# Characterization of CPG15 During Cortical Development and Activity Dependent Plasticity

By

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## ABSTRACT

Regulation of gene transcription by neuronal activity is thought to be key to the translation of sensory experience into long-term changes in synaptic structure and function. Here we show that *cpg15*, a gene encoding an extracellular signaling molecule that promotes dendritic and axonal growth and synaptic maturation, is regulated in the somatosensory cortex by sensory experience capable of inducing cortical plasticity. Using in situ hybridization, we monitored *cpg15* expression in 4-week-old mouse barrel cortex after trimming all whiskers except D1. We found that *cpg15* expression is depressed in the deprived barrels and enhanced in the barrel column corresponding to the spared D1 whisker. Induction of *cpg15* expression is significantly diminished in adolescent as well as adult CREB knockout mice. *cpg15*'s spatio-temporal expression pattern and its regulation by CREB are consistent with a role in experience-dependent plasticity of cortical circuits. Our results suggest that local structural and/or synaptic changes may be a mechanism by which the adult cortex can adapt to peripheral manipulations.

The balance between proliferation and apoptosis is critical for proper development of the nervous system. Yet, little is known about molecules that regulate apoptosis of proliferative neurons. Here we identify a soluble, secreted form of CPG15 expressed in embryonic rat brain regions undergoing rapid proliferation and apoptosis, and show that it protects cultured cortical neurons from apoptosis by preventing activation of caspase 3. Using a lentivirus-delivered small hairpin RNA, we demonstrate that endogenous CPG15 is essential for the survival of undifferentiated cortical progenitors in vitro and in vivo. We further show that CPG15 overexpression in vivo expands the progenitor pool by preventing apoptosis, resulting in an enlarged, indented cortical plate and cellular heterotopias within the ventricular zone, similar to the phenotypes of mutant mice with supernumerary forebrain progenitors. CPG15 expressed during mammalian forebrain morphogenesis may help balance neuronal number by countering apoptosis in specific neuroblasts subpopulations, thus influencing final brain size and shape.

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# Contents

# Chapter 1

# Introduction

**Cell death is an essential component of neural development.**

A central question in developmental neurobiology is how does the cerebral cortex go from a thin layer of neuroepithelial cells lining the ventricle to create the diversity of cell types arranged in a stereotypical pattern to form the circuitry of the mature functional brain (Stern, 2001). It is now known that formation of the cerebral cortex involves the proliferation of neural progenitor cells along the anatomically defined ventricular zone (1970). Traditionally it was believed that neural progenitors lining the ventricle would divide symmetrically to produce two progenitor cells, thereby expanding the progenitor pool, and divide asymmetrically to produce a progenitor cell and a newly generated neuron (Chenn and McConnell, 1995; Takahashi et al., 1996). It is now known that in addition to the traditionally viewed neuron/progenitor producing asymmetric division, neurons are also generated by asymmetric divisions of progenitors that produce a radial glial progenitor cell, and an intermediate progenitor that moves up into the subventricular zone and divides symmetrically to produce two neurons (Noctor et al., 2004). The next step in the development of the brain is migration of these newly postmitotic neurons to the cortical plate where they continue to differentiate and eventually make synaptic connections (Bayer et al., 1991). Traditionally, after cell division a cell was thought to choose one of two fates, either continued proliferation or differentiation into a neuron or glial cell (Bayer and Altman, 1991). This view highlights the assumption that the major factor governing the size of the final neuronal population in the mature brain is the

magnitude and rate progenitor of cell proliferation. It was previously assumed that cell death had a minor role during embryonic cortical development, and that the major wave of cell death in the CNS occurred during target selection (Oppenheim, 1991). However, fairly recently it has been shown that programmed cell death plays an important role in the development of the cerebral cortex(Blaschke et al., 1996; Blaschke et al., 1998; Kuan et al., 2000). While an established role for programmed cell death in cortical development is a relatively new concept, the importance of cell death during the course of normal embryonic development had been known for quite some time (Clarke and Clarke, 1995; Clarke and Clarke, 1996; Jacobson et al., 1997). First observed during amphibian metamorphosis, it was found that stereotyped, and predictable cell death was observed during the development of both invertebrate and vertebrate animals (Truman et al., 1990). Later, it was discovered that not all forms of cell death were created equally and there is a distinction between the cell death observed during development and tissue homeostasis, and the pathological cell death that occurs as a result of trauma or ischemia (Kerr et al., 1974; Kerr et al., 1972). The latter form came to be known as necrosis and is characterized by the swelling and rupturing of the damaged cells causing them to leak their contents into the surrounding environment inducing an inflammatory response (Kerr et al., 1972). In contrast, when cells die during normal development or during tissue homeostasis they usually shrink and condense while maintaining the integrity of the plasma membrane, thus allowing the dying cell to be engulfed by surrounding cells before any of its contents are leaked into the surrounding environment. One of the hallmarks of cells undergoing this controlled cell death is the fragmentation of its nuclear DNA as the cell is packaged for disposal (Kerr et al., 1972; Vaux and Korsmeyer, 1999;

Wyllie et al., 1980; Yuan et al., 1993). This process came to be known as apoptosis, coined from the ancient greek word that describes the 'falling off' of flower petals or leaves from trees (Jacobson et al., 1997; Kerr, 1995; Kerr et al., 1972).

In a study using an optimized *in situ* end labeling plus technique (ISEL+) that labels fragmented nuclear DNA of dying cells, it was shown that cortical neuroblasts in the proliferative ventricular zone undergo programmed cell death (Blaschke et al., 1996). In fact using this particular technique it was observed that in the developing telencephalon as many as 70% of all cells are undergoing apoptosis at embryonic day 14, the rate of apoptosis declines to approximately 50% by embryonic day 18. The finding of such a high degree of cell death was highly controversial, and it remains unclear whether the ISEL+ is detecting cells in the early phase of apoptosis or, transient DNA breaks in cells not committed to apoptosis. While the accuracy of the ISEL+ and its ability to detect cells that were actually undergoing programmed cell death was highly controversial, with the magnitude of apoptosis in the developing cortex still subject to debate (Kuan et al., 2000; Voyvodic, 1996), this study did help shed light upon the idea that programmed cell death may be playing an important role during cortical development.

**The Genetics of Programmed Cell Death**

Genetics studies in the roundworm *Caenorhabditis elegans* demonstrated that animal cells have a built in cell suicide program, and that certain genes appear to be dedicated to the death program and its control. During the development of an adult worm 131 out of the 1090 total cells that make up the organism undergo programmed cell death in a lineage specific and mostly cell autonomous manner (Kuan et al., 2000; Metzstein et

al., 1998). Through genetic screening in the worms three groups of cell death genes have been identified. The first group of genes including egl-1, ced-4, and ced-3 and ced-9 are required for all somatic programmed cell death to occur (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Trent et al., 1983). The next group includes the genes ces-1 and ces-2 and is necessary for cell death of specific types of cells (Yuan and Horvitz, 2004; Yuan and Horvitz, 1990). The last group of genes which includes ced-1, ced-6, ced-7, ced-2, ced-5, ced-10 and nuc-1, are involved in degradation of DNA and phagocytosis of the remains of the apoptotic cell (Metzstein et al., 1998). The finding that genes in each of these groups had mammalian homologs raised the possibility that the genetic program for cell suicide and the necessity for its function has been conserved throughout evolution.

The strongest evidence for the necessity of cell death genes during cortical development came from mutant mice deficient in the pro-apoptotic ced-3 homologues that comprise a family of cysteine-containing, apartate-specific proteases called caspases. All of the caspase deficient mice that have been examined (Caspases 1, 2, 3, 8, 9, and 11) appear to have a cell type specific death defects, while none appears to have global defects in apoptosis (Cecconi et al., 1998; Hakem et al., 1998; Kuan et al., 2000; Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). The result from these caspase mutants indicates that the individual members of the caspase family appear to regulate cell death in a tissue specific or stimulus specific manner (Kuan et al., 2000). Surprisingly Casp3 and Casp9 null mutants had severe programmed cell death defects in the brain that lead to a malformed cortex with multiple indentations and periventricular masses consisting of supernumerary neurons(Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996). The mutants are embryonic or perinatal lethal with a reduction in pyknotic cells observed

specifically in the brain. These mutants thus provided the strongest evidence for the necessity of programmed cell death programs for proper cortical development. The similarity of the phenotypes of Casp3 and Casp9 mutants raised the possibility that they may be functioning within the same pathway in the developing brain. In fact biochemical studies would reveal that caspase 9 functions as an upstream activator of caspase 3 in the cell death pathway (Kuan et al., 2000; Li et al., 1997). It still remains unclear what factors lie downstream of caspase 3 and leads to apoptosis in during embryonic cortical development. One known downstream target of caspase 3 is DNA fragmentation factor 45 (DFF45), which is responsible for causing the fragmented DNA that characterizes apoptotic cells. However, mice with homozygous mutations in DFF45 are viable with no discernable defects in brain development (Zhang et al., 1998). The finding that there are a small number of Casp3 null mice that survive to adulthood without obvious defects (Kuan et al., 2000), would indicated that there are additional compensatory pathways for developmental apoptosis that remain to be identified .

**Neurotrophins are Extrinsic Factors Regulating Cell Death**

Over fifty-five years ago Viktor Hamburger and Rita Levi-Montalcini made the observation that the sensory ganglia of the neck are small when compared to the brachial ganglia which are larger because they innervate the additional mass of the limb in the embryonic chick (Levi-Montalcini, 1987). In addition they found that if the embryonic chick limb bud is removed early on, neuroblasts in the corresponding spinal ganglia will degenerate. These results suggested that neurons were competing for a limited supply of peripherally derived trophic factors and their death is the result of the

competition of surplus neurons for limited amounts of these factors. This 'neurotrophic hypothesis' went on to become one of the most influential concepts in developmental neurobiology (Aloe, 2004; Kuan et al., 2000; Levi-Montalcini, 1987). Levi-Montalcini and colleagues would eventually go on to discover the first diffusible survival factor that would eventually become known as Nerve Growth Factor (NGF) (Cohen and Levi-Montalcini, 1956). Eventually a larger family of molecules structurally related to NGF would be identified. Four members of the family have been found to be expressed in birds and mammals: NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). The characterization of NGF and later brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) revealed the essential role for cellular interactions in numerous phases of neural development (Huang and Reichardt, 2001). The expression of these molecules in the developing cortex also gave further evidence for the necessity of apoptosis for proper cortical development, and that cell survival could potentially be regulated by an extrinsic trophic factor in a manner similar to that observed in the spinal cord ganglia (Ernfors et al., 1992). Studies of the spatio-temporal distributions of NT-3 and BDNF, shows that these transcripts are expressed in the cortical ventricular/subventricular zones, coinciding with the onset of neurogenesis (Maisonpierre et al., 1990), along with their preferred receptors tyrosine kinase receptors TrkB and TrkC (Fukumitsu et al., 1998). The onset of neurotrophin expression may mark the time period when they first become generally required to maintain survival of neural precursors, and postmitotic neurons and their localization supports the notion that the mechanisms proposed in the neurotrophic hypothesis extends into early development of the brain where neurotrophins play

developmental roles of particular importance to brain morphogenesis. Survival factor dependence in a proliferating CNS population has previously been demonstrated in the generation of oligodendrocytes, where 50% of the cells die soon after they leave the cell cycle and begin to differentiate (Barres et al., 1992). In this case cell death appears to be functioning to regulate the number of oligodendrocytes to match the size of the axon population that needs to be myelinated. For CNS neural progenitors the largest body of evidence to support the existence of an exogenous factor has been observed *in vitro*. Multiple studies using cultures of neural precursors at the onset of neurogenesis have been used to demonstrate that NT-3 and BDNF are essential for progenitor cell survival. Studies in cell culture showed that the survival of CNS precursors is regulated by neurotrophins (Poser et al., 2003) and the use of function blocking antibodies against BDNF and NT-3 in cortical neural progenitor cultures causes a drastic reduction in their survival and proliferation and an inhibition of neurogenesis (Barnabe-Heider and Miller, 2003).

