronal traits in mouse fibroblasts, but additional factors such as Brn2 and Myt1l are necessary to facilitate neuronal conversion and maturation (Vierbuchen et al., 2010). In our microarray hybridization assays of P19T1A2 cells treated with Dox, we also see an increase of Myt1l expression after Ascl1 induction, supporting the conclusion that while Ascl1 is sufficient to induce immature neuronal features, expression of other downstream factors are necessary to generate mature neurons with high efficiency.

Neuronal differentiation *in vivo* is the result of extrinsic cues such as retinoic acid, bone-morphogenic antagonists, and cell surface molecules activating and altering intrinsic genetic programs within neural stem and progenitor cells. The bHLH proteins, such as Ascl1, function at critical points in these genetic programs to generate fully

differentiated neurons at the proper developmental time and anatomical position within the embryo (Rodriguez et al., 1990; Guillemot et al., 1993; Horton et al., 1999). The P19T1A2 cells in which Ascl1 is tightly regulated by Dox will allow for more detailed elucidation of Ascl1 regulated genetic programs. While microarray hybridization assays are beneficial for discerning gene regulatory networks of neuronal differentiation, the importance of determining global changes in protein expression could also significantly enhance the fidelity of network modeling. Protein translation and stability are often regulated separately from mRNA, and as such there is often a lack of correlation between changes in protein levels and changes in mRNA levels. Furthermore, post-translational modifications such as phosphorylation, proteolysis, and ubiquitination can drastically alter protein function but are outside the scope of microarray hybridization studies. The ease with which the P19T1A2 cells can be grown, and their reliability to yield a high percentage of cells expressing neuronal proteins makes this cell line a robust system for proteomic analysis as well as for high-throughput chemical and RNAi screening. In the long term, such studies may help to guide the development of differentiation strategies for human embryonic stem cells in the treatment of human neurodegenerative diseases.

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Figure 3.1. Generation of rtTA-stable P19 cells. A. Relative luciferase activity in five putative P19 clones stably transfected with the rtTA expression plasmid pUS2-TetOnAdv. Compared to the negative control of wild-type P19 cells, clones P19T3, P19T1, and P19T6 showed significant induction of luciferase activity, but only P19T1 showed sustained induction after multiple passages. Results are shown as mean firefly luciferase expression levels relative to renilla luciferase controls ± s.d. **B**. Expression of the rtTA protein in the three P19 clones that showed strong Dox regulation of luciferase activity in (A). Western blot analysis (50 μg of protein/lane) using an antibody against rtTA showed that clone P19T1, which had the highest sustained induction of luciferase activity in the presence of Dox, also expressed the most protein.

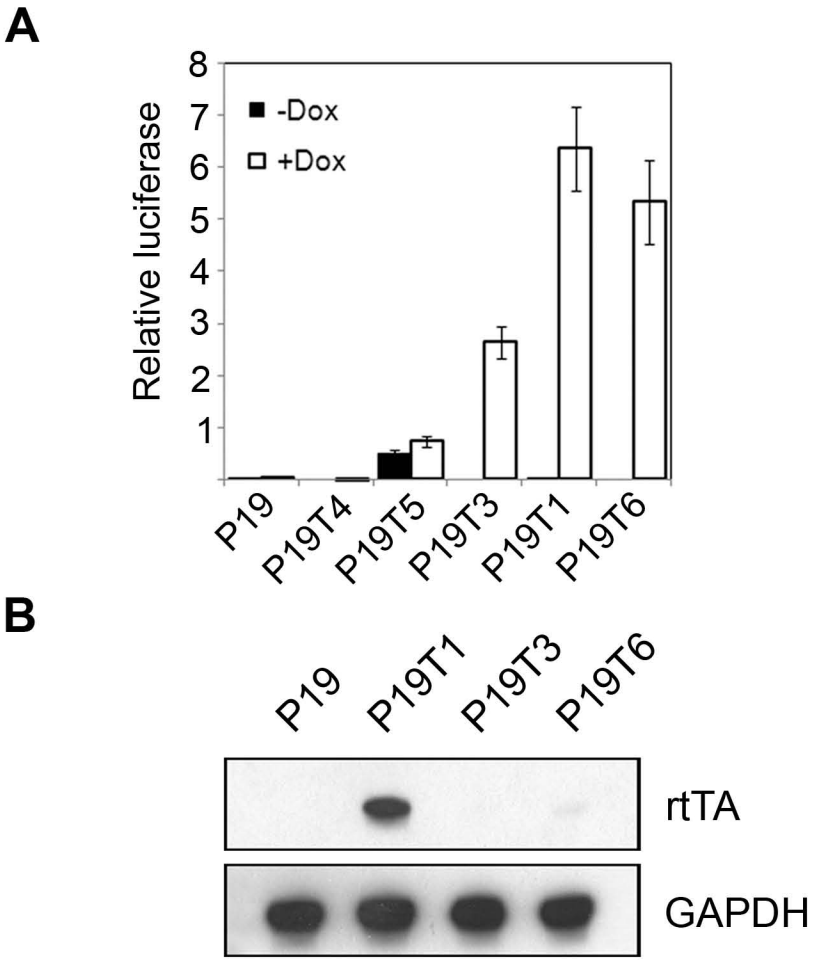
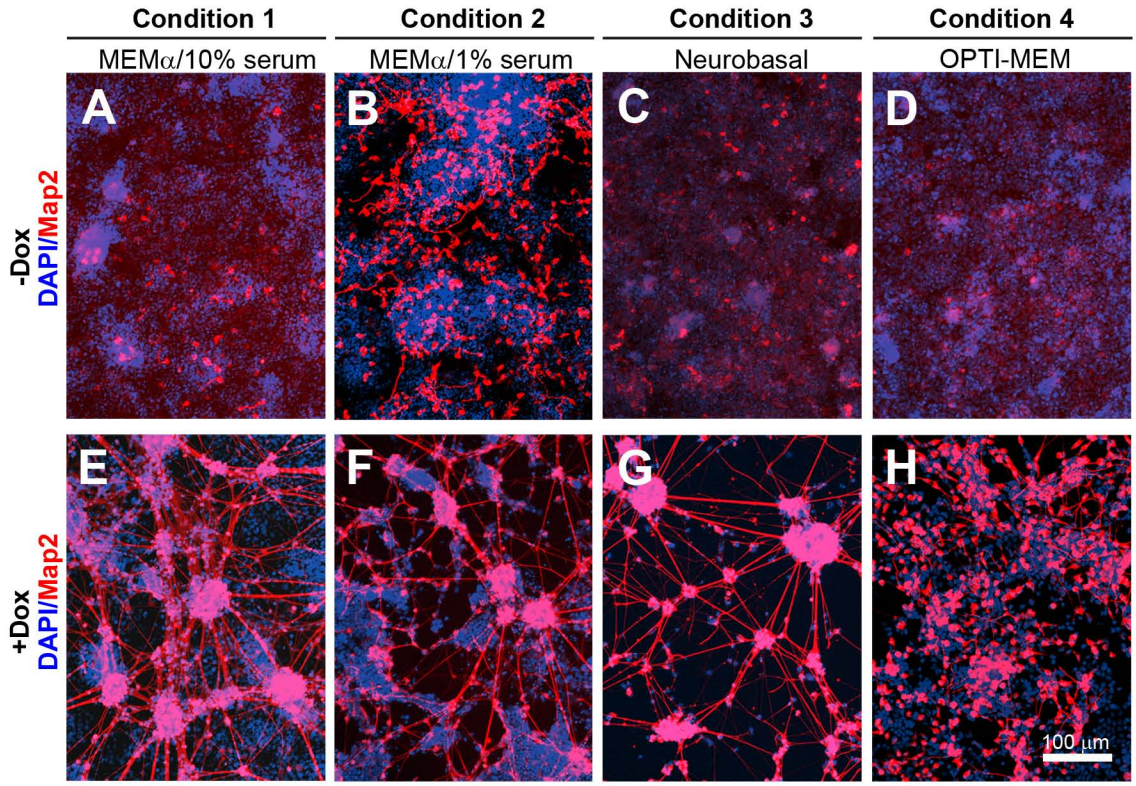

A
Relative luciferase
8
7
6
5
4
3
2
1
-Dox
+Dox
P19
P19T4
P19T5
P19T3
P19T1
P19T6
B
P19
P19T1
P19T3
P19T6
rtTA
GAPDH

Figure 3.2. Optimization of growth conditions for differentiation. A-H. Map2 (red) staining of P19T1M2 cells treated ± Dox at 0.5 μg/ml for 10 days under the following growth conditions: (1) MEMα (7.5% CS, 2.5% FBS), (2) MEMα (1% FBS), (3) MEMα (7.5% CS, 2.5% FBS) for the first three days of differentiation, followed by a switch to Neurobasal media (B27, GlutaMAX), and (4) OPTI-MEM (1% FBS). Nuclei were visualized with DAPI and appear blue. Under the first three growth conditions, cells differentiated into neurons that were immunoreactive to Map2 in the presence of Dox (E-G), although cells grown in reduced serum were immunoreactive to Map2 protein in the absence of Dox (B). Cells grown in OPTI-MEM resulted in Map2-positive cells that were morphologically less differentiated than the other conditions (H). Scale bar = 100 μm. **I**. Western blotting for expression of Map2 protein under the growth conditions shown in (A-H). The highest amount of Map2 protein was observed in cells cultivated in Neurobasal media (Condition 3).


Condition 1
Condition 2
Condition 3
Condition 4
MEMα/10% serum
MEMα/1% serum
Neurobasal
OPTI-MEM
A
B
C
D
-Dox
DAPI/Map2
E
F
G
H
+Dox
DAPI/Map2
100 μm

I

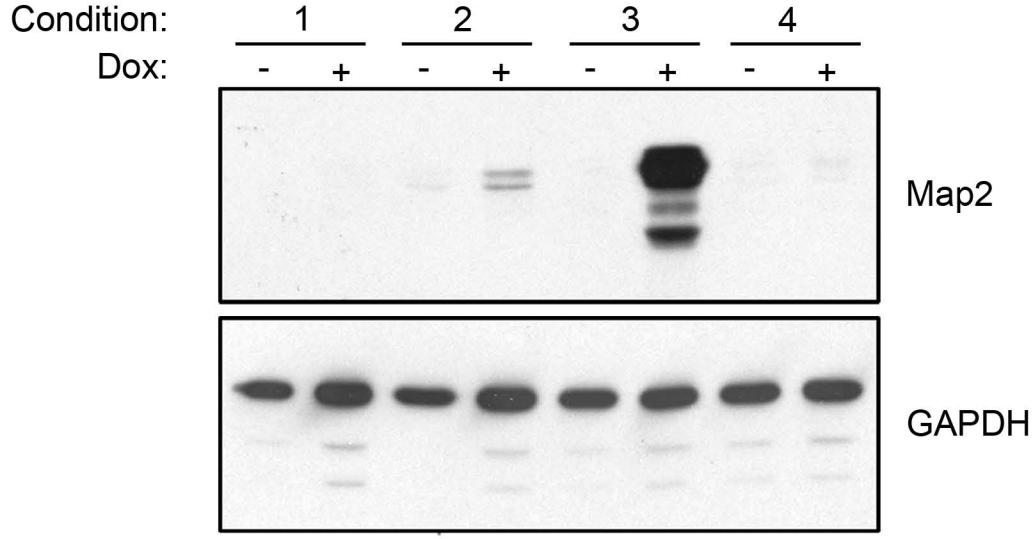

Condition:
1
2
3
4
Dox:
- + - + - + - +
Map2
GAPDH

Figure 3.3. Time course of differentiation with P19T1M2 cells. A. Map2 (green) staining of P19T1M2 cells treated ± Dox at 0.5 μg/ml for the indicated days. Nuclei were visualized with DAPI and appear blue. In the absence of Dox, no Map2-positive cells were observed. In the presence of Dox, Map2-positive cells became evident after three days of treatment with Dox. Cells expressing Map2 underwent neuritogenesis by day five, and by day eight, the somas of Map2-positive cells began to cluster together, while the neurites became elongated and better defined. Scale bar = 100 μm. **B**. Western blot for expression of Map2 and Ascl1 protein during the time course of differentiation of P19T1M2 cells. **C**. Western blot for expression of cleaved PARP protein in P19T1M2 cells.