While *in vitro* evidence would suggest that neurotrophins regulate the size of the precursor pool and neurogenesis, analyses of various neurotrophin and Trk receptor knockouts has provided little evidence to suggest that neurotrophin function is essential for normal development in these cell populations during embryonic cortical development (Huang and Reichardt, 2001). Studies utilizing gene targeting technology revealed mice with deletions in several genes encoding neurotrophins displayed varying deficits in the CNS and PNS (Brady et al., 1999; Fan et al., 2000; Fundin et al., 1997; Kernie et al., 2000; Minichiello and Klein, 1996; Stucky et al., 1998), but, interestingly, mutations in the most well known neurotrophins BDNF, NT-3, and NT-4 don't exhibit severe

perturbations in normal embryonic cortical development (Snider, 1994), possibly due to complex compensation mechanisms and redundancy of other neurotrophic molecules (Brady et al., 1999; Minichiello et al., 1998). Another possibility is that although the classical neurotrophins are important for survival in the PNS, they may regulate other aspects of neuronal development, and survival in the CNS is regulated by the ever widening spectrum newly discovered molecules that have been shown to have roles in survival (Henderson, 1996).

**Additional Functions of Neurotrophins During Cortical Development**

In addition to their proposed roles for promoting survival of specific neuron and progenitor populations neurotrophins have also been shown to effect the differentiation and growth of neurons in the developing brain. As discussed previously several neurotrophins are expressed in the neocortex and hippocampus during development, and this expression continues in the adult animal (Ernfors et al., 1992; Ernfors et al., 1990; Maisonpierre et al., 1990). It is known that cortical progenitor cells express BDNF and NT-3 as well as their receptors TrkB and TrkC. It has also been shown that these neurotrophins play an important role in regulating cell cycle exit of cortical progenitors, and promoting a neural cell fate upon exiting the cell cycle (Barnabe-Heider and Miller, 2003; Ghosh and Greenberg, 1995). In cell culture BDNF, NT-3 and NT-4 have been shown to promote differentiation of hippocampal neuron precursors, and NT-3 has been shown to promote differentiation of rat cortical precursors.

**Neurotrophin functions during developmental and adult plasticity**

*In situ* hybridization studies of neurotrophins and their receptors have shown that they are developmentally regulated, where mRNA levels for TrkB and TrkC transiently peak between postnatal day 1 (P1) and P14 correlating with times of maximal neuronal growth, differentiation and synaptogenesis (Ernfors et al., 1990). In the adult cortex BDNF and NGF mRNA have been shown to be regulated by epileptiform activity in the hippocampus and the cerebral cortex (Ernfors et al., 1991; Gall and Isackson, 1989). Changes in the levels of sensory stimuli such as light, or whisker activity also regulate the levels of BDNF and TrkB (Castren et al 1992, Schoups et al 1995, Rocamora et al., 1996). These activity dependent spatiotemporal patterns of expression are consistent with a role for neurotrophins during developmental and adult plasticity.

It is believed that neurotrophins may exert control over developmental and adult changes in cortical circuitry through control of dendritic and axonal arbor growth, as it was NGF's dramatic effects on neurite outgrowth that initially caught the attention of Levi-Montalcini and colleagues (Cohen and Levi-Montalcini, 1956; Levi-Montalcini, 1987). Neurotrophins are believed to exert both tropic and trophic influences on the growth of axons (McAllister et al., 1999). *In vitro* experiments have shown that neurotrophin gradients can mediate chemotropic effects on growth cones of specific populations of dorsal root ganglion cells (McAllister et al., 1999). Local application of BDNF, or application of an antagonist has been shown to increase and decrease respectively the complexity of *Xenopus* retinal ganglion cell dendritic arbors (Cohen-Cory, 1999; McAllister et al., 1999). Applications of BDNF, NT-3 or NT-4 to slices of neonatal cortex have also been shown to regulate the dendritic morphology of pyramidal cells over comparatively short time spans (Horch et al., 1999). In addition to their effect

on structure, neurotrophin can also have effects upon the synaptic properties of neurons. BDNF and NT-4 have been shown to rapidly enhance spontaneous synaptic activity in dissociated hippocampal neurons (Levine et al., 1998). In hippocampal slices acute applications of BDNF or NT-3 potentiates synaptic transmission at Schaffer collateral/CA1 synapses (Kang and Schuman, 1995). Recent studies have shown that BDNF may function to promote synaptic maturation of GABAergic cells and thereby regulate the onset of the visual critical period for ocular dominance plasticity (Huang et al., 1999).

Although they were originally discovered for their survival and growth promoting activities, neurotrophins appear to function in numerous capacities throughout development, and even into the adult animal, by promoting growth and differentiation and even synaptic plasticity of surviving neuronal populations.

**CPG15 as a putative Neurotrophic Factor**

In order to isolate activity dependent effector genes, Nedivi and colleagues used a highly sensitive subtractive and differential cloning procedure (Nedivi et al., 1993). This screen isolated over 300 candidate plasticity genes (CPGs). Sequence analysis showed that 120 of the cloned CPGs encode known proteins, and approximately 70 correspond to expressed sequence tags, while more than 100 are novel. The known CPGs can be classified into distinct functional categories such as, transcription factors, components of second messenger pathways, growth factors, and structural proteins. Amongst the novel CPGs a potential growth factor named Candidate Plasticity Gene 15 (CPG15) was discovered. *cpg15* encodes a small highly conserved protein with a secretion signal and a

consensus sequence for membrane attachment via a glycosylphosphatidylinositol (GPI) link [Nedivi, 1993 #4; Hevroni, 1998 #48 Naeve, 1997 #156; Nedivi, 1998 #219]. CPG15 has been shown to function non-cell autonomously to coordinately regulate growth of apposing dendritic and axonal arbors, and to promote synaptic maturation in the developing *Xenopus* optic tectum (Cantallops et al., 2000; Hevroni et al., 1998). As an activity-regulated gene, late *cpg15* expression is contemporaneous with critical periods for activity-dependent plasticity and requires action potential activity. However, *cpg15* is also expressed in an activity-independent manner during early brain development prior to circuit formation and maturation, (Corriveau et al., 1999; Lee and Nedivi, 2002), suggesting that it may play an additional role at this stage.

**Summary**

While neurotrophins are the most widely studied molecules with important roles in brain morphogenesis and synaptic development, there are obviously numerous other extrinsic factors that regulate neuron development and function. CPG15 may be a molecule with multiple roles in nervous system function similar to those attributed to the classical neurotrophins. In Chapter 2, I characterize the activity dependent expression of *cpg15* in the rodent somatosensory cortex. I will demonstrate that *cpg15* expression is regulated by sensory activity that has been shown induce receptive field plasticity in the mouse barrel cortex. I further demonstrate that the activity-dependent expression of *cpg15* is at least partially regulated by the transcription factor CREB. In Chapter 3, I characterize the activity independent expression of *cpg15* in the embryonic brain, and show that a soluble form of CPG15 functions to promote survival of neurons and neural

progenitors *in vivo*. In Chapter 4, I discuss the role of CPG15 as a molecule with pleiotropic functions in development and plasticity that are dependent upon the cellular context in which it is expressed.

# Chapter 2

## Regulation of *cpg15* Expression during Single Whisker Experience in the Barrel Cortex of Adult Mice


### Abstract

Regulation of gene transcription by neuronal activity is thought to be key to the translation of sensory experience into long-term changes in synaptic structure and function. Here we show that *cpg15*, a gene encoding an extracellular signaling molecule that promotes dendritic and axonal growth and synaptic maturation, is regulated in the somatosensory cortex by sensory experience capable of inducing cortical plasticity. Using in situ hybridization, we monitored *cpg15* expression in 4-week-old mouse barrel cortex after trimming all whiskers except D1. We found that *cpg15* expression is depressed in the deprived barrels and enhanced in the barrel column corresponding to the spared D1 whisker. Changes in *cpg15* mRNA levels first appear in layer IV, peak 12 h after deprivation, and then decline rapidly. In layers II/III, changes in *cpg15* expression appear later, peak at 24 h, and persist for days. Induction of *cpg15* expression is significantly diminished in adolescent as well as adult CREB knockout mice. *cpg15*'s spatio-temporal expression pattern and its regulation by CREB are consistent with a role in experience-dependent plasticity of cortical circuits. Our results suggest that local structural and/or synaptic changes may be a mechanism by which the adult cortex can adapt to peripheral manipulations.


### Introduction

The rodent vibrissae system is a useful model for studying activity dependent plasticity in the adult cortex. Each vibrissa projects to a discrete aggregate of neurons in

layer IV of the somatosensory cortex, known as a barrel (Woolsey and Van der Loos, 1970). Neurons in a particular barrel column are maximally excited by a single principal whisker and to a weaker extent by surround whiskers (Armstrong-James and Fox, 1987; Simons, 1978). Neuronal receptive fields in the barrel cortex are plastic and change in response to sensory experience even in the adult brain (Fox, 2002). Simply trimming whiskers on the mystacial pad results in a profound depression of responses in the contralateral barrel cortex corresponding to the deprived whiskers (Armstrong-James et al., 1994; Diamond et al., 1993). When all the large whiskers are trimmed except a single ("spared") whisker, responses to the spared vibrissa are potentiated in the neighboring, deprived barrels following the initial depression caused by trimming of their primary whiskers ((Glazewski et al., 1996). Both synapse formation and elimination (Trachtenberg et al., 2002) and long term potentiation (LTP)-like mechanisms (Hardingham et al., 2003) have been implicated in barrel receptive plasticity. Yet the molecular mechanisms underlying cortical receptive field plasticity are largely unknown.

Molecules involved in learning and memory, LTP, long term depression (LTD) and in visual developmental plasticity have also been implicated in receptive field plasticity of barrel cortex (Glazewski et al., 1999; Glazewski et al., 1996). One such molecule is cyclic-AMP (cAMP) responsive element binding protein (CREB), a transcription factor that regulates gene expression by binding to $Ca^{2+}$/cAMP-responsive element (CRE) promoter sites (Glazewski et al., 1999; Glazewski et al., 1996). In response to single whisker experience, potentiation of the spared vibrissa response in adult α, δ CREB mice is reduced relative to wild-type animals (Glazewski et al., 1999). In transgenic mice carrying a lacZ reporter gene driven by a promoter containing CRE

sites, single whisker experience induces lacZ expression in the barrel corresponding to the spared whisker (Barth et al., 2000). CREB activation during single whisker experience is likely to be on e of the first steps in a transcriptional program leading to long term changes I synaptic properties. *cpg15* is a CREB target gene and a potential downstream effector in an activity-dependent transcriptional program. *cpg15* (also known as Nrn1) was isolated in a screen for seizure-induced genes in the hippocampal dentate gyrus (Hevroni et al., 1998; Nedivi et al., 1993) and was subsequently shown to be regulated by light in the visual system, where it is expressed in correlation with critical periods for activity-dependent plasticity (Corriveau et al., 1999; Lee and Nedivi, 2002). *cpg15* contains CRE sites in its promoter region and its activity-dependent regulation in cultured cortical neurons is partially regulated by CREB (Fujino et al., 2003). CPG15 (also known as neuritin-1) encodes a small highly conserved protein that in membrane bound form coordinately regulates growth of apposing dendritic and axonal arbors, and promotes synaptic maturation (Cantallops et al., 2000; Nedivi et al., 1998).

Here we use *in situ* hybridization to monitor experience dependent expression of *cpg15* in barrel cortex of wild type and CREB knockout mice. We find that *cpg15* expression is modulated by sensory experience capable of inducing receptive field plasticity and that this expression is regulated by CREB.

## Results

### Experience-Dependent Expression of *cpg15* in Barrel Cortex

We investigated whether *cpg15* is expressed in barrel cortex and whether this expression is regulated by sensory experience known to induce plasticity. *in situ*

hybridizations with a *cpg15* probe were conducted on tangential sections through the barrel cortex of four week old mice with normal whisker experience or after unilateral deprivation of all the large whiskers (A1-A4), B1-B4, C1-C5, D2-D5, E1-E5, α, β, γ and δ) except D1 on the right side of the muzzle (referred to as single whisker experience). Previous studies have shown that, at this age single whisker experience induces significant experience dependent plasticity in extragranular layers (Fox, 1994; Glazewski and Fox, 1996). The patterned distribution of *cpg15* mRNA in barrel-like compartments was readily apparent in tangential sections through layer IV of somatosensory cortex in mice with intact whisker fields (Figure 2-1A). *cpg15* mRNA distribution was characterized by regions of low intensity signal corresponding to cell sparse barrels, separated by regions of high intensity signal corresponding to the relatively cell dense septa surrounding each barrel (Figure 2-1A). A quantitative comparison showed that in control animals, mRNA signal intensity in the D1 barrel and surrounding septa was similar to the mRNA signal in other barrels (large and small) and their surround septa (Figure 2-1D, blue bars). Twelve hours of single whisker experience resulted in increased *cpg15* signal intensity within the D1 barrel and its septal rim as compared to the small barrels with septa corresponding to the spared anterolateral small vibrissae (Figure 2-1B aligned with 1C quantified in 1D, D1/Spared). There was no apparent change in *cpg15* expression in these small barrels located three to four arcs away from the D1 barrel. Most barrels and their surrounding septa corresponding to the deprived whisker rows showed a marked decrease in *cpg15* signal intensity when compared to the small spared barrels and their septal rims (Figure 2-1D, Deprived/Spared). Since changes in *cpg15* expression in the barrels and their septal rims were qualitatively similar, we continued to measure

expression in each barrel and its septal rim a single unit (henceforth referred to as barrel unit). The difference in the D1/Deprived ration between the control and single whisker experience mice was large and highly significant (Figure 2-1D, D1/Deprived). The D1/Deprived ratio provides a measure of net change in *cpg15* expression by combining the increased *cpg15* mRNA expression levels in the D1 barrel unit with the diminished *cpg15* expression in the adjacent deprived barrel units. These results demonstrate that *cpg15* expression in the barrel cortex can be regulated by changes in sensory experience known to induce receptive field plasticity.

**Layer Localization of Activity-Dependent *cpg15* Expression in Barrel Cortex**

During adolescence and in the adult, receptive field plasticity in barrel cortex is manifested mainly in the superficial layers (reviewed in Fox, 2002). To determine if changes in *cpg15* expression correspond with the location of electrophysiologically measured receptive field plasticity, we analyzed *cpg15* distribution within serial sections tangentially cut through barrel cortex of 4-week-old mice after unilateral single whisker experience. We found that after 12h of single whisker experience, *cpg15* expression was highest in layer IV and layers II/III (Figure 2-2B-F). Although barrels were not anatomically apparent in layers II/III, *cpg15* expression in these layers was similar to the pattern seen in layer IV, with increased expression in the region corresponding to the spared D1 barrel unit and decreased levels in the surrounding area representing the deprived whiskers. *cpg15* expression in layers I, V and VI was low and remained relatively unchanged by whisker manipulation (Figure 2-2G-I). These results show that

changes in *cpg15* expression due to single whisker experience are localized to layers of barrel cortex that undergo electrophysiological changes during receptive field plasticity.

**Time Course of Activity-Dependent *cpg15* Expression in Barrel Cortex**

Single unit studies have shown that changes in whisker responses can begin after 16h of single whisker experience and continue for weeks thereafter (Barth et al., 2000). If these long-term changes result from alterations in molecular constituents within cortical neurons, one would expect that physiological changes be preceded by changes in gene expression. To investigate the onset and time course of *cpg15* expression, we monitored mRNA levels through all cortical layers of mice after single whisker experience of 6h, 12h, 24h, 3d or 7d. In layer IV, the typical *cpg15* pattern in response to single whisker experience could be seen as soon as 3h after whisker trimming and was clearly visible until 12h after onset of single whisker experience (Figure 2-3, left). In layers II/III, onset of the *cpg15* response was first evident at 6h after whisker trimming, but was apparent up to 3d after onset of single whisker experience (Figure 2-3, right). To quantify these results and to assess whether *cpg15* elevation in D1 and depression in the surrounding barrel field follow a similar time course, we compared the D1/Spared, Deprived/Spared and D1/Deprived whisker ratios at each time point (Figure 2-4). *cpg15* induction in the D1 barrel unit (D1/Spared) peaked after 12h of single whisker experience in layers II/III as well as IV (Figure 2-4A,B) *cpg15* depression in the surrounding barrel field (Deprived/Spared) showed a similar trend, but was significant only in layer IV at 12h (Figure 2-4C,D). Although *cpg15* induction in D1and depression in the deprived barrel units are significant only at 12h (likely due to small sample size at other time points), the

patterns are somewhat different for the two layers, suggesting that with additional sampling it may be possible to discriminate whether *cpg15* induction in the spared D1 barrel unit and depression in the surrounding deprived barrel units differ in their time course between layer IV and the superficial layers. When the net change in *cpg15* expression was quantified by combining the increased *cpg15* mRNA levels in the D1 barrel unit with diminished *cpg15* expression in the adjacent deprived barrel units (D1/Deprived), the difference in the time course of *cpg15* expression was after 3h of single whisker experience, with levels markedly elevated only until 12h (Figure 2-4E). In layers II/III, onset of the *cpg15* response occurred later. A significant increase in the D1/Deprived signal ratio was detectable only after 12h of single whisker experience (Figure 2-4F). However, in contrast to the rapid return to basal expression seen in layer IV, change in *cpg15* levels in the layer II/III region corresponding to the D1 barrel units was maintained for at least 3d after onset of single whisker experience (Figure 2-4F). These laminar differences in the *cpg15* response to single whisker experience are consistent with an initial activity-dependent response in layer IV, the primary thalamic input layer, subsequently progressing to the superficial layers where changes in receptive field properties then persists for days (Glazewski and Fox, 1996; Huang et al., 1998).

**Decreased *cpg15* Induction on α/δ CREB Knockout Mice**

The transcription factor CREB is known to be required for experience-dependent receptive field plasticity in response to whisker manipulation (Glazewski et al., 1999). The requirement for CREB is likely due to its regulation of downstream effector genes

that mediate changes in synaptic structure and function. Studies of *cpg15* regulation in cultured cortical neurons indicate that CREB is a mediator of *cpg15* activity-dependent expression *in vitro* (Fujino et al., 2003). To determine if *cpg15* is a CREB-regulated effector gene that is induced *in vivo* during receptive field plasticity, we examined *cpg15* expression in barrel cortex of α/δ CREB knockout mice after 12h of single whisker experience. Since previous studies using the spared whisker paradigm have shown that plasticity in layers II/III of these mutants is relatively unaffected in adolescent animals (1-2 months) but is significantly diminished in adult (>6 months) mice) (Glazewski et al., 1999), we tested both adolescent and adult knockout α/δ CREB mice.

When comparing cytochrome oxidase staining of layer IV sections from cortices of the CREB mutants with those of their wild-type counterparts, it was apparent that the size of the barrel field was smaller in the mutants (Figure 2-5A, B). CREB mutants were previously found to have a thalamus significantly smaller than normal (Pham et al., 2001), suggesting that CREB may have a role in development of sensory brain structures. However, organization of the individual barrels and their relative size and position within the field is normal. The CREB mutants show no overt behavioral abnormalities and the mutation does not seem to cause sensory or motor performance deficits (Glazewski et al., 1999).

In response to single whisker experience, the qualitative pattern of changes in *cpg15* expression was similar in the wild-type and CREB mutants; *cpg15* levels in the spared D1 barrel unit were increased while levels in the surrounding barrel field were decreased (Figure 5C, D). However, the magnitudes of the increase in the D1 barrel unit and the decrease in barrels representing the large deprived whiskers were both less in the

α/δ CREB mutants relative to wild-type mice. To quantify the difference in *cpg15* expression between wild-type and α/δ CREB knockouts, we compared the ratio of *cpg15* signal intensity in the D1 to its level in the surrounding deprived barrel units in control hemispheres or after single whisker experience. We found that *cpg15* levels in control hemispheres of both adolescent 4-week-old mice and 6-month old adults were not significantly different between the wild-type and α/δ CREB knockouts (Figure 5E, F). The change in the D1/Deprived ratio in response to single whisker experience was 16% smaller in the 4-week-old α/δ CREB knockouts relative to their wild-type control counterparts (Figure 5E, $*p < 0.05$), and 23% smaller when 6-month-old mice were compared (Figure 5F, $*p < 0.01$). These results show that lack of α/δ CREB isoforms diminishes activity-dependent changes in *cpg15* expression in response to the spared whisker experience.

## Discussion

We measured the experience-dependent expression of *cpg15*, a gene encoding a signaling molecule that promotes growth of dendritic and axonal arbors and synaptic maturation in developing neurons, in the adult barrel cortex. We found that the spatial and temporal patterns of *cpg15* expression, and its regulation by CREB, are consistent with a role in mediating adult receptive field plasticity.

### Spatial and Temporal Features of Cortical Plasticity

Layer II/III neurons are the primary substrate for receptive field plasticity in the adult barrel cortex (Glazewski and Fox, 1996; Glazewski et al., 1998). After deprivation,

depression of deprived whisker responses, likely due to changes at synapses of layer IV onto layer II/III neurons (Allen et al., 2003; Shepherd et al., 2003), occurs rapidly (Glazewski and Fox, 1996). Potentiation of the response to the spared whisker in deprived barrels happens later. Previous studies in *Xenopus* and feline visual systems have suggested that *cpg15* expression is presynaptic to the connections undergoing activity-dependent plasticity (Corriveau et al., 1999; Nedivi et al., 2001). Thus, the spatio-temporal patterns of *cpg15* expression we observe are consistent with experience-dependent physiological plasticity in barrel cortex. Early up-regulation (down-regulation) of *cpg15* in layer IV may relate to strengthening (weakening) of layer IV to layer II/III synapses, while its subsequent expression in the superficial layers relates to modification of horizontal connections within these layers. It is interesting to note that *cpg15* expression is regulated in both the barrel and its surrounding septal region since the structures receive input through parallel subcortical pathways (Koralek et al., 1988; Lu and Lin, 1993), and give rise to two distinct intracortical circuits (Kim and Ebner, 1999). The layer IV barrel columns receive input from the ventral posteriormedial nucleus of the thalamus (VPM), and within somatosensory cortex project short distances to superficial layers of the same barrel or immediately neighboring barrels (Koralek et al. 1988; Lu and Lin, 1993; Kim and Ebner, 1999). These observations indicate that modifications in both types of circuits occur, or they may be required for physiological changes in barrel representation.

**Molecular Analysis of Receptive Field Plasticity in Barrel Cortex**

Only a handful of studies have addressed the immediate effect (on the scale of hours) of sensory experience in the adult on expression of candidate proteins (Barth et al., 2000; Bisler et al., 2002; Filipkowski et al., 2000; Rocamora et al., 1996; Staiger et al., 2002). Here we show that, by altering sensory experience for a few hours, we can produce significant changes in *cpg15* expression within barrel cortex. Transcriptional activation is usually a good indicator of a subsequent increase in protein levels. Indeed following kainic acid seizure in the hippocampus, both the *cpg15* transcript and the CPG15 protein are dramatically induced (Naeve et al., 1997; Nedivi et al., 1998). Other than transcription factors *cpg15* and BDNF are the only genes shown to be rapidly regulated by activity in a manner spatially and temporally correlated with experience-dependent synaptic modification in adult barrel cortex (Rocamora et al., 1996). Moreover, *cpg15* is the first activity-dependent effector gene demonstrated to be regulated downstream of CREB in the context of barrel cortex plasticity *in vivo*.