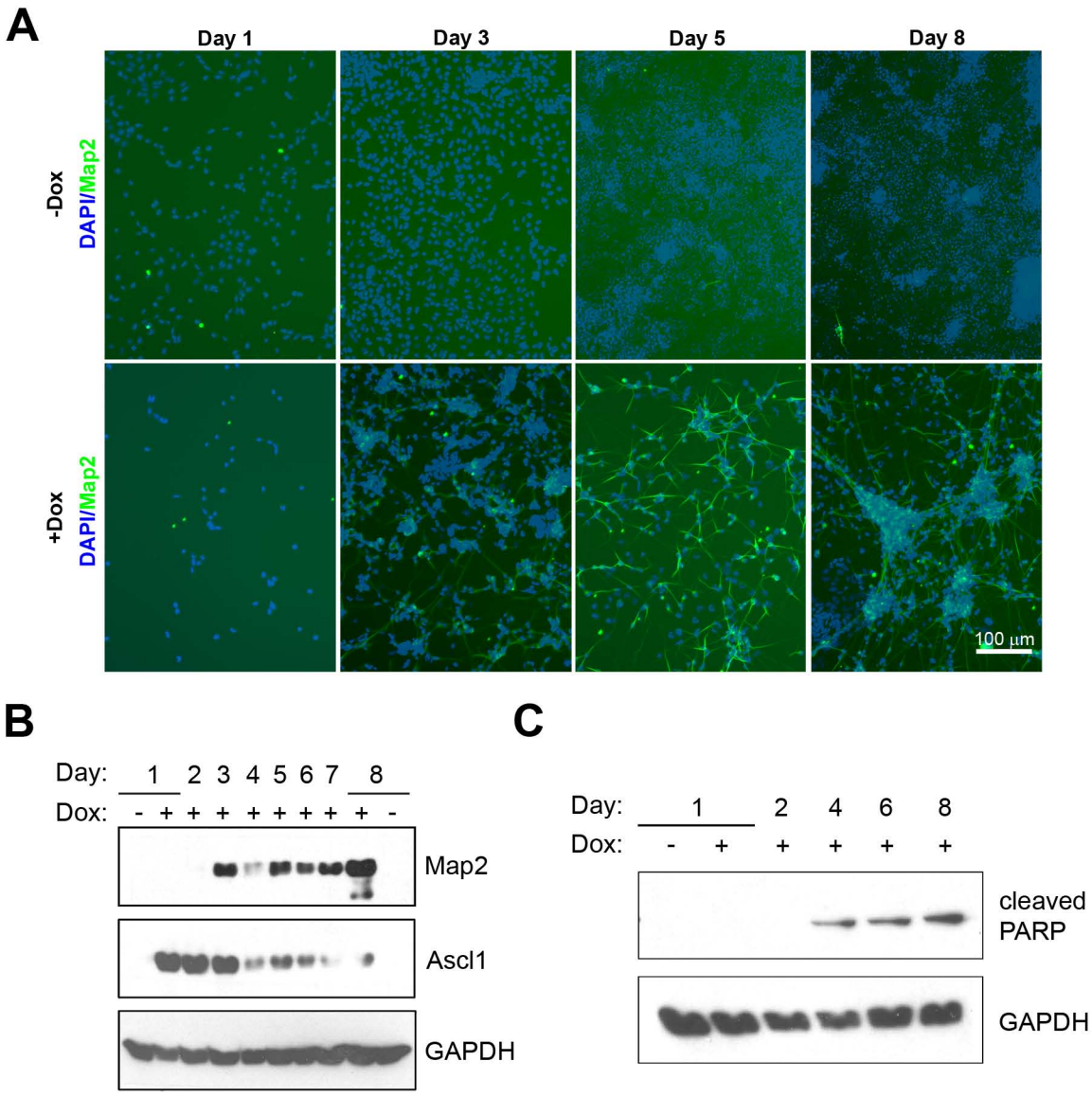

A
Day 1
Day 3
Day 5
Day 8
-Dox
DAPI/Map2
+Dox
DAPI/Map2
100 μm
B
Day: 1 2 3 4 5 6 7 8
Dox: - + + + + + + + + -
Map2
Ascl1
GAPDH
C
Day: 1 2 4 6 8
Dox: - + + + + +
cleaved PARP
GAPDH

Figure 3.4. P19T1M2 cells express Ascl1 and differentiate in response to Dox in a dose-dependent manner. A. Ascl1 (red) staining of P19T1M2 cells treated with the indicated concentrations of Dox for 24 h. Treatment of P19T1M2 cells with as low as 3 ng/ml of Dox resulted in sporadic Ascl1-positive cells, and the number and intensity of Ascl1-positive cells increased with greater concentrations of Dox. **B**. TuJ1 (red) images P19T1M2 cells treated with the indicated concentrations of Dox. P19T1M2 cells expressed TuJ1 in a dose-dependent manner. Furthermore, in the highest concentrations of Dox, the total number of cells was reduced due to the inhibition of proliferation resulting from neuronal differentiation. Scale bars = 100 μm. **C, D**. Western blot analysis for expression of Ascl1 (C) and β-III-tubulin (D) protein showed that protein expression correlates with the results shown in (A and B).

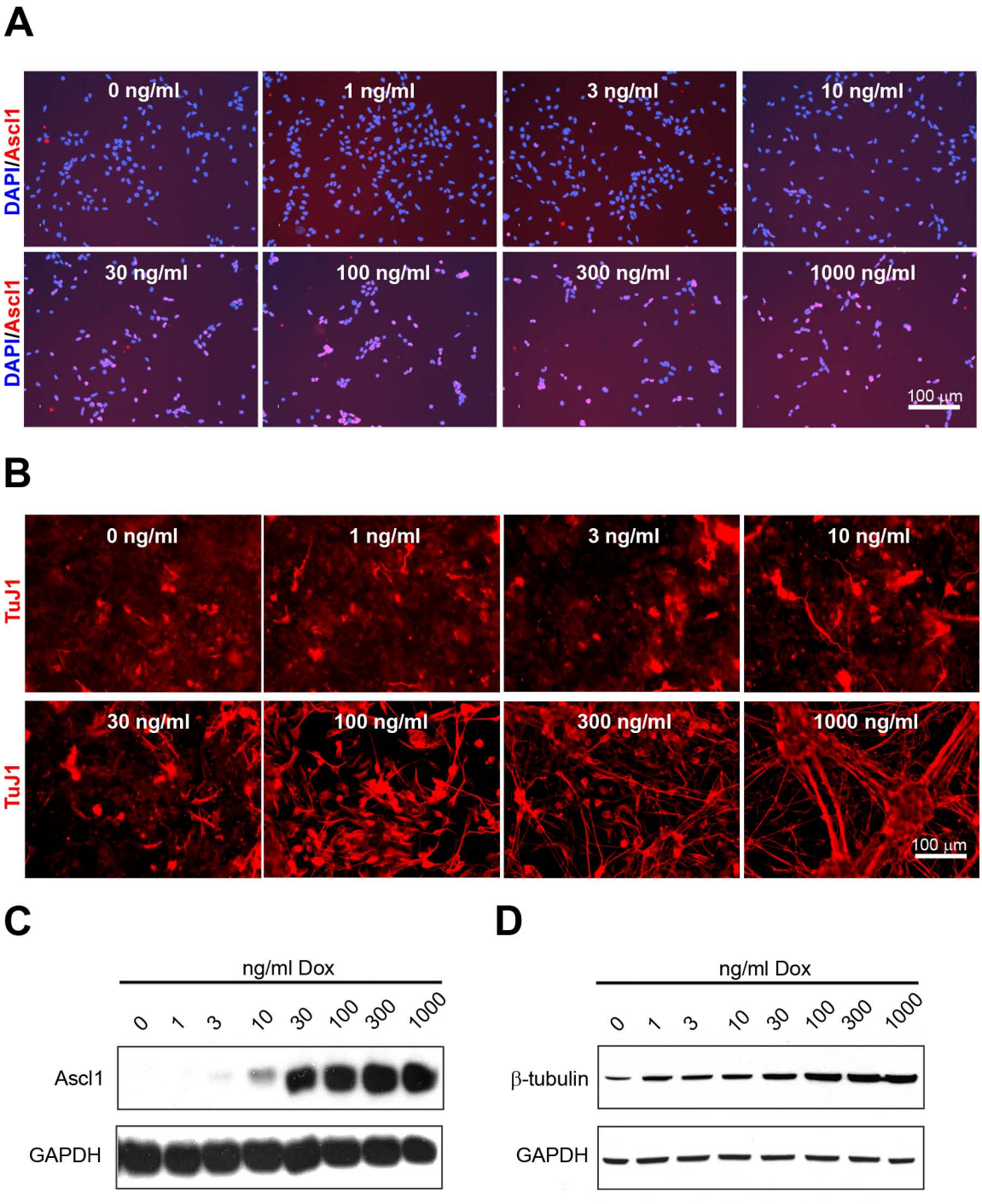

A
DAPI/Ascl1
0 ng/ml
1 ng/ml
3 ng/ml
10 ng/ml
DAPI/Ascl1
30 ng/ml
100 ng/ml
300 ng/ml
1000 ng/ml
100 μm
B
TuJ1
0 ng/ml
1 ng/ml
3 ng/ml
10 ng/ml
TuJ1
30 ng/ml
100 ng/ml
300 ng/ml
1000 ng/ml
100 μm
C
ng/ml Dox
0 1 3 10 30 100 300 1000
Ascl1
GAPDH
D
ng/ml Dox
0 1 3 10 30 100 300 1000
β-tubulin
GAPDH

Figure 3.5. P19T1M2 cells show characteristics of mature neurons. A. Western blot analysis examining the protein expression of selected Ascl1-induced target genes identified in the microarrays. As predicted from the microarray results, expression of Gap43, Isl1, Synaptophysin (Syp), and Tau proteins increased in response to Dox-induced overexpression of Ascl1. **B**. P19T1M2 cells have the electrophysiological properties of neurons. Shown is a representative action potential-like waveform recorded from a P19T1M2 cell grown in the presence of Dox for six days. **C, C'**. P19T1M2 cells are polarized. Immunostaining for expression of the dendritic marker Map2 (red) and the axonal marker Neurofilament-L (NF-L, green). Nuclei were visualized with DAPI staining and appear blue. The boxed area in (C) is enlarged in panel (C'), with dendritic varicosities indicated by white arrowheads.