Previous studies have shown that barrel plasticity in α/δ CREB knockout mice is significantly reduced, although not eliminated (Glazewski et al., 1999). Our findings regarding *cpg15* expression in these mice are consistent with these studies, in that activity-dependent *cpg15* induction within the spared whisker barrel unit is diminished, but not completely abolished. *In vitro* studies of *cpg15* regulation in cultured cortical neurons indicate that CREB is a mediator of *cpg15* activity-dependent expression, but is by no means the only transcriptional activator involved (Fujino et al., 2003). Thus CREB likely works in concert with other transcription factors to modify expression of *cpg15* and other genes that contribute to synaptic plasticity. Another possible explanation for the partial effect of α/δ CREB isoform deletion on both receptive field plasticity and *cpg15*

expression in barrel cortex is that in the mutants there is compensation for the loss of the α/δ CREB isoforms by up-regulation of the β isoform (Glazewski et al. 1999), or other CREB-like transcription factors (Hummler et al., 1994). These factors may act to partially rescue cortical receptive field plasticity.

**Potential Role of *cpg15* in Adult Receptive Field Plasticity**

In the developing *Xenopus* tectum (Nedivi et al., 1998; Cantallops et al. 2000), *cpg15* functions as an intercellular signaling molecule that promotes process outgrowth and synaptogenesis. While the role of *cpg15* in adult barrel cortex has not been defined, it would be intriguing to consider that it performs a similar function. Recent *in vivo* imaging studies have not revealed changes in dendritic arbor morphology of barrel cortex neurons during receptive field plasticity (Trachtenberg et al., 2002). Yet these same studies provide evidence for increased synapse turnover in response to experience-dependent receptive field plasticity and demonstrate that synapse formation and elimination is associated with sprouting and retraction of dendritic spines (Trachtenberg et al., 2002). Expression of *cpg15* in the context of receptive field plasticity could be related to its involvement in small scale synaptic changes that consolidate adaptive cortical responses to peripheral manipulation.

**Methods**

**Animal manipulations and tissue isolation**

All animal work was approved by the Massachusetts Institute of Technology and Cold Spring Harbor Laboratory Committees on Animal Care and conforms to NIH

guidelines for the use and care of vertebrate animals. C57BL/6 (+/+) (Charles River, Wilmington, MA) or CREB α, δ knockout mice (-/-) (Bourtchuladze et al., 1994; Hummler et al., 1994) were used at 4-5 weeks or 6 months of age. For the single whisker experience paradigm, mice were gently restrained by hand and all the large whiskers (A1-A4), B1-B4, C1-C5, D2-D5, E1-E5, α, β, γ, and δ) except D1 on the right side of the muzzle were trimmed close to the face (<1 mm) using microspring scissors. Whiskers on the left muzzle were left intact, and their corresponding hemispheres served as controls. After 12h of sensory deprivation, mice were sacrificed by guillotine decapitation, and brains immediately removed. At 4-5 weeks n = 8 brains per group (+/+ or -/-). At 6 months, n = 6 for +/+ and n = 5 for -/-. For a time course of the cpg15 response to the single whisker experience, additional 4-5 week old mice were harvested after 3h, 6h, 24h, 3d, or 7d of sensory deprivation (n = 2 brains per time point). For long deprivation periods, whiskers were trimmed every 2d. Cortical hemispheres were rapidly dissected from the freshly removed brains and flattened between two clean microscope slides, rapidly frozen in dry ice and stored at -80°C.

### *In situ* hybridizations

Flattened cortices were tangentially sectioned by cryostat (12um), thaw-mounted on Superfrost/plus microscope slides (VWR Scientific, West Chester, PA), dried, fixed in 4% paraformaldehyde, washed in PBS, dehydrated in ethanol, air-dried, and stored desiccated at −80°C. Before hybridization, slides were pretreated (at room temperature, unless otherwise stated) with 0.2 M HCl (20 min), double distilled water (DDW) (5 min), 2× SSC (30 min at 70°C), and DDW (5 min). The next prehybridization treatments, from

pronase (type XIV) (Sigma) to air-drying slides for 1 hr, were conducted as described previously (Hogan et al., 1994). RNA probes were synthesized with an RNA transcription kit (Stratagene, La Jolla, CA) and $^{35}$S-UTP (800 Ci/mmol; Amersham Biosciences, Piscataway, NJ), using linearized *cpg15* cDNA as a template. Hybridizations were done as described previously (Nedivi et al., 1996). Posthybridization wash conditions were as follows: 3 hr at 50°C in 50% formamide and 1× salt solution (Hogan et al., 1994) with 10 mM DTT; 15 min at 37°C in TNE (10 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM EDTA); 30 min at 37°C in TNE containing RNase A (20 μg/ml; Sigma); 30 min at 37°C in TNE; and finally overnight at 50°C in 50% formamide and 1× salt solution. Slides were dehydrated with 0.3 M $NH_4Ac$ in ethanol, air-dried, and processed for autoradiography as described previously (Hogan, 1994), using autoradiographic emulsion type NTB-2 (Eastman Kodak) diluted 1:1 with 2% glycerol, and exposed for 3-5 d at 4°C.

**Quantitative Analysis**

*In situ* hybridizations on brains of age-matched wild type and mutant animals were always carried out in parallel. Brains from the 4-week wild type and mutant animals with 12h of single whisker experience were processed in four different experiments containing two brains from each group. Brains from the 6-month wild type and mutant animals were processed in two experiments, each containing one brain from each time point. Prehybridization treatments hybridizations, posthybridization washes, processing for autoradiography, counterstaining and mounting were done as previously described (Lee and Nedivi, 2002). In all experiments, alternate slides were stained by cytochrome

oxidase to provide a clear anatomical map of the barrel field in layer IV (Wong-Riley, 1979). On the *in situ* hybridized slides, two to three sections from each brain contained barrel fields sufficiently intact for quantitative analysis. Dark field images of these layer IV sections were imported into Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA) with a Diagnostic Instruments (Sterling Heights, MI) Spot 2 digital camera mounted on a Nikon (Tokyo, Japan) Eclipse E600 using a 1x/0.04 Plan ultra-wide objective. Images were saved as gray-scale Tiff's and imported in NIH Image (version 1.62). Mean pixel density measurements were taken from four areas on each slide: the area representing the D1 whisker (barrel and septae), the area representing the other large whiskers (A1-A4, B1-B4, C1-C5, D2-D5, E1-E5, α, β, γ, and δ) (deprived area during single whisker experience), the area corresponding to the anterolateral small vibrissae (spared in single whisker experience), and the background outside of the section. Pixel density was measured on a 0-255 scale, in which 255 is white. To ensure that measurements were unbiased, image files from wild type and mutant animals were coded and measured blind to age and genotype. The background served as a zero labeling negative control and was subtracted from the mean pixel densities of the D1, large barrel (Deprived), and small barrel (Spared) regions to yield the net mean pixel density for each region. The net mean pixel density of the D1 barrel was divided by the net mean density f the large or small barrel regions on the same section. Measurements from 2-3 sections per layer were averaged for each brain. Statistical significance was determined by unpaired Student's *t* test for all experiments except the time course. The time course data was compared using ANOVA post-hoc analysis with the Bonferroni/Dunn method. In all experiments, n represents number of brains.

**Figure 2-1. *cpg15* expression in barrel cortex is regulated by single whisker experience**

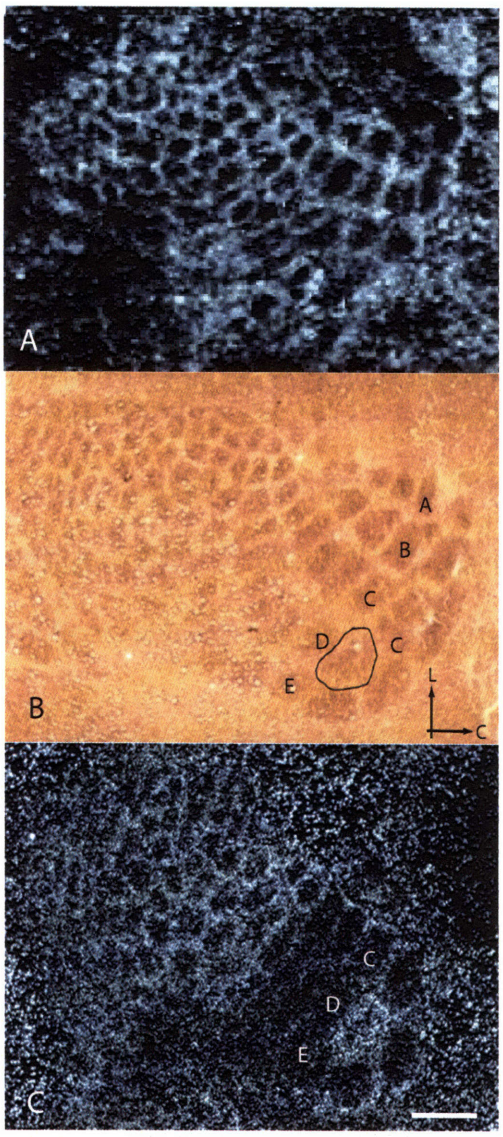


**Figure 2-2. Activity dependent *cpg15* expression is localized to layers II/III and IV of barrel cortex.**

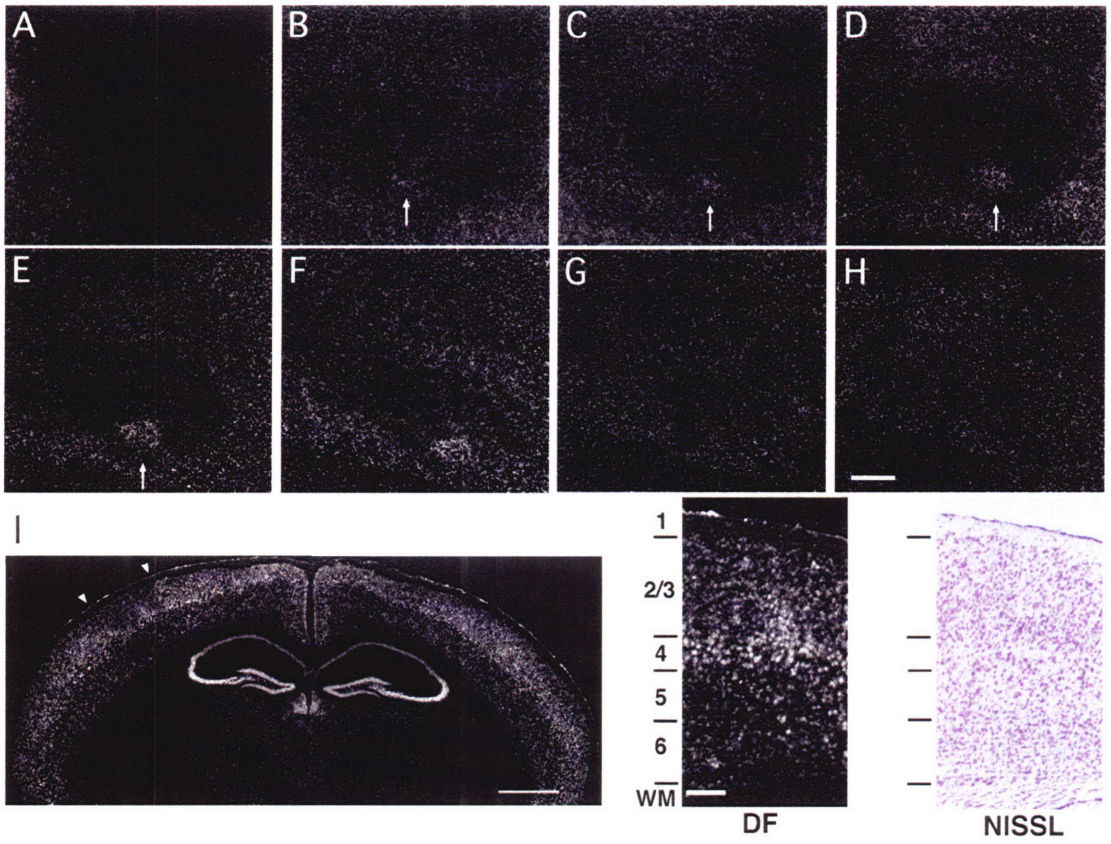


**Figure 2-3. Time course of *cpg15* expression in barrel cortex after spared whisker experience.**

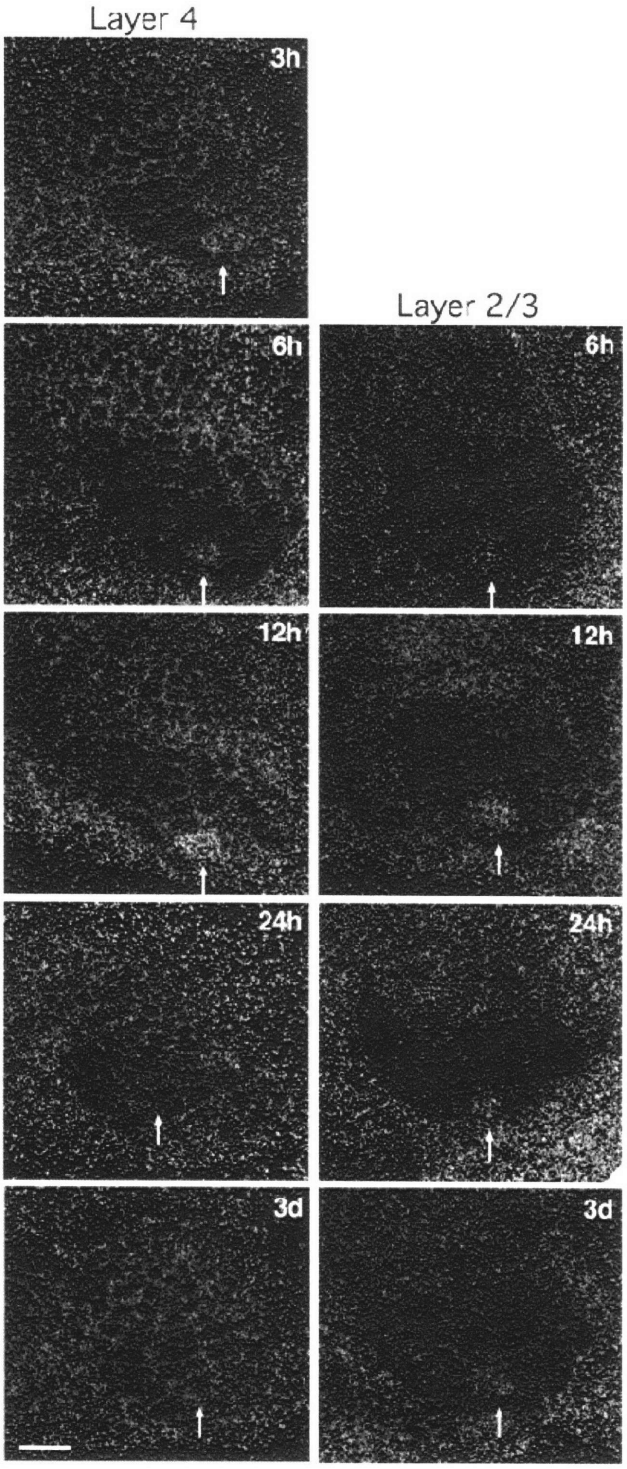


**Figure 2-4. Quantification of the time course *cpg15* expression after single whisker experience.**

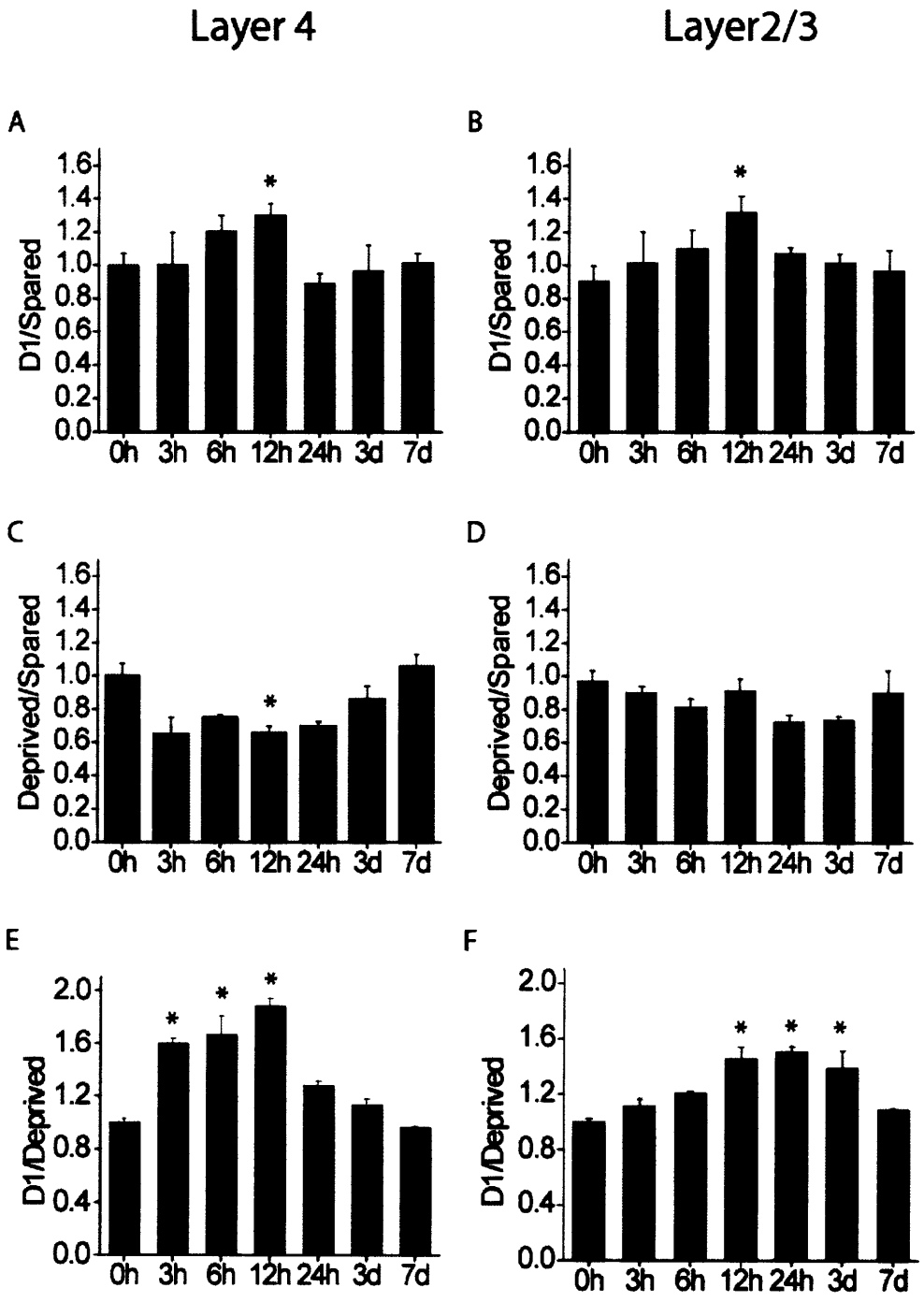
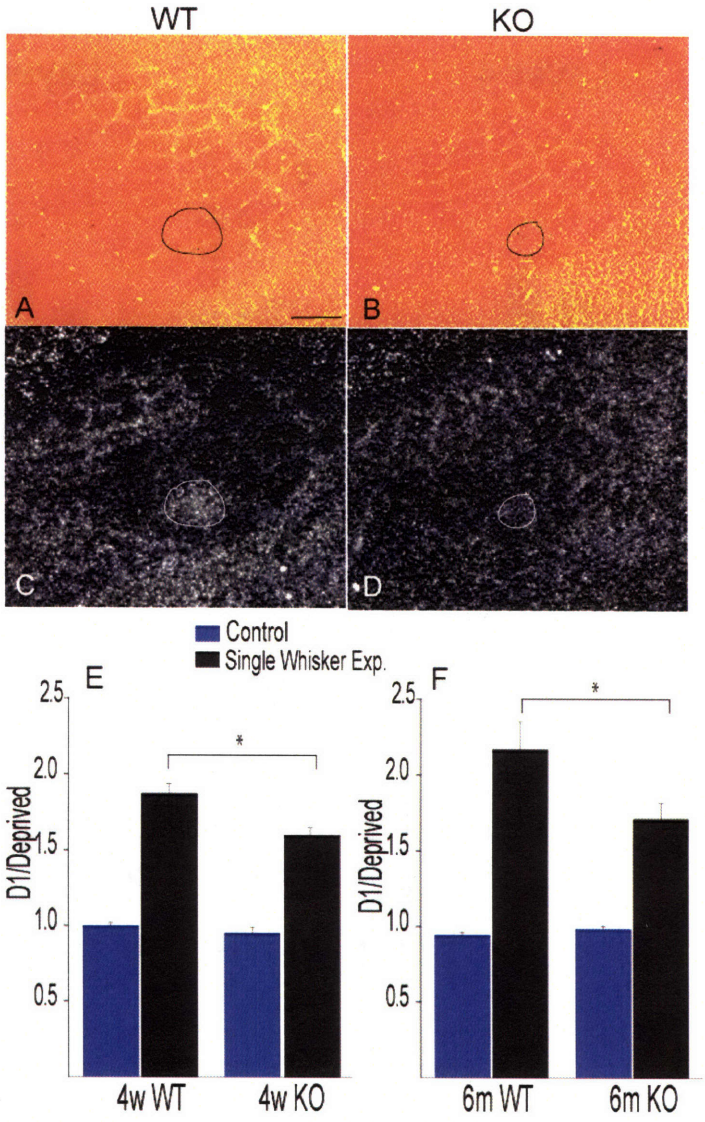


**Figure 2-5. Induction of *cpg15* is diminished in CREB mutant mice.**



## Figure Legends

**Figure 2-1.** *cpg15* expression in barrel cortex is regulated by single whisker experience. Representative dark-field photomicrographs of *in situ* hybridizations for *cpg15* mRNA in mice: (A) with normal whisker experience. (C) after 12h of single whisker experience. (B) Cytochrome oxidase staining of a section adjacent to (C) with the region representing the spared D1 whisker outlined. Arrows mark lateral (L) and caudal (C) orientation. (D) A quantitative comparison of sections such as shown in (A) and (C) (n = 8 brains per group, 2-3 sections per brain). In response to single whisker experience, *cpg15* levels are increased in the barrel and septal region (barrel unit) representing the D1 whisker when compared with barrel units of the small (untrimmed in single whisker experience) whiskers in the same section (Spared), and decreased in the barrel units of the large whiskers (trimmed in single whisker experience) (Deprived). The D1/Deprived ratio represents the net change in *cpg15* levels, combining the increase in the D1 barrel unit with the decrease in the surrounding deprived barrel units. *$p < 0.05$, **$p < 0.001$. Scale bar, 350μm.