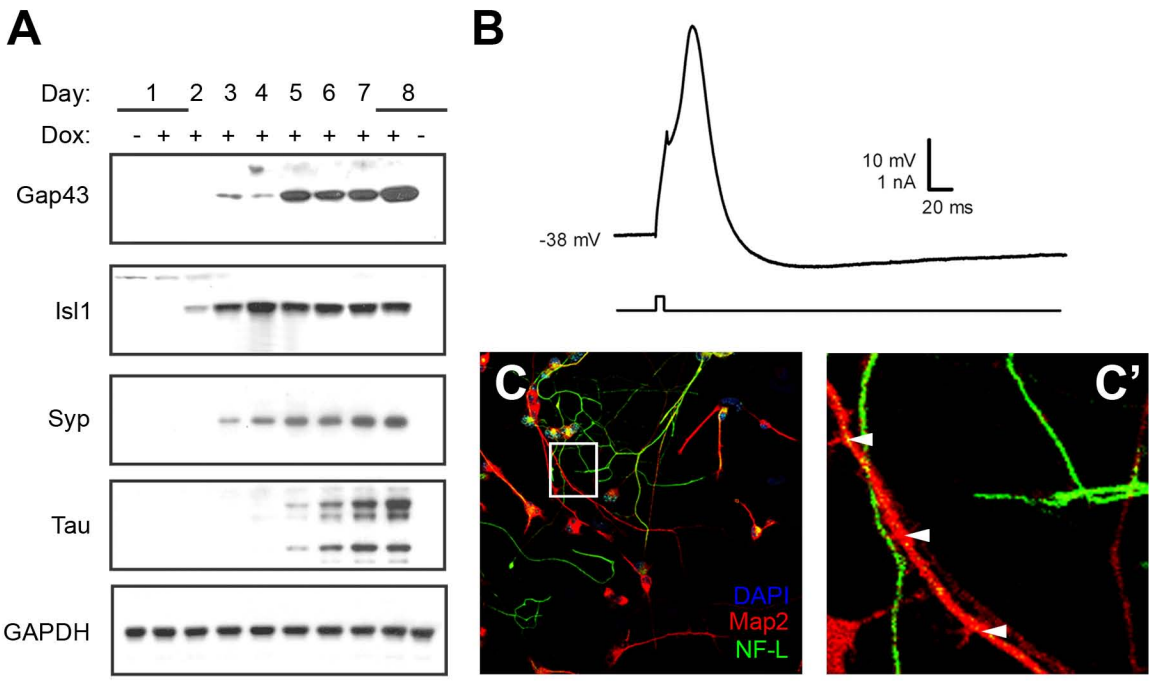

A
Day: 1 2 3 4 5 6 7 8
Dox: - + + + + + + + -
Gap43
Isl1
Syp
Tau
GAPDH
B
10 mV
1 nA
20 ms
-38 mV
C
DAPI
Map2
NF-L
C'

Figure 3.6. Early Ascl1-expression in P19M1T2 cells. Western blot analysis of P19T1M2 cells treated with Dox for the indicated hours. Ascl1 protein was induced as early as 3 h after the addition of Dox. Ascl1 expression became saturated at 9 h and remained elevated for the duration of the time course.

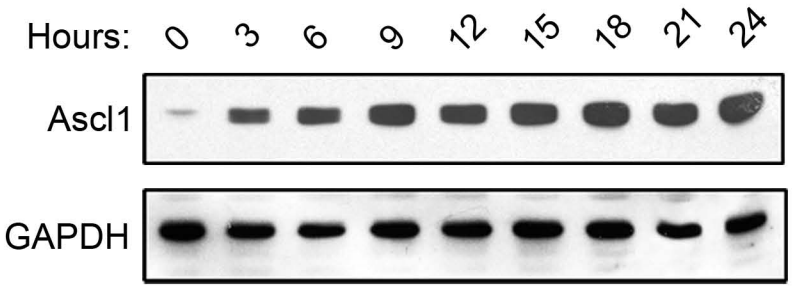

Hours:
0
3
6
9
12
15
18
21
24
Ascl1
GAPDH

Figure 3.7. Gadd45γ-immunoreactive protein is induced during P19T1A2 neuronal differentiation. A, B. RT-PCR analysis of early (A) and late (B) stages of Ascl1-induced neuronal differentiation showed that Gadd45γ mRNA expression increased in response to Ascl1, remained elevated through day four, and then declined. Results are shown as the mean ± s.d. normalized to GAPDH levels. **C**. Western blot analysis showing induction of Gadd45γ protein expression during Dox-induced differentiation of P19T1M2 cells.

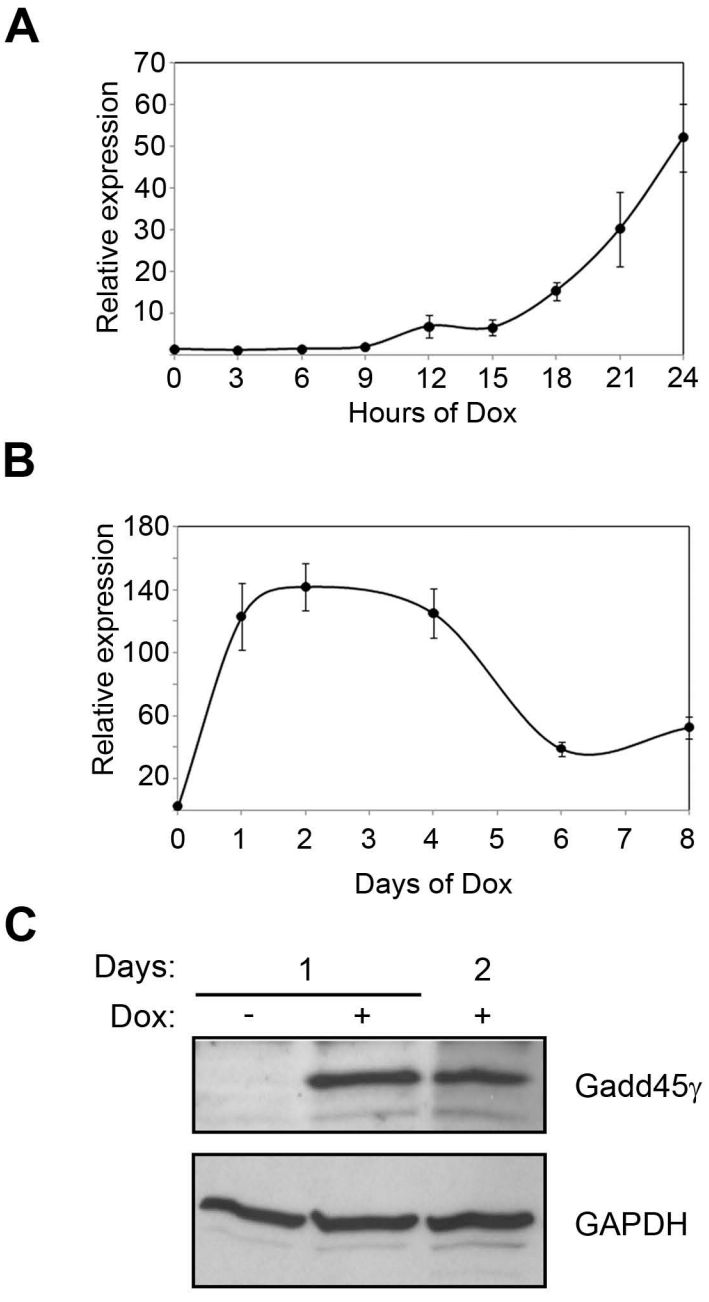

A
Relative expression
70
60
50
40
30
20
10
0 3 6 9 12 15 18 21 24
Hours of Dox
B
Relative expression
180
140
100
60
20
0 1 2 3 4 5 6 7 8
Days of Dox
C
Days: 1 2
Dox: - + +
Gadd45γ
GAPDH

Figure 3.8. Direct transcriptional regulation of Gadd45γ by Ascl1. A. Transcriptional activity of the Gadd45γ promoter increased 12-fold in the presence of Ascl1. Results are expressed as mean relative luciferase activity, with error bars denoting standard deviation. *$p < 0.01$. **B**. Transcriptional activity of 5' Gadd45γ deletion reporters in response to Ascl1. Schematics of pEL2 reporter constructs used are shown on the y-axis. Deletion analyses localized the necessary Ascl1 regulatory element to within 281 bp upstream of the transcriptional start site, and mutational analysis showed that augmentation of Gadd45γ transcription was dependent on the presence of two proximal E-boxes. * $p < 0.01$ as compared to the wild-type 281-EL2 reporter. **C**. Transcriptional activity of the Gadd45γ promoter increased 5.2-fold in the presence of Neurog2. Results are expressed as mean relative luciferase activity, with error bars denoting standard deviation. *$p < 0.01$.

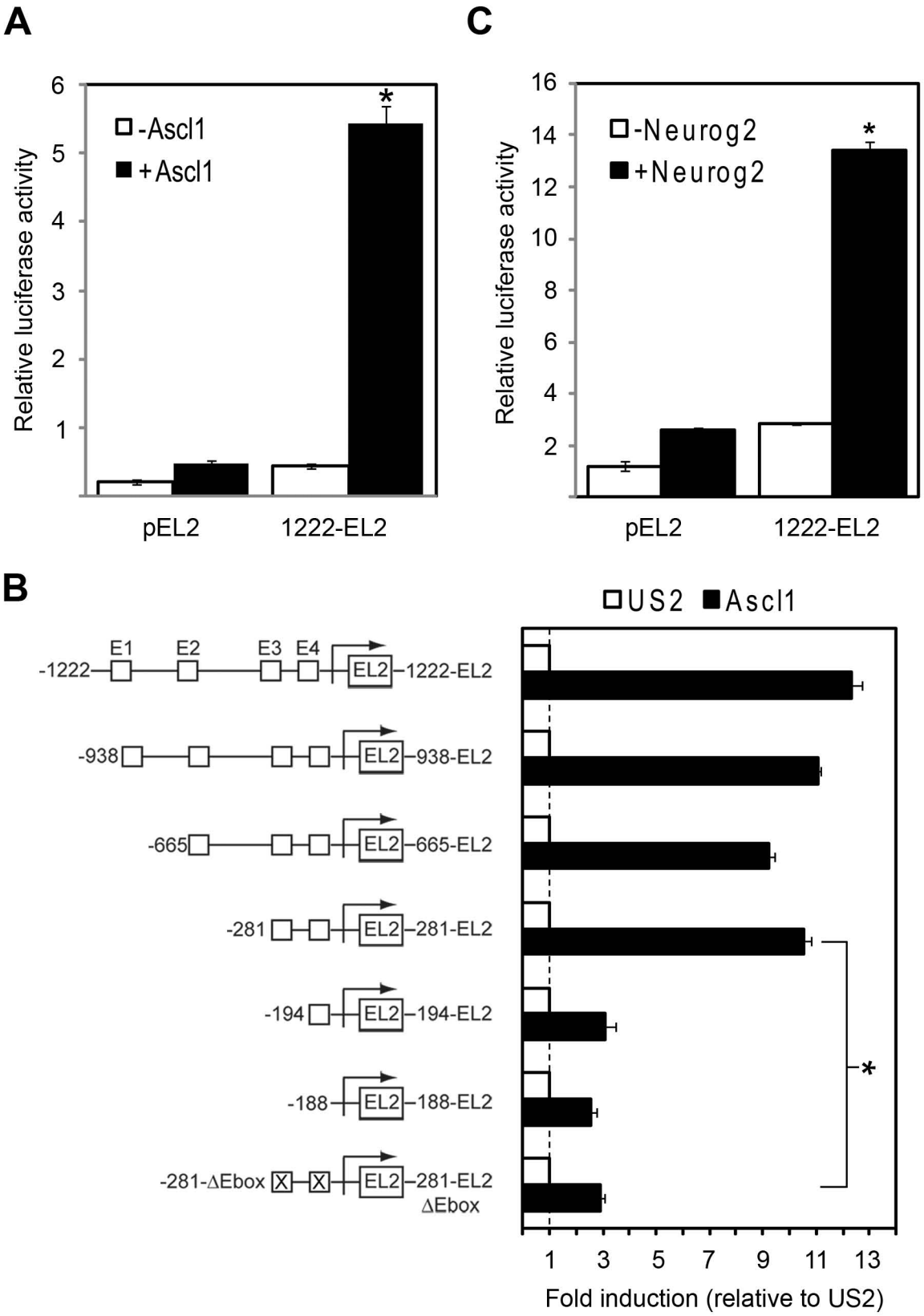

A
Relative luciferase activity
-Ascl1
+Ascl1
pEL2
1222-EL2
C
Relative luciferase activity
-Neurog2
+Neurog2
pEL2
1222-EL2
B
US2
Ascl1
E1
E2
E3
E4
EL2
-1222
1222-EL2
-938
938-EL2
-665
665-EL2
-281
281-EL2
-194
194-EL2
-188
188-EL2
-281-ΔEbox
281-EL2 ΔEbox
Fold induction (relative to US2)