**Figure 2-2.** Activity-dependent *cpg15* expression is localized to layers II/III and IV of barrel cortex. Representative dark-field photomicrograph of *in situ* hybridization for *cpg15* mRNA on serial tangential sections through flattened cortex of a 4-week-old mouse after 12h of single whisker experience. The sections progress from layer I (A) to layer VI (H). Arrows mark the cortical region representing the spared whisker in layers II through IV (B-F). Scale bar, 0.5mm. (I) *cpg15* mRNA localization is shown in a coronal section through the brain of a mouse treated as above. The contralateral hemisphere to the

spared whisker shows *cpg15* up-regulation in the region representing the D1 whisker, with a concomitant down-regulation in the surrounding barrel field. White arrowheads indicate the region of the barrel field shown at high magnification on the right. Scale bar, 1mm. Dark-field view shown side by side with a bright-field view of an adjacent Nissl stained section delineating cortical layers. Scale bar, 0.1mm.

**Figure 2-3.** Time course of *cpg15* expression in barrel cortex after spared whisker experience. Representative dark-field photomicrographs of *cpg15* expression in layer IV (left) and layers II/III (right) of the barrel field after different lengths of spared whisker experience. Scale bar, 0.5mm. Arrows mark the spared D1 barrel unit.

**Figure 2-4.** Quantification of the D1/Spared, Deprived/Spared, and D1/Deprived ratios in layer IV (A, C, E), and II/III (B, D, F) as a function of single whisker experience time (n = 2 brains per time point, 2-3 sections per brain). Compared to 0h controls, $*p < 0.0024$ (5% significance level). Although *cpg15* induction in D1 (A, B) and depression in the deprived barrels (C, D) are significant only at 12h (likely due to the small sample size at other time points), the net change in *cpg15* expression, combining induction in D1 and depression in the deprived neighboring barrel units (D1/Deprived) shows a significantly different time course in layers IV and II/III.

**Figure 2-5.** Induction of *cpg15* is diminished in CREB mutant mice. Cytochrome oxidase staining of the barrel field of WT (A) and α/δ CREB knockout mice (B) with the D1 barrel unit outlined. Corresponding *in situ* hybridizations for *cpg15* mRNA after 12h

single whisker in WT (C) and α/δ CREB knockout mice (D). (E) Quantification of the D1/Deprived ratios show that *cpg15* regulation in response to single whisker experience is significantly reduced in both 4-week-old ($n = 8$ +/+; $n = 8$ -/-; $^*p < 0.05$) and 6-month-old animals ($n = 6$ +/+; $n = 5$ -/-; $^*p < 0.01$).

# Chapter 3

**Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis.**


## Abstract

The balance between proliferation and apoptosis is critical for proper development of the nervous system. Yet, little is known about molecules that regulate apoptosis of proliferative neurons. Here we identify a soluble, secreted form of CPG15 expressed in embryonic rat brain regions undergoing rapid proliferation and apoptosis, and show that it protects cultured cortical neurons from apoptosis by preventing activation of caspase 3. Using a lentivirus-delivered small hairpin RNA, we demonstrate that endogenous CPG15 is essential for the survival of undifferentiated cortical progenitors *in vitro* and *in vivo*. We further show that CPG15 overexpression *in vivo* expands the progenitor pool by preventing apoptosis, resulting in an enlarged, indented cortical plate and cellular heterotopias within the ventricular zone, similar to the phenotypes of mutant mice with supernumerary forebrain progenitors. CPG15 expressed during mammalian forebrain morphogenesis may help balance neuronal number by countering apoptosis in specific neuroblasts subpopulations, thus influencing final brain size and shape.


## Introduction

During mammalian evolution, the cerebral cortex has greatly expanded through a tremendous increase in the number of cortical neurons. The surface of the cortical plate has extended and become indented and convoluted as a result of neuron addition in columnar radial units (Rakic, 1995). At the onset of cortical neurogenesis, the

proliferative population of founder cells is confined to the ventricular zone of the embryonic cerebral wall (Takahashi et al., 1997). Even modest alterations in the size of this progenitor population during its early exponential growth phase can markedly affect final neuronal numbers (Caviness et al., 1995; Rakic, 1995). Thus, it has been proposed that cellular mechanisms that influence founder cell number may underlie the telencephalic expansion and sculpting that are characteristic of mammalian forebrain development and evolution (Rakic, 1995). Apoptosis within the founder population is one putative mechanism for influencing eventual brain size and shape (Haydar et al., 1999; Kuan et al., 2000).

It has recently been recognized that the role of apoptosis in brain development extends beyond matching of neuronal populations with their appropriate target fields, as specified in the 'neurotrophic hypothesis' (de la Rosa and de Pablo, 2000; Kuan et al., 2000). Caspase 3, a key enzyme in the mammalian apoptotic pathway, is expressed at high levels in the mouse cerebral wall around embryonic day 12 (E12) (Pompeiano et al., 2000), when dying cells are prevalent in proliferative zones of the cerebral cortex(Blaschke et al., 1996; Blaschke et al., 1998; Thomaidou et al., 1997). Consistent with these observations, mutant mice deficient in the pro-apoptotic genes Casp3, Casp9 and Apaf1 show gross nervous system malformations resulting from improper expansion of specific neural progenitor populations (Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). The excess neurons in some of these mutants are added as extra radial units, expanding the surface of the cortical plate, rather than influencing its thickness. The cortical plate, with increased size, forms convolutions resembling the gyri and sulci of the primate brain. In addition, later generated cells accumulate below the

cortical plate, forming heterotopic cell masses within the ventricles (Kuida et al., 1998; Kuida et al., 1996). Despite the essential role of the core apoptotic pathway in brain morphogenesis, little is known about the signals regulating apoptosis of proliferative neurons. Identification of the molecules involved is vital to understanding the complex morphogenetic processes that shape the mammalian brain.

*cpg15* (also known as Nrn1) was identified in a screen for activity-regulated genes involved in synaptic plasticity (Hevroni et al., 1998; Nedivi et al., 1993) and encodes a small, highly conserved protein (also termed neuritin-1) (Naeve et al., 1997; Nedivi et al., 1998). In a membrane-bound form attached by a glycosylphosphatidylinositol (GPI) link, CPG15 has been shown to function non–cell autonomously to coordinately regulate growth of apposing dendritic and axonal arbors, and to promote synaptic maturation (Cantallops et al., 2000; Nedivi et al., 1998). As *cpg15* is an activity-regulated gene, late *cpg15* expression is contemporaneous with critical periods for activity-dependent plasticity and requires action potential activity. However, *cpg15* is also expressed in an activity-independent manner during early brain development before circuit formation and maturation (Corriveau et al., 1999; Lee and Nedivi, 2002), suggesting that it may have a different role at this stage. We hypothesized that, like the neurotrophic factors, CPG15 has multiple roles during nervous system development. In addition to its previously characterized role as a growth and differentiation factor that affects process outgrowth and synaptic maturation, CPG15 may also function as a survival factor during early brain development. Here we describe the identification of a soluble CPG15 expressed in the embryonic brain that regulates survival of cortical progenitors by preventing caspase-mediated apoptosis.

## Results

### *cpg15* is expressed in embryonic proliferative zones

To examine localization of early, activity-independent *cpg15* expression, we performed in situ hybridizations on sections from embryonic rat brains. At the earliest times tested, embryonic days 14 (E14) and 15 (E15), *cpg15* mRNA is present in the cortical plate, in the ventricular zone of the dorsal thalamus and in retinal ganglion cells (Figure 3-1a–f). At E17–E19, *cpg15* is expressed in the telencephalic and dorsal diencephalic subventricular zones (Figure 3-1g–l), is expressed in the hippocampal primordia (Figure 3-1i–j), and at postnatal day 7 (P7) appears in the external granular layer of the cerebellum (Figure 3-1m). In all these regions early *cpg15* expression is temporally correlated with expansion of the progenitor pool and apoptotic elimination of superfluous neuroblasts (Blaschke et al., 1998). *cpg15* is not expressed in all proliferative zones and is markedly absent from the olfactory epithelium and ganglionic eminence (Figure 3-1a,b,e,f,i,j), suggesting that its function may be cell type specific. *cpg15* is also expressed when target-derived trophic support is crucial for protection from apoptosis used to match neuron number with target size. From E19 to P7, *cpg15* mRNA is present in the trigeminal ganglia, sensory thalamus and various brainstem nuclei (Fig. 3-1i,j,m), at times of afferent ingrowth, target selection and synaptogenesis in these structures. From P0, *cpg15* expression in the cerebral cortex is downregulated to undetectable levels (Lee and Nedivi, 2002), coincident with cessation of apoptosis in this region (Blaschke et al., 1996; Thomaidou et al., 1997). At P14, *cpg15* mRNA re-appears, not in the ventricular or subventricular zones, but in the differentiated cortical layers (Fig. 3-1n), where activity-dependent plasticity is thought to occur postnatally. *cpg15* mRNA patterns

are thus consistent with an early role as a survival factor during brain morphogenesis, and a later role in structural remodeling and synaptic maturation associated with developmental and adult plasticity.

**CPG15 is primarily expressed in a soluble, secreted form**

To generate CPG15 for testing in a survival assay, we cloned full-length CPG15 tagged with a Flag epitope into a vector containing an internal ribosomal entry site (IRES) for enhanced green fluorescent protein (EGFP) coexpression (pIRES-EGFP-CPG15-FLAG, (Figure 3-2a) and expressed it in HEK293T cells. The Flag tag allowed detection and subsequent affinity purification of the CPG15 protein, whereas EGFP marked transfected cells. Consistent with CPG15's GPI link to the cell surface (Naeve et al., 1997; Nedivi et al., 1998), immuno-histochemistry with a monoclonal antibody to Flag (anti-Flag) showed membrane staining of transfected cells (data not shown). Notably, we observed CPG15 staining in untransfected EGFP-negative cells, suggesting intercellular transfer of CPG15 from transfected cells to their untransfected neighbors. To test whether cell-to-cell contact is necessary for intercellular transfer of CPG15, we cocultured transfected and untransfected HEK293T cells on the same coverslip but in distinct locations without physical contact (Fig. 3-2b). Anti-Flag immunohistochemistry showed CPG15 membrane staining of all untransfected cells (Figure 3-2c), suggesting that a soluble form of CPG15 can diffuse between isolated cells. Cells cocultured with cells expressing vector alone (data not shown) or a control Flag-tagged cytoplasmic protein showed no membrane staining (Figure 3-2d).

We verified the presence of soluble CPG15 in supernatants from CPG15-transfected HEK293T cells by western blot analysis (Figure 3-2e). Anti-Flag staining showed two CPG15-specific bands of distinct molecular weights in whole-cell extracts. The lower-molecular-weight protein was also present in the supernatant fraction. Treatment of CPG15-transfected cells with phospholipase C (to promote cleavage of GPI anchors and release GPI-anchored proteins from the cell surface) resulted in a disappearance of the higher molecular weight protein from the cell extracts and its concurrent appearance in the supernatant fraction (Figure 3-2e). The lower-molecular-weight protein remained unaffected by phospholipase C treatment. These results suggest that the higher-molecular-weight protein represents membrane-bound GPI-linked CPG15 and that the smaller protein is a soluble form of CPG15.

To determine whether both CPG15 forms are expressed in vivo and at what developmental times, we prepared membrane and soluble protein fractions from brains of E14, E18 and adult rats and examined them by western blot analysis using an antibody against CPG15 (Nedivi et al., 1998). In both embryonic brains and adult cortex, CPG15 was detected predominantly in the soluble protein fractions, with low levels of the membrane-bound protein detected only in the adult (Figure 3-2f). As the prevalent form of CPG15 in vivo is soluble, this form is likely the primary mediator of CPG15's early role during embryonic brain development and possibly of its later role as an activity-regulated growth and differentiation factor (Cantallops et al., 2000; Nedivi et al., 1998).

**CPG15 rescues cultured cortical neurons from apoptosis**

The presence of soluble CPG15 in brain extracts at early developmental times and the localization of its mRNA to specific proliferative populations and to neurons at their

target selection phase led us to test whether it may serve as a survival factor that protects against apoptosis. We examined whether soluble CPG15, affinity purified from supernatants of CPG15-Flag–transfected HEK293T cells, was capable of preventing cultured cortical neurons from undergoing apoptosis. Using Hoechst 33324 staining to identify cells with fragmented nuclei, we counted the number of apoptotic neurons in untreated cortical cultures and in cultures after growth factor deprivation (starvation), with or without addition of purified CPG15 (Figure 3-3a–c). Growth factor deprivation more than doubled the percentage of apoptotic neurons in the cultures, from approximately 15% to 40%. The increased apoptosis could be completely prevented by addition of soluble CPG15, but not by addition of affinity column elution buffer (Figure 3-3d). To confirm that CPG15 was rescuing neurons from apoptotic rather than necrotic cell death, the treated and control cultures were immunostained with an antibody specific to the p17 subunit of activated caspase 3, a key component of the apoptotic pathway in brain development (Kuida et al., 1996). All neurons containing pyknotic nuclei visualized by Hoechst staining also stained positive for the p17 cleavage product of activated caspase 3 (Figure 3-3e–j). Independent quantification of immunostained neurons expressing cleaved caspase 3 showed that starvation more than doubled their number. CPG15 provision completely prevented the increased caspase 3 activation induced by starvation (Figure 3-3k). We conclude that soluble CPG15 protects cortical neurons from apoptosis by preventing activation of caspase pathways induced by growth factor deprivation.

**Lentivirus-delivered *cpg15* shRNA reduces CPG15 levels**

To test whether CPG15 also promotes survival of cortical progenitors, we used lentivirus delivery (Lois et al., 2002; Rubinson et al., 2003), combined with RNA interference (RNAi) (McManus and Sharp, 2002) to knock down CPG15 expression in vitro and in vivo. We generated lentiviruses expressing CPG15-Flag, a *cpg15* shRNA (small hairpin RNA), and a control scrambled *cpg15* shRNA with four of the hairpin nucleotides inverted (Figure 3-4a). shRNAs are processed to small interfering RNAs (siRNAs) that guide the specific cleavage and elimination of their cognate mRNAs. When primary cortical cultures were coinfected with the *cpg15*-FLAG and *cpg15* shRNA lentiviruses, both the exogenous *cpg15*-FLAG and the endogenous *cpg15* mRNAs were severely reduced as detected by northern blotting (Figure 3-4b), but they were unaffected by coinfection with the scrambled *cpg15* shRNA control virus. The *cpg15* shRNA lentivirus was also effective in knocking down cellular levels of the CPG15 protein as detected by immunocytochemistry (Figure 3-4c–f) and by western blotting (Figure 3-4g).

**CPG15 knockdown increases cortical progenitor apoptosis**

To address the role of CPG15 in progenitor cell survival, we first examined the effect of CPG15 knockdown on cultured cortical progenitors that were isolated from E14–E15 embryonic rat cortex and plated in the presence of basic fibroblast growth factor (bFGF). At plating, cells were infected with *cpg15* shRNA, scrambled *cpg15* shRNA or EGFP lentiviruses. After 4 d in culture the vast majority of cells in uninfected, EGFP-infected or scrambled hairpin–infected control cultures were positive for nestin, a marker of neural progenitors (Figure 3-5a,b red staining). In *cpg15* shRNA–infected cultures, the number of progenitors was greatly reduced, whereas the number of

differentiated neurons (marked by neurofilament-M (Nf-M) staining, blue) was unchanged (Figure 3-5c). Quantification of these results shows that the decrease in progenitor numbers in *cpg15* shRNA–infected cultures is similar to that seen in cultures deprived of bFGF from plating (Figure 3-5d,e). Progenitor loss in the *cpg15* shRNA–infected cultures was accompanied by a marked increase in apoptotic cell death identified by Hoechst staining (Figure 3-5f). These results demonstrate that depletion of endogenous CPG15 results in increased apoptosis of neuronal progenitors, suggesting that CPG15 is required for their in vitro survival. As acute CPG15 loss has no immediate affect on the number of Nf-M positive neurons, our results further suggest that CPG15 primarily affects progenitor survival.

Our finding that CPG15 is required for survival of cortical progenitors in culture led us to predict that depletion of endogenous CPG15 at early developmental times would increase apoptosis and reduce survival within the cortical progenitor population in vivo. To test this hypothesis, we delivered *cpg15* shRNA lentiviruses or control lentiviruses into E15 embryonic brains by direct ventricular injection. Embryos were harvested at E22 and their brains sectioned. Nissl staining showed moderate shrinkage in the size and ventricular volume of *cpg15* shRNA lentivirus–infected brains when compared with uninfected (data not shown), EGFP lentivirus–infected or scrambled shRNA lentivirus–infected brains (Figure 3-6a–e), suggesting a decrease in neuronal number. We stained alternate sections by TUNEL and quantified apoptotic neurons in the neocortex, where CPG15 is highly expressed during embryonic development, and in the diencephalon, where CPG15 expression is low in early development (Figure 3-1). When compared to uninfected (Figure 3-6f), EGFP lentivirus–infected (Figure 3-6g) and

scrambled shRNA lentivirus–infected brains (Figure 3-6h), the *cpg15* shRNA brains (Figure 3-6i) showed an increase in apoptotic cells in the neocortex. Overlay of TUNEL staining and EGFP staining on the same sections (Figure 3-6j–m) demonstrated comparable infection levels by the different lentiviruses. Quantification of TUNEL-stained cells showed that *cpg15* shRNA increases apoptosis in the neocortex (Figure 3-6n) but not in the diencephalon (Figure 3-6o). These results demonstrate that decreasing endogenous CPG15 levels during embryonic development results in increased apoptosis and diminished survival of cortical neurons. Reduced neuronal number is likely to cause shrinkage of the cortical plate and its contraction around the lateral ventricles, as seen in the deformed *cpg15* shRNA lentivirus–infected brains (Figure 3-6a–e). The specific effect of CPG15 depletion on neocortical neurons suggests that it is not essential for survival in all progenitor populations. Alternatively, the RNAi intervention may be past the critical time when CPG15 is necessary for survival of the diencephalic progenitors. In any case, the lack of effect of the *cpg15* shRNA lentivirus on apoptosis in the developing diencephalon demonstrates that the lentivirus-delivered RNAi has no deleterious effect in brain regions surrounding the ventricles.

**CPG15 overexpression results in an enlarged cortical plate**

In a complementary study, we further examined the role of CPG15 in vivo by overexpressing CPG15-Flag in the developing brain. CPG15-Flag lentivirus was injected into the ventricles of E15 embryonic brains harvested as described above. When compared with control EGFP lentivirus–infected brains, brains overexpressing CPG15 were significantly larger in diameter, with enlarged ventricles but with no alteration in

cortical thickness (Figure 3-7a–e). Closer examination of the enlarged brains showed sulcus-like indentations (Figure 3-7f–k; boxes in a–c are shown at higher magnification in f,h,i, and two additional examples from different CPG15-Flag lentivirus–infected brains are shown in j,k), consistent with a larger surface area resulting from addition of supernumerary neurons in radial units (Haydar et al., 1999). Sections from control EGFP lentivirus–infected brains (Figure 3-7l) and from brains overexpressing CPG15 (Figure 3-7m–p) were double-labeled with nestin (blue) and Nf-M (red), showing heterotopic cell masses within the proliferative ventricular zone. Thus, CPG15 overexpression results in the expansion and involution of the cortical plate, and in heterotopias and discontinuities of the ventricular zone. Malformations of this type are typically seen in mutant mice with an increase in forebrain progenitor numbers (Chenn and Walsh, 2002; Chenn and Walsh, 2003; Kuida et al., 1998; Kuida et al., 1996) and are consistent with an expansion of the progenitor pool.

**CPG15 expands the progenitor pool by reducing apoptosis**

To directly measure an affect of CPG15 on progenitor number, we used BrdU to label proliferating neurons. Pregnant dams were injected with BrdU 3d after intraventricular injection of the CPG15-Flag lentivirus into embryonic brains. Embryos were harvested 2 or 24h after BrdU injection (Figure 3-8a,b), earlier than for previous experiments to avoid confounding secondary effects of the deformed cortical plate. Low-magnification views showed that 2 h after BrdU injection, the CPG15-Flag lentivirus–infected brains are indistinguishable from uninfected control brains, with normal cellular lamination and cellular distribution within the ventricular and

subventricular zones (Figure 3-8c,d). However, a higher-magnification view showed a small but significant increase in BrdU-labeled cells within the ventricular zone of CPG15-Flag lentivirus–infected brains, suggesting an increase in progenitor number (Figure 3-8e–g).

Expansion of the progenitor pool can result from increased mitotic rates, a decrease in cell cycle re-entry, decreased cell death or any combination of these factors. To test for changes in mitotic rates of dividing progenitors, embryos injected with the CPG15-Flag lentivirus and harvested 24 h after BrdU injection at E19 (Figure 3-8b) were double stained for BrdU, to mark cells in S phase of the cell cycle, and for phospho-histone-H3 (p-H3), to mark cells in M phase undergoing mitosis (Figure 3-8h,i). We found no significant difference between un-infected control and CPG15-Flag lentivirus–infected brains in the ratio of dividing progenitors at M phase to progenitors in S phase (Figure 3-8j), indicating that CPG15 does not increase mitotic rate. We next tested whether CPG15 affects the number of progenitors that re-enter the cell cycle rather than progressing towards terminal differentiation. E15 embryos were injected with the CPG15-Flag lentivirus, followed by BrdU injection at E18 (Figure 3-8b). Twenty-four hours later, embryos were harvested and double-stained for BrdU, to mark cells that were dividing at the time of injection, and Ki67, to mark progenitors. We identified cells that had exited the cell cycle within the 24 h of BrdU labeling as BrdU+ and Ki67- (red) and divided progenitors in the cell cycle as BrdU+ and Ki67+ (yellow) (Figure 3-8k,l). We found that in the CPG15-Flag lentivirus–infected embryos, there was no significant change in the proportion of cortical progenitors exiting the cell cycle as compared to uninfected controls (Figure 3-8m).

Finally, to examine whether CPG15 overexpression decreases progenitor apoptosis, we performed TUNEL staining on E18 brains from uninfected control (Figure 3-8n) and CPG15-Flag lentivirus–infected (Figure 3-8o) embryos 3d after viral delivery. Counts of TUNEL-stained cells in the cortex of CPG15-Flag lentivirus–infected brains showed a significant decrease in the numbers of apoptotic cells when compared to uninfected control brains (Figure 3-8n–p). Together, these studies indicate that the observed increase in progenitor pool size and the expanded cortices seen in CPG15-Flag lentivirus–infected brains are not due to increased progenitor mitotic rates or decreased cell cycle exit, but rather are likely to be a result of decreased apoptosis within the progenitor population.

**Discussion**

*cpg15* expression patterns in the embryonic brain concomitant with periods of rapid cell proliferation and apoptosis, and during circuit formation and target selection, led us to test whether early in development CPG15 might function as a survival factor, similarly to neurotrophins. We found that CPG15 is able to rescue cortical neurons from starvation-induced apoptosis by preventing caspase 3 activation and is also crucial for survival of cortical progenitors. Consequently, manipulating in vivo CPG15 levels in utero had a profound effect on the size and shape of the neocortical plate. Decreasing CPG15 levels caused increased apoptosis of cortical progenitors and shrinkage of the cortical plate, whereas overexpression resulted in decreased progenitor apoptosis and an expanded and convoluted cortical plate.