Figure 3.9. Ascl1 occupies the regulatory regions containing E-boxes E3 and E4 of Gadd45γ in P19T1M2 cells. A. P19T1M2 cells treated with or without Dox for 24 h were subjected to ChIP assays using anti-Ascl1 antibody (+Ab) or control IgG (-Ab) followed by real-time PCR assays to detect the Gadd45γ and RPL30 promoter DNAs. Representative ChIP-PCR analyses were stopped in the linear amplification range and run on an agarose gel for visualization with ethidium bromide. Input (In) samples were loaded as a control. **B**. ChIP-PCR analysis of the binding of Ascl1 to Gadd45γ enhancers in P19T1M2 cells using primers specific to Gadd45γ (black bars) or a control gene (RPL30; white bars). Data are presented as the mean percentage of input ± s.d. of the results from three independent experiments.

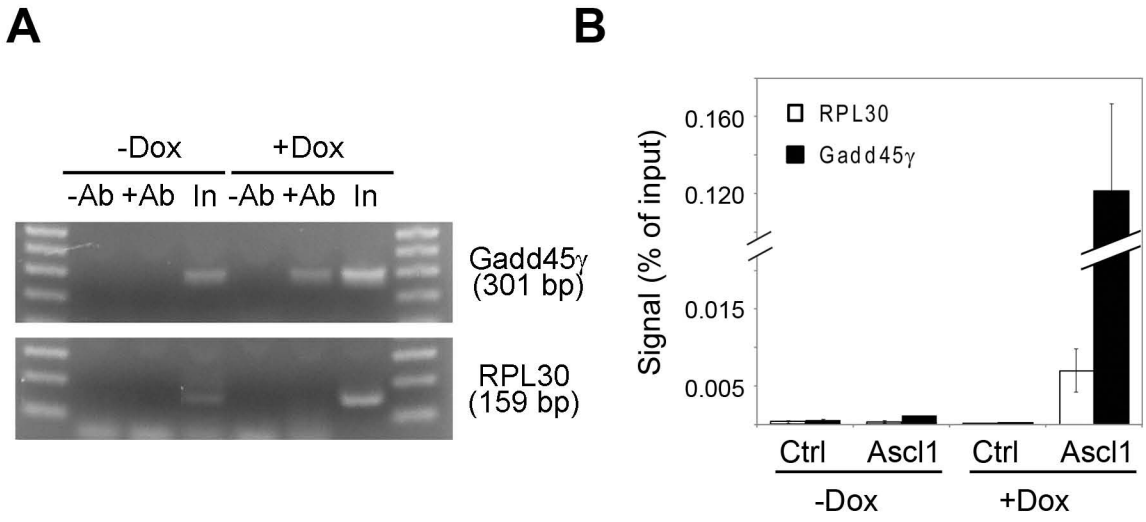

A
-Dox
+Dox
-Ab +Ab In -Ab +Ab In
Gadd45γ
(301 bp)
RPL30
(159 bp)
B
RPL30
Gadd45γ
Signal (% of input)
0.160
0.120
0.015
0.005
Ctrl
Ascl1
Ctrl
Ascl1
-Dox
+Dox

Figure 3.10. Overexpression of Gadd45γ is sufficient to induce a neuronal-like phenotype. A-B. TuJ1 (red) staining of P19T3GIE2 cells one day (A) and eight days (B) after treatment with Dox. Nuclei were visualized with DAPI and appear blue. Eight days after treatment with Dox, P19T3GIE2 cells adopted a neuronal morphology and expressed TuJ1. Scale bar = 50 μm. **C**. Western blotting for protein expression in P19T3GIE2 cells treated with Dox for the indicated days. Gadd45γ was induced one day post-Dox, and then declined. P19T3GIE2 cells also showed induction of proteins characteristic of a P19T1M2 induction, e.g. β-III-tubulin and Gap43. **D**. Western blotting using antibodies against β-III-tubulin and Gap43 showed that overexpression of Gadd45γ was sufficient to induce expression of neuronal protein markers in a Dox-dependent manner. **E**. Western blot analysis for Ascl1 expression in P19T1M2 and P19T3GIE2 cells. Ascl1 protein expression is strongly induced in P19T1M2 cells one day after treatment with Dox and then declines. No Ascl1 expression was detected in P19T3GIE2 cells at any time point.

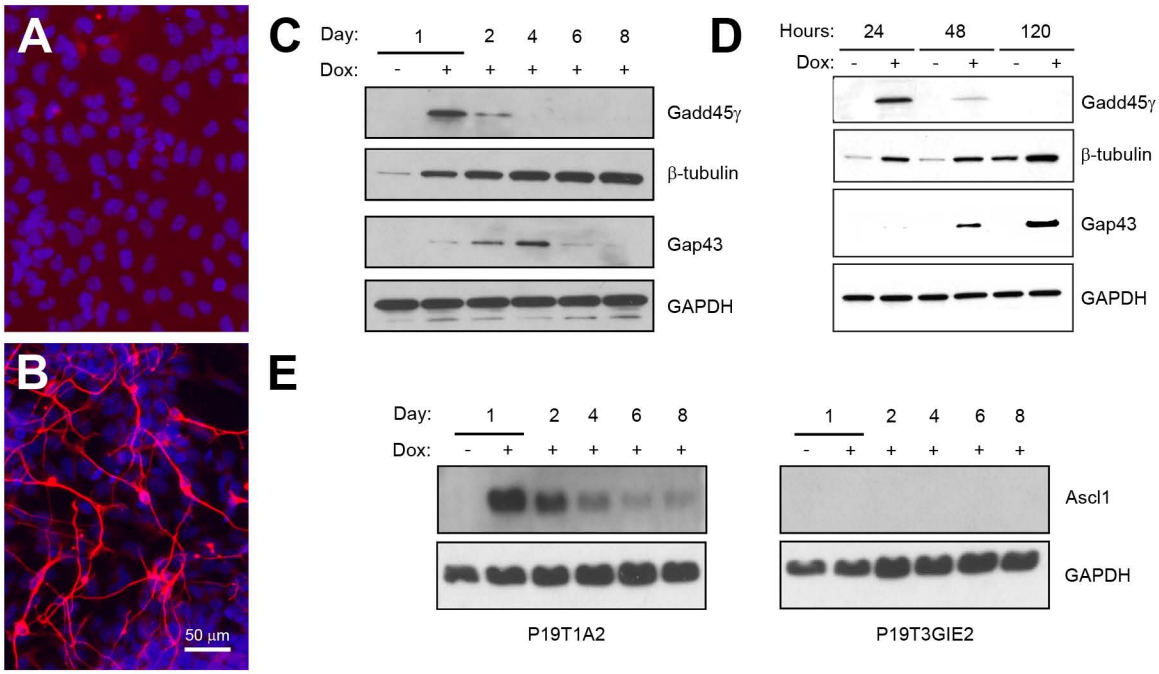

A
B
50 µm
C
Day: 1 2 4 6 8
Dox: - + + + + +
Gadd45γ
β-tubulin
Gap43
GAPDH
D
Hours: 24 48 120
Dox: - + - + - +
Gadd45γ
β-tubulin
Gap43
GAPDH
E
Day: 1 2 4 6 8
Dox: - + + + + +
Ascl1
GAPDH
P19T1A2
1 2 4 6 8
- + + + + +
Ascl1
GAPDH
P19T3GIE2

Table 3.1. Selected gene expression changes by microarray analysis at eight days following Ascl1 induction

Gene Symbol	Gene Name(s)	Function/Process	Fold Change(s)
Igfbp5	Insulin-like growth factor binding protein 5	Growth factor binding	(75, 59, 59)
Dner	Delta/notch-like EGF-related receptor	Notch binding	(65)
Mapt	Microtubule-associated protein tau	Negative regulation of microtubule depolymerization	(35)
Isl1	ISL1 transcription factor	DNA binding; neuron differentiation	(31)
Chgb	Chromogranin B	Protein binding	(27)
Map2	Microtubule-associated protein 2	Cytoskeletal regulatory protein binding	(24)
Igf2	Insulin-like growth factor 2	Growth factor activity	(23)
Syp	Synaptophysin	Syntaxin-1 binding; synaptic transmission	(22)
Slc17A6	Solute carrier family 17; Vglut2	L-glutamate transmembrane transporter activity	(19, 18)
Svop	SV2 related protein	Ion transmembrane transporter activity	(19)
Syt4	Synaptotagmin IV	Calcium ion binding; neurotransmitter secretion	(17)
Gap43	Growth associated protein 43	Calmodulin binding; axon guidance	(7.5)
Dnmt3b	DNA methyltransferase 3B	DNA (cytosine-5-)-methyltransferase activity	(0.29, 0.15)
Folr1	Folate receptor 1	Folic acid transporter activity	(0.19, 0.11)
Pou5f1	POU domain, class 5, transcription factor 1	DNA binding; cell fate commitment	(0.13, 0.10)
FGF5	Fibroblast growth factor 5	Growth factor activity; cell proliferation	(0.11)
Lefty	Left right determination factor 1	Growth factor activity; anterior/posterior axis specification	(0.11)
Npy	Neuropeptide Y	G-protein coupled receptor binding; neuropeptide signaling	(0.05)

Table 3.2. Selected gene expression changes by microarray analysis at one day following Ascl1 induction

Gene Symbol	Gene Name(s)	Function/Process	Fold Change(s)
Gadd45γ	Growth arrest and DNA-damage-inducible 45 gamma	Protein binding; regulation of cell cycle	(33, 16)
Ankrd1	Ankyrin repeat domain 1	Transcription corepressor activity	(25, 16)
Unc45b	Unc-45 homolog B	Binding; cell differentiation	(19, 1.5)
Rgs16	Regulator of G-protein signaling 16	GTPase activator activity; GPCR signaling	(17)
Igfbp5	Insulin-like growth factor binding protein 5	Growth factor binding	(15, 13, 9)
Cdk5r1	Cyclin-dependent kinase 5; regulatory subunit 1 (p35)	Kinase activity; axon guidance	(15)
Ngfr	Nerve growth factor receptor (p75NTR)	Receptor activity; apoptosis; axon guidance	(14, 1.2, 1.1)
Cdh3	Cadherin 3 (P-cadherin)	Calcium ion binding; hemophilic cell adhesion	(13, 11, 5, 1.8)
Idb2	Inhibitor of DNA binding 2	Transcription regulator activity	(13)
Idb1	Inhibitor of DNA binding 1	Transcription regulator activity; BMP signaling	(12)
Nsg2	Neuron specific gene family member 2	Dopamine receptor binding; dopamine signaling	(11, 9, 1.0, 1.0)
Mfng	MFNG glucosaminyltransferase (manic fringe)	Glycosyl transferase activity; organismal development	(9.4, 4.0)
Nanog	Nanog homeobox	DNA binding; embryonic development	(0.21, 0.61)
Slc35d3	Solute carrier family 35, member D3 (fringe-like 1)	Sugar:hydrogen symporter activity; transport	(0.21)
Glra2	Glycine receptor, alpha 2	GABA-A receptor activity; chloride channel activity	(0.19)
Tdgf1	Teratocarcinoma-derived growth factor 1 (Cripto-1)	Growth factor activity; activation of MAPK activity	(0.19)
Eras	ES cell-expressed Ras (HRAS2)	GTP binding; small GTPase signal transduction	(0.17)
Trh	Thyrotropin releasing hormone	Hormone activity; hormone-mediated signaling	(0.16)

Table 3.3. Selected gene expression changes by microarray analysis at eight days following Gadd45γ induction

Gene Symbol	Gene Name(s)	Function/Process	Fold Change(s)
Igfbp5	Insulin-like growth factor binding protein 5	Growth factor binding	(20, 20, 15)
Dner	Delta/notch-like EGF-related receptor	Notch binding	(9)
Map2	Microtubule-associated protein 2	Cytoskeletal regulatory protein binding	(6)
Slc17A6	Solute carrier family 17; Vglut2	L-glutamate transmembrane transporter activity	(15, 13)
Svop	SV2 related protein	Ion transmembrane transporter activity	(5)
Syt4	Synaptotagmin IV	Calcium ion binding; neurotransmitter secretion	(8)
Pou5f1	POU domain, class 5, transcription factor 1	DNA binding; cell fate commitment	(0.18, 0.17)
FGF5	Fibroblast growth factor 5	Growth factor activity; cell proliferation	(0.16)
Lefty	Left right determination factor 1	Growth factor activity; anterior/posterior axis specification	(0.06)
Npy	Neuropeptide Y	G-protein coupled receptor binding; neuropeptide signaling	(0.06)

Table 3.4. Oligonucleotides used in the experimental methods

Section	Primer Name	Sequence
Gadd45γ-IRES2-EGFP		
	hGadd45γ CDS 5'	GGGGAATTCACCATGACTCTGGAAGAAGTCCGC
	hGadd45γ CDS 3'	GGGTCTAGAACTCGGGGAGGG TGATGCTG
pEL2 Reporter Plasmids		
	Firefly Luc 5'	GGGCTGTACCAGATGGAAGACGCCAAAAACATAAAG
	Firefly Luc 3'	GGGGCGGCCGCTTACACGGCGATCTTTCCGCC
	1222-EL2 5'	GGGAAGCTTAAAGATTCCGAGCTGCATTACCTA
	1222-EL2 3'	GAAGAAGTCCGCGGCCAGGGGATCCCCC
	938-EL2 5'	GGGAAGCTTCCCACCCCACCCGGCGG
	665-EL2 5'	GGGAAGCTTCGCTCGGGACGCTAGTGGT
	281-EL2 5'	GGGAAGCTTCATCCCCCGCACCCCTCC
	194-EL2 5'	GGGAAGCTTGGCGCGCAGCCTCGCGC
	188-EL2 5'	GGGAAGCTTGCGGCGCCGCACCCACC
	pEL2ΔEbox3 5'	GCCTTCTGGCACGAGCCGC
	pEL2ΔEbox3 3'	ACGCGTGCGCGAGGCTGCGCGCCT
	pEL2ΔEbox4 5'	ACGCGTGCGGCGCCGCACCCACCA
	pEL2ΔEbox4 3'	GAAGAAGTCCGCGGCCAGGGGATCCCCC
RT-PCR Analysis		
	GAPDH 5'	AGGTCGGTGTGAACGGATTTG
	GAPDH 3'	TGTAGACCATGTAGTTGAGGTCA
	Gadd45γ 5'	CGCACAATGACTCTGGAAGA
	Gadd45γ 3'	AAGTTCGTGCAGTGCTTTCC
ChIP Analysis		
	Gadd45γ Pro 5'	AAAAAGAAAGGCGAGATGAAA
	Gadd45γ Pro 3'	CCCGAGCGCAGGTAAAGATT
	RPL30 5'	TACCTTTGAGGCACGTTGTGTGAC
	RPL30 3'	ACCATCACCTCCATAATCCAGCCA

CHAPTER IV

CONCLUSIONS

Summary of results

Transcription factors regulate many biological processes, including cell-fate determination and differentiation during embryonic development. Many groups have proposed elements of a core regulatory network of transcription factors that are important for controlling pluripotency, self-renewal, and differentiation of ES cells into neurons (reviewed in Jaenisch and Young, 2008). With the completion of genome sequences in many organisms, a major challenge remaining is to globally define transcriptional regulatory networks underlying complex biological processes. This requires the identification of primary targets directly controlled by each transcription factor, and then defining how expression of these targets is regulated with the proper specificity in a particular biological context.

As reviewed in Chapter 1, proneural bHLH transcription factors such as Ascl1 are key regulators of vertebrate neurogenesis. While the expression and activities of the proneural bHLH factors have been extensively characterized in many organisms, the molecular mechanisms underlying their ability to regulate neurogenesis are not well understood. This is in large part because primary target genes and transcriptional programs that are directly regulated by proneural bHLH proteins have not been

systematically defined. It is also not clear which regulatory sequence features enable proneural bHLH proteins to distinguish among many potential targets in the genome to specifically activate targets relevant to neurogenesis.

The goal of the research described in this dissertation was to determine critical changes in gene expression that mediate the neuronal differentiation of P19 cells, particularly those triggered by the bHLH protein Ascl1. P19 cells have long been used as a facile model system for studying neuronal differentiation, as they exhibit gene expression changes similar to neuronally differentiating embryonic stem cells and primary neural progenitors (Thomson and Marshall, 1998; Yu and Thomson, 2008). They have a distinct advantage over the latter, though, as they are more easily and robustly induced towards a neuronal phenotype. This makes them ideal for performing large scale biochemical analyses. Using the same biological samples helps to make studies integrative over multiple levels of analysis, allowing for the development and testing of a highly coordinated gene regulatory network.

PKIβ is necessary for Ascl1-mediated neuronal differentiation

PKA is critical in phenotypic specification and transition in the adult and developing nervous system, but its role in neuronal differentiation remains controversial with contradictory roles emerging depending on cell type. In SH-SY5Y human neuroblastoma cells, PKA activity blocks the initial steps of neurite elongation in adenosine 2A receptor-mediated neuritogenesis (Canals et al., 2005). In PC12 cells, whereas PKA activity is not required for the induction of sodium channel mRNA, it is

required for the expression of fully functional sodium channels. *In vivo*, PKA effectively inhibits the progression of retinal neurogenesis in zebrafish (Masai et al., 2005). Almost all retinal cells continue to proliferate when PKA is activated, suggesting that PKA inhibits the cell-cycle exit of retinoblasts. Contrary to these data, however, other research shows that in SH-SY5Y cells the inhibition of PKA blocks the initial steps of cAMP-induced neurite elongation (Sanchez et al., 2004). Similarly, in hippocampal HiB5 cells, treatment with a cAMP analog results in a dramatic increase in neurite outgrowth (Kim et al., 2002). In NG108-15 cells, inhibition of PKA activity accelerates neuritogenesis and neurite outgrowth rate, but decreases the number of varicosities and the frequency of post-synaptic miniature current, resulting in a suppression of synaptogenesis (Tojima et al., 2003). Despite these incongruities, these data suggest that the level of active PKA expressed in a neuronal cell can have profound effects on the excitability of a cell and its ability to generate and transfer electrical signals within the nervous system.

Our laboratory has made significant contributions to the understanding of the *in vitro* and *in vivo* roles of the PKI proteins in regulating PKA activity, including studies of the structure and function, tissue specific expression, evolutionary conservation, and the subcellular location of the PKI proteins (Olsen and Uhler, 1991; Olsen and Uhler, 1991b; Scarpetta and Uhler, 1993; Baude et al., 1994; Baude et al., 1994b; Gamm and Uhler, 1995; Seasholtz et al., 1995; Collins and Uhler, 1997). Despite the number of *in vitro* studies examining purified or overexpressed PKI, little is currently known about the physiological roles of PKIs *in vivo*.

Due to their activity as direct inhibitors of the catalytic subunit of PKA and their ability to facilitate nuclear export (Figure 4.1), research has long speculated that PKIs

serve to reset the basal activity of PKA in preparation for the next round of stimulation (Grove et al., 1989; Wiley et al., 1999). The presence of three isoforms with unique patterns of tissue expression suggests that they play specific, non-overlapping roles in the modulation of the cAMP-PKA signaling cascade. However, ablation of the PKIα gene in PKIα knockout mice resulted in little detectable effect on phenotype (Gangolli et al., 2000). No compensatory up-regulation of other PKI isoforms was observed, but a significant up-regulation of the RIα protein was observed. Phosphorylation of CREB was significantly reduced in these mice, which is counterintuitive to the characterized activities of PKI.

Similar to the PKIα knockouts, PKIβ and PKIα/β double knockout mice both exhibited normal fertility and had no apparent perturbation of PKA activity and regulation (Belyamani et al., 2001). Although the PKIβ knockout mice showed a loss of PKI activity in testis compared with wild-type mice, a substantial amount of residual inhibitory activity remained, suggesting that PKIγ may be able to compensate for the other two isoforms. While PKIγ knockout mice have yet to be generated, antisense knockdown of PKIγ in osteoblastic osteosarcoma cells showed that PKA catalytic subunit export from the nucleus was dependent on PKIγ, but this export was not required for the termination of PKA signaling (Chen et al., 2005). Together, these findings challenge the prevailing view that PKIs solely function to maintain low basal PKA activity, and suggest that they may have a yet undiscovered function in the regulation of gene expression and transcription factor phosphorylation.

In our studies, following overexpression of Ascl1, microarray hybridization showed that P19 cells undergo a transient increase in all three isoforms of PKI, each

displaying a unique temporal pattern of expression. We verified these results using RT-PCR and found that, in agreement with the microarray hybridization analysis, the PKIβ transcript was the most highly induced, exhibiting a 2500-fold increase in expression (compared to an 18-fold and 6-fold expression for PKIα and PKIγ, respectively). shRNA constructs targeting each isoform were evaluated for their ability to knockdown expression of all three PKI genes, and although we successfully identified a number of effective shRNAs for each isoform, only those targeting the PKIβ gene prevented neuronal differentiation. We confirmed that the induction of PKIβ mRNA expression was accompanied by a significant increase in PKIβ protein levels, and that the shRNA constructs that proved effective in reducing PKIβ mRNA levels were also effective in reducing levels of protein. Most importantly, this shRNA-mediated reduction in PKIβ protein prevented normal differentiation of P19 cells in response to Ascl1 expression. We were able to partially rescue this effect by overexpressing PKIβ protein, and found that this rescue of neuronal differentiation was dependent on the binding of PKIβ to PKA. Our results strongly suggest a requirement for PKIβ and its association with PKA during the neuronal differentiation of P19 cells (Figure 4.1). Very few systems have been described in which PKI gene transcription is regulated, making this Ascl1-P19 system a potential new model to study the physiological regulation of PKIs.

It has been previously reported that PKIs bind to PKA via a conserved pseudosubstrate sequence, and once bound cause a conformational change that exposes its nuclear export signal (NES). This conformation change shuttles the PKI-PKA complex out of the nucleus, thereby preventing nuclear accumulation and activity of the catalytic subunit of PKA (Meinkoth et al., 1993; Taylor et al., 2005). Given that regulation of PKA

appears to be critical for proper Ascl1-mediated neuronal differentiation, a more complete understanding of PKIβ and its role in subcellular localization is important for understanding its functional roles.

We are currently developing a mutant PKIβ expression vector where the NES is rendered non-functional by mutation of critical leucine residues within this region to alanine residues (PKIβNESmut). The inhibitory activity of this construct will be assayed using the PepTag kinase assay, similar to the experiments conducted in Chapter 2 (see Figure 2.7). It will also be of interest to determine the rate of export of the catalytic subunit of PKA, which could be done by expressing Flag-tagged PKIβ or Flag-tagged PKIβNESmut and scoring its subcellular distribution by fluorescence microscopy. The addition of the Flag epitope tag is necessary in our studies, as antibodies that are specific for PKIβ are extremely limited, and the extent of their specificity and sensitivity is not clear. The availability of more antibody sources would facilitate further immunocytochemical studies, which would in turn provide useful information about the cellular and subcellular localizations of PKIβ.

The studies described in Chapter 2 could be strengthened by taking into consideration alterations in regulatory subunit levels as an indication of modifications in PKA activity and cAMP signaling. Of the four regulatory subunits, RIIβ is thought to be the dominant isoform expressed in the brain (Ventra et al., 1996; Brandon et al., 1997; Brandon et al., 1998). Furthermore, the specificity of PKA signaling is believed to arise at least in part from compartmentalization of PKA to specific subcellular locations (Buxton and Brunton, 1983; Rich et al., 2001; Zaccolo and Pozzan, 2002). In particular the RIIβ subunit has been shown to play a role in localization of PKA in neurons via binding to

AKAPs such as Map2 (Zhong et al., 2009). Supporting a role for the RIIβ subunit in neurons, our microarray studies indicate that this is the only regulatory subunit isoform to increase in expression during Ascl1-induced differentiation of P19 cells. However, RIIβ induction is preceded by the expression of mature neuronal markers such as Map2 and β-III-tubulin. Therefore, it unlikely that the upregulation of RIIβ expression participates in neuronal fate determination, but it is conceivable that RIIβ may play a role in the development of a mature neuronal phenotype. Since compensatory up-regulation of other PKI isoforms has been reported previously (Belyamani et al., 2001), our studies would have been enhanced by additional experiments testing whether exogenous PKIα or PKIγ expression could rescue the phenotype conferred by antisense knockdown of PKIβ. Additionally, an alternative approach we could have taken to our studies would have been to use a cell line that stably expresses mutant regulatory subunits of PKA that are deficient in cAMP binding (described in Correll et al., 1989). This would render recipient cells insensitive to stimulation by cAMP, allowing us to assess the requirement for PKAs in Ascl1 responses.

Although P19 cells provide a tractable model system for studying neuronal differentiation due to the ease of their handling, and despite research showing that they are easily transfected to recapitulate the intrinsic and extrinsic cues responsible for the differentiation of neurons *in vivo* (see Chapter 2; Farah et al., 2000), a complicating factor with these transient transfection studies is that the level and timing of Ascl1 expression is difficult to control, making detailed studies of early events triggered by bHLH expression challenging. In order to address this issue, we developed an experimental system where P19 cells stably express Ascl1 under control of the tetracycline repressor protein.

Microarray hybridization analysis was carried out using this Ascl1-stable cell line, and we found that it recapitulated the transient increases in PKI isoform expression, with the PKIβ transcript still being the most strongly induced (455-fold at 36 h). This novel model system also allowed for detailed study of earlier events mediating Ascl1-induced neuronal differentiation, as discussed in Chapter 3.

Gadd45γ is a direct transcriptional target of Ascl1

Using this novel model system of Ascl1-inducible P19 cells that differentiate into a homogenous population of neurons, we identified a direct transcriptional target of Ascl1. Gene expression changes between untreated (undifferentiated) versus Dox-treated (differentiated) P19 cells were determined by microarray hybridization analysis, and the results were validated using RT-PCR. Using this approach, we identified over 270 genes whose expression increased four-fold or greater after eight days of differentiation, some of which were novel Ascl1-responsive genes (i.e. *Npy*, *Fgf5*, and *Igf2*). We examined the earliest changes in gene expression following Ascl1 induction, and found the Gadd45γ gene to be the most highly induced (33-fold) after 24 h of treatment with Dox. Like PKIs, Gadd45γ can function as an inhibitor of the CDK protein kinase family (Zhan et al., 1999; Vairapandi et al., 2002).

Gadd45 proteins play a role in cell cycle regulation, cell survival, and cell death (Zhang et al., 2001; Candal et al., 2004; Rai et al., 2008; Ma et al., 2009). All of these processes (Figure 4.2) are critical for proper neuronal development, and we showed that Gadd45γ is direct transcriptional target of Ascl1 during neurogenesis. We identified

evolutionarily conserved sequences within the promoter region of Gadd45γ that successfully drove expression of a reporter vector (pEL2) in the presence of Ascl1. We furthermore narrowed the location of the core promoter elements necessary for driving the transcriptional response to Ascl1 to the proximal 281 bp fragment. Mutations in the two E-box sites within this promoter region significantly reduced its activity, and this reduction in reporter activity was specifically due to loss of Ascl1 interaction with the core promoter region. Ascl1 was shown to bind directly to this promoter in P19 cells, as ChIP assays using Ascl1 antibody showed a significant enrichment for the Gadd45γ promoter sequence containing the two most proximal E-boxes. Finally, we examined the downstream transcriptional events initiated by Gadd45γ. Using an inducible, Gadd45γ-stable P19 cell line (P19T3GIE2), we showed that forced expression of Gadd45γ was sufficient to induce some genes characteristic of an Ascl1-induced differentiation program (i.e. β-III-tubulin and Gap43), and that these transcriptional responses were shown to be independent of Ascl1 expression (see Figure 3.10).

To extend our microarray hybridization analyses, it will be important to examine the physiological role of Gadd45γ upregulation in the context of Ascl1-induced neuronal differentiation. A study in N1E-115 neuroblastoma cells showed that treatment with valproic acid (VPA) upregulates the expression of Gadd45α, which through the effector MAPK/ERK kinase kinase 4 (MEKK4) activates the downstream c-Jun N-terminal kinase (JNK) signaling cascade to induce neurite outgrowth (Figure 4.2; Yamauchi et al., 2007). In our studies, overexpression of Gadd45γ induced a neuron-like phenotype, with cells adapting a neuronal morphology and expressing neuronal markers such as β-III-tubulin and Gap43. Both of these are involved in neurite formation and outgrowth, and it

therefore seems plausible that Gadd45γ could be mediating this process in Ascl1-differentiated P19 cells. It will be a significant contribution to determine whether Gadd45γ initiates a similar pathway, and whether the activation of the JNK cascade is responsible for induction of an Ascl1-like phenotype. This would point to a role for Gadd45γ as a regulator of neurite outgrowth in P19 cells, but not necessarily a regulator of neuronal differentiation.

Future directions

My discussions of ongoing and future research projects will be grouped into the following areas: 1) further examination of the physiological roles of the PKI isoforms, 2) elucidation of binding partners of PKIβ that may also be required for Ascl1-induced neuronal differentiation, 3) development of a Gadd45γ knockdown model to determine downstream effectors of this protein, 4) elucidation of the physiological role of Gadd45γ in neuronal differentiation, and 5) methods for identifying other protein kinases important in neurogenesis.

Examination of the physiological roles of the PKI isoforms

After treatment with high concentrations of RA and aggregation, P19 cells can differentiate into neurons, glia, and fibroblast-like cells (Jones-Villeneuve et al., 1982). These cell types appear in a reproducible manner temporally: fibroblast-like cells emerge first, followed by neurons and glial cells. This temporal pattern is similar to that seen in

rat embryo brain explant cultures, and cells with similar morphology are also seen in cultures of cells from the central and peripheral nervous system (Jones-Villeneuve et al., 1982). These findings suggest that RA-induced P19 neural differentiation in part mimics the development of nervous system. Interestingly, the expression of glial-specific proteins such as GFAP was not found in the Ascl1-transfected P19 cells when compared to RA-induced differentiated cells, suggesting that bHLH-induced differentiation is more specifically targeted to a neuronal fate. To determine whether PKIs are specifically necessary for neuronal differentiation, P19 cells could be treated with RA and simultaneously transfected with PKI shRNAs. Immunocytochemistry using neuronal and glial specific antibodies could be used to make comparisons between Ascl1-induced cell fate and RA induced cell fate.

In the RA method of neuronal differentiation, both RA treatment and cell aggregation are both necessary to complete neural differentiation of P19 cells, and a study done by Teramoto et al., (2005) showed that each regulates a distinct network of gene expression. Recently, research has shown that these treatments change the expression of alternatively spliced isoforms of several genes during neuronal differentiation of P19 cells: some undergo up-regulation of alternative splicing (such as the protein tyrosine kinase *SrcN1* and RARα/γ), while others such as *Mtap2* are down-regulated (Boutz et al., 2007; Alam et al., 2010). The regulation of alternative splicing requires the interaction of *cis*- and *trans*-acting elements (Garcia-Blanco et al., 2004). One of the major types of *trans*-acting factors involved in alternative splicing is serine/arginine (SR)-rich proteins (Krainer et al., 1990; Mayeda and Krainer, 1992). SR proteins are highly phosphorylated and necessary for the initiation of spliceosome

assembly, while their dephosphorylation appears to be necessary for splicing catalysis (Cao et al., 1997) thereby making the regulation of SR phosphorylation levels critical to successful alternative splicing. It would be a significant contribution, therefore, to determine whether RA and Ascl1-mediated neural differentiation result in unique patterns of alternative splicing for the PKIβ gene or other regulators of phosphorylation state.

We have shown that knocking down PKIβ expression results in decreased numbers of TuJ1- and Map2-positive cells following Ascl1 transfection. This suggests that other endogenous isoforms of PKI are not sufficient to compensate for the lack of PKIβ activity. Whether compensatory mechanisms exist in a RA model of differentiation has yet to be determined. Because RA differentiation of P19 cells results in a heterogenous populations of both glial and neuronal cells, this model could be used to test the hypothesis that PKIs are also necessary for glial differentiation. We could compare the number of GFAP-positive cells between P19 cells differentiated with RA in the absence or presence of PKI shRNAs. If the number of GFAP positive cells remains the same or increases, this suggests that PKIs are essential for neuronal differentiation, but not for glial differentiation. If the number of GFAP positive cells also decreases, this means PKIs are necessary for both neuronal and glial differentiation.

One question arising from the results described in Chapter 2 is whether the P19 cell line model recapitulates the behavior of developing neurons *in vivo*. Although P19 embryonic carcinoma cells recapitulate many of the gene expression changes observed by neuronal progenitor cells *in vivo*, their transformed phenotype and genetic drift during passage in culture could limit their relevance to *in vivo* neuronal differentiation. In order to gain insight in the neuronal gene expression changes that are both conserved with and

distinct from P19 cells, we could examine PKI gene expression in embryonic cortical progenitor cells in culture. We would look at gene expression changes in primary neuronal cultures, and make a comparison between global gene expression changes during P19 and embryonic cortical progenitor cell neuronal differentiation. Studies using mouse embryonic stem cells induced to differentiate via forced expression of Ascl1 have shown that PKIβ is induced with the same temporal pattern as in Ascl1-differentiated P19 cells (unpublished data). The results of these will build upon the foundation provided in Chapter 2 and contribute to a comprehensive and detailed understanding of the contribution(s) of PKIs to neuronal differentiation.

If we find that the patterns of PKI expression are significantly different in P19 cells versus embryonic cortical progenitor cells, this would suggest that these two systems of modeling neuronal differentiation are fundamentally different for the PKI genes. With the plethora of contradicting research regarding the role of PKA in neuronal differentiation, this will not be a surprising result since the intracellular signaling pathways that initiate neuronal differentiation are complex and far from understood (Weisenhorn et al., 1999). One possible explanation is that in embryonic cortical progenitor cells, compensatory pathways exist that result in PKA-independent neuritogenesis and subsequent neuronal differentiation. It has been shown in a number of cell lines that the cAMP/PKA and MAPK pathways act independently of one another to regulate processes involved in neuronal differentiation (Shi et al., 2006). Therefore, there may be some yet to be determined developmentally regulated factor(s) responsible for differences observed between P19 embryonic carcinoma cells and embryonic cortical progenitor cells.

However, if a close correlation between P19 cells and embryonic cortical progenitor cells is observed, then another proposed experiment would be to utilize progenitor cells from PKIα -/- and PKIβ -/- knockout mice. PKIα knockout mice completely lack PKI activity in skeletal muscle and, surprisingly, show decreased basal and isoproterenol-induced gene expression in muscle (Gangolli et al., 2000). These animals also exhibit reduced levels of the phosphorylated and active form of the transcription factor CREB. This phenomenon stems in part from lower basal PKA activity levels in the mutants, potentially arising from a compensatory 1.6-fold increase in the level of the RIα subunit of PKA (Gangolli et al., 2000). PKIβ knockout mice exhibit a partial loss of PKI activity in testis but remain fertile with normal testis development and function. When both the PKIα and PKIβ genes were ablated, still few detectable phenotypes were observed (Chen et al., 2005). However, knockout mice for the PKIγ gene have not been generated, and compensatory expression of PKIγ in the PKIα or PKIβ knockout lines has not been tested.

We would predict that progenitor cells isolated from PKIα -/- and PKIβ-/- knockout mice should show either reduction in the total number of cells differentiating or a delay in the time course of differentiation. Based on previous research examining the effects of ablating PKIs, it is entirely possible that no pronounced differences will manifest between wild-type and the knockout progenitor cell differentiation. This result would suggest some neural form of compensation similar to that observed in skeletal muscle with the PKIα knockout mice. Our main focus, then, would be to determine what compensatory mechanisms are responsible for the normal progression of neuronal differentiation. Quantitative RT-PCR is a straightforward method for detecting changes in

the most compelling candidates for compensation: other PKI family members or the regulatory subunits of PKA.

Another important experiment to perform is to use a synthetic analog of cAMP to increase intracellular levels of this second messenger, thereby mimicking the lack of inhibition of PKA by PKI by increasing the activity of PKA. However, it is important to consider the distinct features of PKA modulation governed by PKI when compared with cAMP. First, PKI directly interacts with the catalytic subunit of PKA, and can thus inhibit the PKA previously activated by cAMP. Second, unlike cAMP, which is a small diffusible molecule, the intracellular distribution of PKI can be localized to certain structures or organelles. Studies have demonstrated a substantial pool of PKI proteins that are associated with microtubules in the cytoplasm (Tash et al., 1980), and that the NES of PKIs serves as an adapter to target the catalytic subunits of PKA to the nuclear export machinery (Wen et al., 1994; Wiley et al., 1999). These allow for a spatially specific activation/inactivation of PKA within a single cell. Localized PKA activity has been implicated in controlling the regulation of synaptic plasticity by modulating Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity (Blitzer et al., 1998; Brown et al., 2000), and PKA was shown to be directly involved in synaptic plasticity by controlling the delivery and incorporation of the GluR1 and GluR4 subunits of AMPA receptors into synapses (Esteban et al., 2003). Finally, the kinetics of PKA regulation based on the modulation of PKI gene expression are likely to drastically different from those mediated by cAMP. Turnover of cAMP is rapid due to the presence of phosphodiesterases, while transcriptional regulation of PKI would occur with much slower kinetics. These reasons led us to first focus specifically on PKI modulation of

PKA, rather than cAMP induction of PKA activity.

Identification of PKI binding partners

While further investigation into the physiological role of PKI isoforms is important, it is equally essential to isolate and identify protein partners involved in PKI-mediated inhibition of PKA activity. Proteins carry out and regulate the majority of cellular activities and generally interact with neighboring proteins to form multi-protein complexes in a time- and space-dependent manner. Identifying PKI protein complexes will be important in gaining further insight into the cellular role of this protein and determining possible mechanisms by which it has an effect in its molecular environment. A generic protein complex purification strategy, tandem affinity purification (TAP), in combination with mass spectrometry is a straightforward method that allows for the identification of binding partners and the purification of protein complexes (Rigaut et al., 1999; Puig et al., 2001).

The TAP method involves fusion of a TAP tag to proteins of interest. The TAP tag consists of two IgG-binding units of protein A of *Staphylococcus auerus* (ProtA) and a calmodulin-binding domain (CBP), with a cleavage site for the tobacco etch virus (TEV) protease inserted between them (Rigaut et al., 1999). Once the TAP fusion protein is expressed, protein complexes containing the TAP-tagged protein are purified from cell extracts via two specific affinity purification steps. This methodology has a number of advantages for researching protein complex interactions: it enables rapid purification of protein complexes without prior knowledge of their function or structure, and this

purification can occur under native conditions. Furthermore, the purification steps are highly specific and thus reduce background caused by contaminants.

An alternative set of proposed experiments involves the fusion of human PKIβ to a TAP tag at the C-terminus and subsequent stable expression in P19 cells. The PKIβ-TAP system would then be used to purify epitope-tagged protein complexes from crude cell extracts, and identify binding partners of PKIβ. To our knowledge, this experiment has the potential of being the first to show a direct physical interaction between PKI proteins and the catalytic subunits of PKA in living cells.

Generation of a Gadd45γ knockdown model to determine mechanisms of regulation of neuronal differentiation

Another consideration to address is whether Gadd45γ is essential for Ascl1-induced neuronal differentiation. We attempted to knockdown expression of Gadd45γ using a number of shRNAs targeted to different regions of the gene, but none proved to be effective at reducing mRNA transcript levels. As a complementary method to RNAi, we could have utilized the technique of post-translational protein knockdown. This approach fundamentally differs from RNAi by destroying existing copies of the protein of interest, rather than simply precluding new protein synthesis. Proteins with a long half-life may not necessarily be vulnerable to RNAi because preventing new synthesis of a protein would not affect the function of existing copies already present within the cell. This can lead to complex phenotypes due to cells adapting to the slow protein depletion.

One of the first attempts to induce selected protein degradation *in vivo* took

advantage of chimeric proteins that were capable of inducing the degradation of protein targets that are normally very stable. This approach entails the use of an F-box protein engineered to contain a binding domain for the target protein. F-box domain-containing proteins are known to exist as complexes with E3 ubiquitin ligases. Once expressed in the cell, a chimeric F-box protein recruits the target protein to the E3 ligase complex, ultimately leading to ubiquitination and degradation of the target (Scheffner et al., 1992; Zhou et al., 2000). This methodology could also be applied to our studies described in Chapter 2 to validate our shRNA studies and definitively assess the necessity of PKIβ for Ascl1-induced differentiation.

Elucidating the physiological role of Gadd45γ

Regulation of gene expression via epigenetic mechanisms is important during neuronal development, providing potential mechanisms for cellular memory and the inheritance of gene expression pattern information during mitosis. DNA methylation is the prototypical epigenetic marker, and is required for repressing gene transcription, X-inactivation, genomic imprinting, and maintaining chromosome stability. Methylated CpG dinucleotides contribute to gene repression by inhibiting the binding of specific transcription factors (Prendergast and Ziff, 1991) or recruiting proteins that contain methyl-CpG-binding domains and act as transcriptional repressors (Miranda and Jones, 2007). Several neurological disorders (e.g. schizophrenia and Parkinson's) are postulated to arise partly because of mutations in proteins that are involved in methylating DNA or that are recruited to methylated DNA, suggesting that appropriate DNA methylation

within the nervous system is vital (Feng and Fan, 2009; Jowaed et al., 2010).

A series of recent studies have provided evidence supporting a role for Gadd45 proteins in DNA demethylation. Gain- and loss-of-function studies have shown that Gadd45β promotes active DNA demethylation through interactions with nucleotide excision repair endonucleases (Barreto et al., 2007). Another study has shown that active ribosomal DNA methylation is mediated by Gadd45α, raising the possibility that local transcribed RNA may recruit Gadd45 and associated complexes for region-specific DNA demethylation (Yi et al., 2000). Gadd45β was identified as a neural activity-induced immediate early gene in hippocampal neurons, and experiments in Gadd45β -/- mice suggested that its activity as an epigenetic regulator is important in the increased neurogenesis that occurs after seizures or physiological activity (Ma et al., 2009; reviewed in Parent, 2010). However, the role of Gadd45 proteins in epigenetic regulation remains controversial, as some groups challenge these findings: Jin et al. (2008) were unable to substantiate a functional role of Gadd45α in DNA demethylation using gene reactivation and DNA methylation assays. Another study showed that siRNA-mediated knockdown of Gadd45α resulted in increased levels of DNA methylation at specific endogenous loci, but when examined in Gadd45α -/- mice, there was no increase in global or locus-specific methylation (Engel et al., 2009). Using the stable Gadd45γ-expressing cell line described in Chapter 3, it will be informative to examine the global methylation status of genes in response to induction of Gadd45γ. Methylated DNA immunoprecipitation, as well as bisulfite sequencing, are accepted methods for determining the methylation status of specific promoter sequences and would be important approaches for characterizing DNA methylation in Ascl1—and Gadd45γ—

inducible P19 cells (Zilberman and Henikoff, 2007; reviewed in Thu et al., 2010).

Integrative profiling to gain insight into biological signaling networks

All of the above studies require further experimentation to determine the molecular components of their mechanism of action. While genome-wide mRNA profiling provides a snapshot of the global state of the cell under different experimental conditions, it is important to recognize that quantitative measurements of changes in mRNA levels do not necessarily translate directly into an understanding of the regulatory mechanisms responsible for the observed changes (e.g. epigenetic modifications). In order to have a comprehensive understanding of processes responsible for the generation of the diversity of cell types in the nervous system, integrative methods are necessary to globally profile the variable composition of different cellular states (e.g. undifferentiated versus differentiated).

One method for identifying critical regulatory elements is phylogenetic footprinting (also known as sequence conservation analysis), whereby functional regulatory motifs are determined by comparing a non-coding region of interest to its orthologous sequence across different species (Kheradpour et al., 2007; Mereiles-Filho and Stark, 2009). While conservation analysis can be an indicator of the functionality of a regulatory motif—and a straightforward tool for identifying *cis*-regulatory modules—lack of conservation does not necessarily translate to an absence of function. Therefore, another strategy regularly employed to identify regulatory regions at the genomic level is chromatin immunoprecipitation with antibodies against key regulator transcription factors

coupled with microarray hybridization (also known as “ChIP-on-chip” or “ChIP-seq”). The combination of these two techniques allows for high-throughput and genome-wide localization of regulatory elements (Celniker et al., 2009; Visel et al., 2009; Zinzen et al., 2009).

However, post-transcriptional regulation introduced by alternative splicing as well as protein post-translational modifications greatly increases the complexity of the analytical problem of defining gene regulatory networks. Techniques and algorithms have been presented that predict tertiary structure of transmembrane proteins (Yang et al., 2008), the subcellular location of a protein based on amino acid and amino acid pair composition (Habib et al., 2008), as well as the localization of protein binding regions (Wang et al., 2008). Models have been proposed that predict the most influential *cis*-acting elements under a given biological condition, as well as estimating the effects of those elements on gene expression levels (Wang et al., 2008). Since proteins bind to many different partners to regulate and control a wide variety of physiological processes, defining protein networks is becoming increasingly important to understanding transcriptional signaling networks and the function of molecules that comprise them.

An established and widely accessible strategy for protein profiling is two-dimensional gel electrophoresis, which displays changes in protein expression and post-translational modifications (such as phosphorylation state) on the basis of protein staining intensities and electrophoretic mobility. Several studies have used this method to successfully identify novel signal transduction targets by selectively activating or inhibiting pathways and screening the molecular responses (Gerner et al., 2000; Lewis et al., 2000; Kanamoto et al., 2002). Another strategy for protein profiling is

multidimensional liquid chromatography coupled with tandem mass spectrometry—commonly referred to as LC-MS/MS sequencing or "shotgun" proteomics. This method is rapidly emerging as a fundamental approach to protein profiling, and involves solution proteolysis of a complex mixture of proteins, followed by multidimensional chromatographic separation of peptides prior to LC-MS/MS sequencing (McDonald and Yates, 2003). A key advantage to this method is its efficiency in detecting hydrophobic proteins, making it especially appealing for analyzing proteins from intracellular organelles (Aebersold and Mann, 2003).

Several methods for systematically analyzing protein interactions have been developed, including the well established yeast two-hybrid analysis (Mrowka et al., 2001), the previously discussed LC-MS/MS of affinity purified complexes and TAP tag purification, and protein microarray technology (Zhu and Snyder, 2003). MacBeath and Schreiber (2000) described the feasibility of using protein arrays to monitor protein-protein interactions as well as the use of peptide arrays for screening catalytic specificity of protein kinases. Although not as common as genomic microarrays, protein microarrays have enormous potential for mapping pathways and elucidating biochemical activities of individual components in signaling pathways. However, they are limited by the quality of protein targets and the representation of relevant post-translational modifications, as well as unique conditions to preserve proper folding and enzyme activity.

By combining promoter analysis, data from various chromatin immunoprecipitation studies, protein-protein interactions, and kinase-protein phosphorylation reactions collected from the literature, it would be possible to identify and rank candidate protein kinases for knockdown, or other types of functional

validations, based on genome-wide changes in gene expression. This identification could be made even more robust by cross-validation with phosphoproteomics data as well as through a literature-based text-mining approach. Therefore, data integration can produce robust candidate rankings for understanding cell regulation through identification of proteins responsible for gene expression changes, and thus rapidly advancing drug target discovery and unraveling drug mechanisms of action.

Closing

The studies described in Chapters 2 and 3 have defined a novel cellular model for studying neuronal differentiation and generated information pertinent to the gene regulatory model of Ascl1-induced neuronal differentiation. More specifically, these studies have suggested that inhibition of protein kinase activity by the expression of specific genes is required during neuronal differentiation. This information, as well as the cell lines generated should be significant resources for investigators in the field of neuronal differentiation, and should aid in the development of strategies to enhance specific neuronal differentiation of human embryonic stem cells in the treatment of neurologic diseases. Currently, many of the tools available to study the control of gene expression require starting material from a large number of ideally homogenous cells. Thus, although great strides have been made in elucidating mechanisms which involve transcriptional and post-translational modifications in non-neuronal cells, such detailed information with regard to neurons has thus far been technically challenging. The model described in this dissertation should help progress in this area, perhaps in combination

with methods to tag and isolate specific subpopulations of neurons. Results of the proposed research will help determine what interactions between intrinsic factors coordinately regulate progenitor cell division and the onset of differentiation, which is crucial in therapeutic strategies for neural repair.

References to Chapter IV

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Figure 4.1. Cellular mechanisms by which PKIs trigger changes necessary for neuronal differentiation. Ascl1 induces expression of PKIβ, whose transient increase in expression is necessary for neuronal differentiation. PKIβ binds to and inhibits the activity of the catalytic subunit of PKA (Cα). This association may prevent the phosphorylation of nuclear PKA substrates such as CREB, cyclin D1, GFAP, and nNOS, which play roles in neuronal gene transcription, cell cycle regulation, and proper neuronal function. In addition to inhibiting the activity of PKA, PKIβ may play a role in catalytic subunit localization, facilitating the nuclear export of the complex and thereby directing the activity of PKA to cytoplasmic targets (such as GluR1/4) as well as allowing the catalytic subunits to reform an inactive holoenzyme.

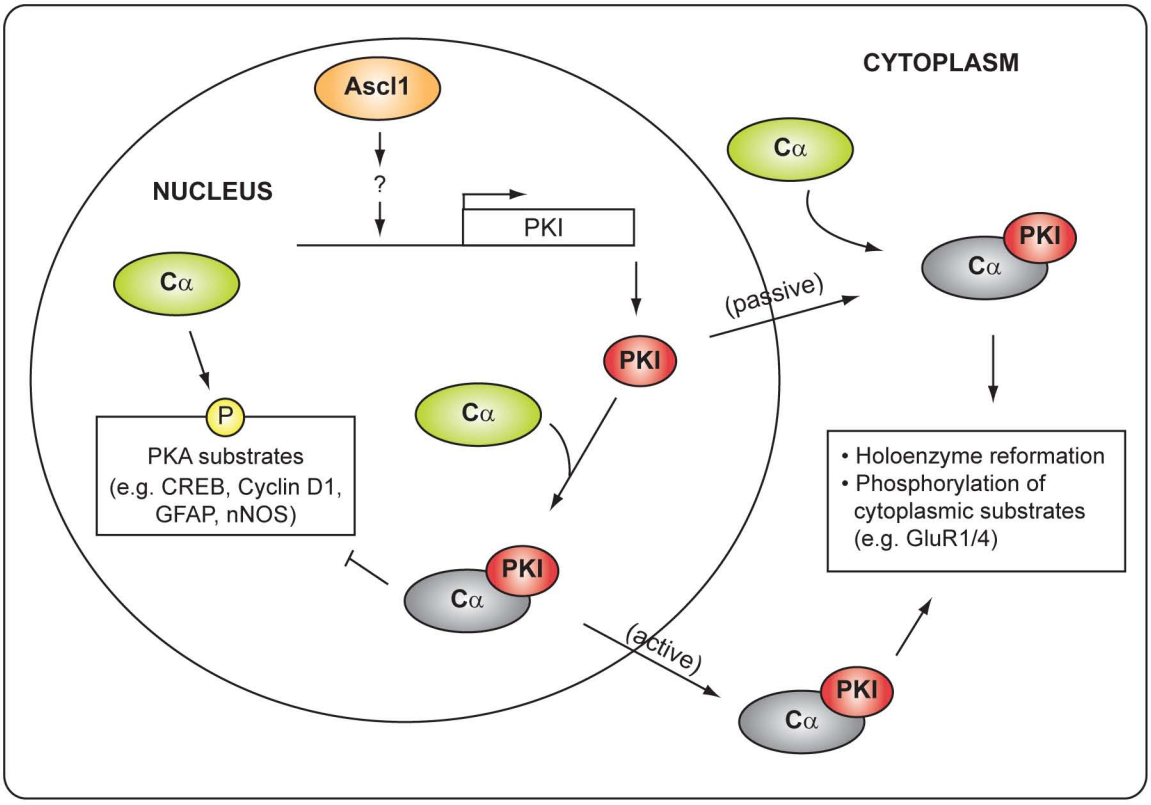
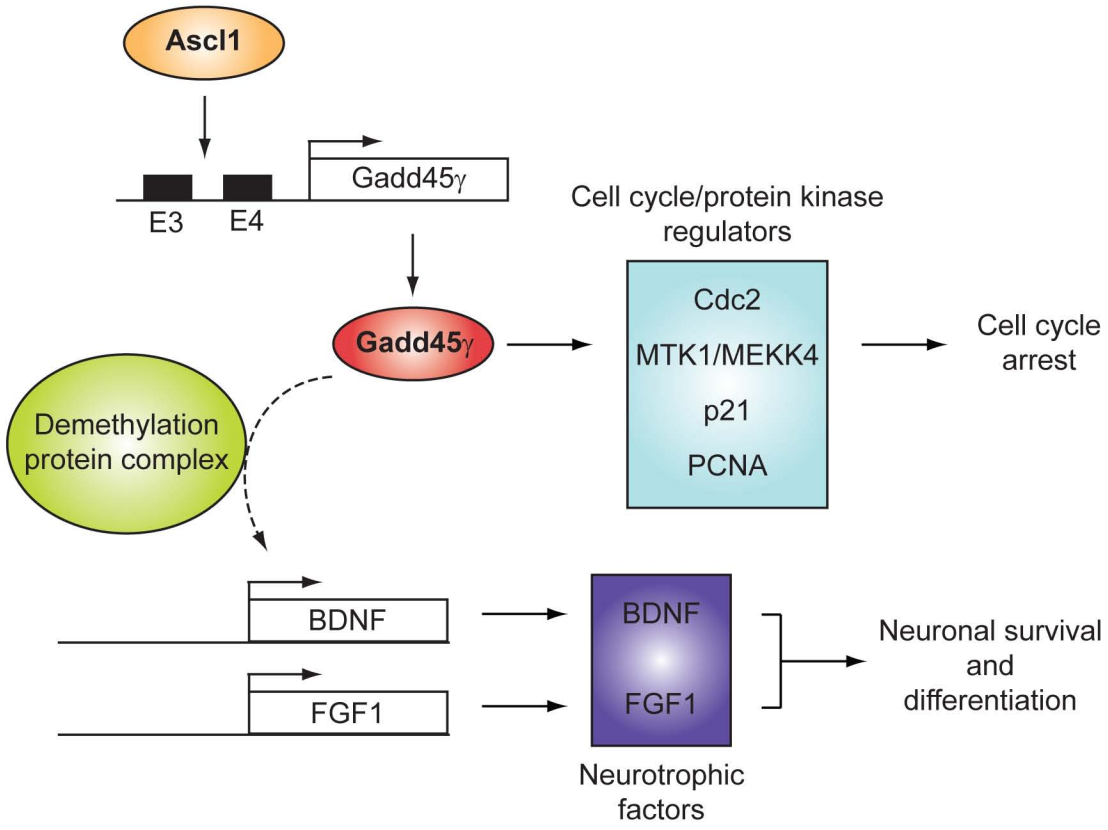

CYTOPLASM
Ascl1
NUCLEUS
?
PKI
Cα
Cα
PKI
Cα
(passive)
PKI
P
Cα
PKA substrates
(e.g. CREB, Cyclin D1,
GFAP, nNOS)
• Holoenzyme reformation
• Phosphorylation of
cytoplasmic substrates
(e.g. GluR1/4)
PKI
Cα
(active)
PKI
Cα

Figure 4.2. Putative substrates of Gadd45γ that are implicated in neuronal development. Ascl1 directly binds to a regulatory region of the Gadd45γ gene containing two necessary E-boxes. Gadd45γ proteins may trigger activation of a protein demethylation complex, which leads to increased expression of specific target genes, including BDNF and FGF1, resulting in increased neuronal survival and differentiation. Experiments in multiple cell types have demonstrated that Gadd45 proteins also function in cell cycle regulation via interactions with Cdc2, MTK1/MEKK4, p21, and PCNA.


Ascl1
Gadd45γ
E3
E4
Cell cycle/protein kinase regulators
Gadd45γ
Cdc2
MTK1/MEKK4
p21
PCNA
Cell cycle arrest
Demethylation protein complex
BDNF
FGF1
BDNF
FGF1
Neurotrophic factors
Neuronal survival and differentiation