It is particularly informative to compare the CPG15 overexpression phenotype to developmental mutants with similar presentations. In the case of mutants in the cell-death cascade, expansion of the cortical plate occurs only when the apoptotic pathway is affected in proliferating neurons rather than in postmitotic neurons and is a direct result of an enlarged progenitor pool (Haydar et al., 1999; Kuan et al., 2000). Gene-targeting studies have identified Bax, Bcl-XL (Bcl2l1), Apaf1, Casp9 and Casp3 as key elements in neuronal apoptosis occurring during brain development (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Motoyama et al., 1995; Roth et al., 2000). By analogy to their counterparts in *Caenorhabditis elegans*, the proteins are likely to form a linear cell-death cascade, with Apaf1 bound to caspase 9, activating caspase 3, and Bcl-XL acting as an upstream anti-apoptotic regulator of Apaf1 that can be blocked by Bax (Kuida et al., 1998; Roth et al., 2000). Epistatic analysis shows that the upstream components of this pathway, Bax and Bcl-XL, are obligatory only in postmitotic neurons, whereas caspase 3 is unique in its effect on apoptosis of neuronal founder cells (Roth et al., 2000; Shindler et al., 1997). Global formation of the nervous system is unaffected in Bax-deficient embryos (Knudson et al., 1995; White et al., 1998), whereas null mutants of Casp3, Casp9 and Apaf1 show severe forebrain malformations that result from hyperplasia and an enlarged neocortical plate (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). This is consistent with the idea that even modest alterations in the size of the progenitor pool during its exponential growth phase can drastically effect final cortical size and shape (Caviness et al., 1995; Rakic, 1995). Hyperplasia and an enlarged neocortical plate can also be seen when the cortical progenitor pool is expanded for reasons other than a decrease in cell death: for

example, in β-catenin transgenic mice, where cortical progenitors fail to exit the cell cycle after mitosis (Chenn and Walsh, 2002). The similar phenotype seen with in vivo CPG15 overexpression suggests that elevated CPG15 expands the progenitor pool, likely by reducing apoptosis. This is supported by results showing that increasing amounts of CPG15 during corticogenesis reduce apoptosis and enlarge the progenitor pool but have no effect on mitotic rates or cell cycle exit. Furthermore, an increased number of apoptotic neurons can be seen in brains with RNAi-mediated knockdown of endogenous CPG15, and in vitro data demonstrates CPG15 function as an anti-apoptotic factor for cortical neurons as well as cortical progenitors.

Other extracellular signaling molecules previously shown to regulate cerebral cortical size through their effect on the progenitor pool are basic fibroblast growth factor (Dono et al., 1998; Ortega et al., 1998; Vaccarino et al., 1999), pituitary adenylate cyclase-activating polypeptide (Suh et al., 2001) and, more recently, lysophosphatidic acid (Kingsbury et al., 2003). Basic fibroblast growth factor and pituitary adenylate cyclase–activating polypeptide, respectively, expand or shrink the progenitor pool by acting as mitogenic or anti-mitogenic signals. Lysophosphatidic acid expands the progenitor pool by increasing terminal mitosis and reducing caspase-mediated cell death, and is thus the only factor other than CPG15 that has been shown to decrease apoptosis of cortical progenitors (Kingsbury et al., 2003). Studies of cultured cortical progenitors suggest that BDNF and NT-3 can mediate progenitor cell survival in vitro (Barnabe-Heider and Miller, 2003). Yet direct intrauterine, intraventricular application of neurotrophins into the embryonic brain does not affect proliferation or apoptosis of cortical progenitors (Brunstrom et al., 1997). Furthermore, classic neurotrophins are

absent in most parts of the embryonic brain (Ernfors et al., 1992), and prenatal CNS defects related to neuronal number have not been seen in neurotrophin single- or double-knockout mice or in animals lacking their receptors(Conover and Yancopoulos, 1997). Thus, CPG15 is one of few molecules shown to be essential for in vivo survival of undifferentiated cortical progenitors. CPG15 expression is specific to a subset of progenitor populations in the developing brain and seems to be required only in these populations. We propose that by countering early apoptosis in specific progenitor subpopulations, CPG15 has a role in regulating brain size and shape during morphogenesis of the mammalian forebrain.

As CPG15 is also expressed in some differentiated neurons during target selection and circuit formation and is able to rescue cortical neurons from starvation-induced apoptosis, it is possible that CPG15 may also function as a target-derived survival factor for differentiated neurons analogous to neurotrophins. It remains to be examined whether CPG15 is required for survival in various populations of differentiated neurons and whether this function of CPG15, as well as its later activity-dependent function as a growth and differentiation factor, are mediated by the soluble form described here. Another possible future direction will be analysis of a *cpg15* knockout mouse. As it is technically difficult to intervene by intrauterine delivery of an shRNA earlier than E14–E15, the CPG15 knockdown using RNAi can only affect the very last rounds of cortical progenitor division. In the knockout, *cpg15* deletion at an earlier step may result in a more extreme phenotype. However, deleting a gene from the onset of embryogenesis could also lead to compensation and lack of a discernible phenotype, something that can be avoided with an acute knockdown using RNAi at a critical developmental step (Gotz,

2003). For example, mice with targeted deletion of the doublecortin gene (Dcx) seem to develop a normal neocortex (Corbo et al., 2002), whereas electroporation of plasmids encoding shRNA against the doublecortin protein *in utero* disrupts radial migration in the rat neocortex, resulting in a malformed cerebral cortex (Bai et al., 2003). In this case, acute intervention using RNAi results in a phenotype more similar to the double cortex syndrome seen in humans with mutations in the Dcx gene than is seen with the complete Dcx knockout. Thus, RNAi approaches may be complementary to gene knockouts in functional studies of genes important for brain development.

## Methods

### *In situ* hybridization

*In situ* hybridizations were performed as described in the previous chapter.

### CPG15 immunocytochemistry and protein purification

HEK293T cells were grown to 80 - 90% confluence in 100 mm culture dishes containing 15 ml media (10% calf serum, 50 U Penicillin, 50 μg Streptomycin, 4 mM L-glutamate in Dulbeccos Modified Eagles medium (BioWhittaker)), then transfected with 8 μg of the pIRES-EGFP-CPG15-FLAG plasmid (Figure 2A) using Lipofectamine 2000 (Invitrogen). For immunocytochemistry, cells were fixed in 4% paraformaldehyde and stained with anti-FLAG antibody (Sigma). For protein purification, the medium was harvested 4 days later and debris removed by centrifugation (3,000 rpm, 15 min, 4°C). The medium was then incubated with 40 μl anti-FLAG antibody coupled to agarose (EZview Red ANTI-FLAG M2 Affinity Gel, Sigma) for 12 hrs at 4°C on a rotator. The

agarose was pelleted by centrifugation for 10 min at 2,000 rpm and washed 3 times with TBS. The tagged CPG15 protein was eluted from the anti-FLAG antibody by incubation with 30 μg of 3x FLAG peptide (Sigma) diluted in 200 μl TBS for 4 hrs at 4°C and then recovered by centrifugation for 5 min at 2,000 rpm. Protein concentration in the supernatant was determined by the Bradford assay.

**Preparation of membrane and soluble fractions from brain, and Western blotting**

Membrane and soluble fractions from brains were prepared as described (Kim et al., 1998) with the following modifications. Briefly, brains from E14 and E18, and cortices from adult Sprague-Dawley rat were homogenized using a Teflon homogenizer in 5 ml ice-cold buffer (320 mM sucrose, 10 mM Tris-HCl, pH7.4, 5 mM EDTA, 1:100 Protease Inhibitor Cocktail (Sigma)) per 1 g of tissue. The homogenates were centrifuged at 700 x g for 10 min at 4°C, followed by centrifugation of the supernatant at 28,000 x g for 16 min at 4°C. The high-speed pellet was resuspended in 500 μl ice-cold TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4). Protein concentration of the pellet and supernatant were determined using the Bradford assay (Gibco). Samples containing 20-40 μg protein were diluted 1:1 in loading buffer (Laemmli, 1970), boiled for 5 min, then separated on 16.5% Tris-Tricine gels (Schagger and von Jagow, 1987). Western blots were incubated with a polyclonal anti-CPG15 antibody (Nedivi et al., 1998). Clean fractionation of soluble versus membrane compartments was confirmed by staining with a monoclonal antibody specific for the membrane associated protein Transferrin-receptor (1:1000, Zymed), followed by a goat anti-mouse HRP secondary antibody (1:5000, Sigma), and a polyclonal antibody specific for the soluble protein Akt (1:1000, Cell

Signaling Technology) followed by a goat anti-rabbit HRP secondary antibody (1:2500, Jackson Immuno Research). Prior to antibody staining the blot was incubated for 2 min with Ponceau S solution (Sigma) to determine protein content as a loading control.

**Primary cortical cultures**

Cultures were done essentially as described (Zhou and Tang, 2002). E18 Sprague-Dawley rat embryos were collected in ice-cold Hank's buffered salt solution (HBSS, Sigma). Cortices were dissected out and digested for 15 min at 37°C in 0.25% trypsin (Gibco) and 0.1% DNase (Sigma) in HBSS. After digestion, the tissue was washed 3 times in ice-cold HBSS and then triturated with fire-polished Pasteur pipettes of decreasing pore size in HBSS with 0.1% DNase. After centrifugation for 10 min at 1,000 rpm, the cell pellet was resuspended in plating media consisting of Neurobasal medium (Gibco) supplemented with B27 (Gibco), L-glutamine (500 μM), and L-glutamate (25 μM). Cells were then counted and plated at 0.75 x $10^5$ cells/well in twelve well plates with 1 ml plating media, each well containing one 10 mm glass coverslip (Assistent) that had been preincubated overnight in 40 μg/ ml poly-D-lysine (Fischer) and 2.5 μg/ml laminin (Fischer), rinsed 3 times in water, and then incubated in plating medium. After 4 days in vitro (DIV), half the plating media was replaced with feeding medium (plating medium minus L-glutamate). Cultures were maintained in a humidified 37°C incubator with a 5% $CO_2$ atmosphere.

**Growth factor deprivation and apoptosis assays**

After 6 DIV, cortical neurons were washed 3 times with Neurobasal medium without supplements, then incubated for 12 hrs in the unsupplemented medium with or without 50 ng/ml purified CPG15 protein. After an additional 12 hrs in feeding medium, cells were fixed in 4% formaldehyde/PBS for 30 min at 4°C before Hoechst staining or immunocytochemistry. Fixed cells were incubated 30 min with Hoechst 33342 (1:1000, in PBS, Sigma), rinsed 3 times in PBS and mounted onto slides with Fluoromount G (Southern Biotechnology). For immunocytochemistry, fixed cells were washed with PBS for 5 min, then permeabilized with 0.3% Triton X-100 for 5 min at 4°C. Neurons were washed again with PBS, incubated with blocking solution (10% goat serum, 0.1% Triton X-100 in PBS) for 1 hr at 4°C, and then incubated with an anti-cleaved caspase 3 antibody (1:100, Cell Signaling Technology) in blocking solution overnight at 4°C. After rinsing 3 times with PBS, an anti-rabbit secondary antibody coupled to rhodamine (1:500, Jackson Immuno Research) was added for 1 hr at RT. Chromatin staining with Hoechst was done simultaneously, and neurons were rinsed and mounted as described above.

For quantification, fragmented apoptotic nuclei as well as healthy nuclei were counted blind to experimental treatment using a fluorescence microscope with a UV filter setting for the Hoechst staining (excitation 330-380; emission 420) and rhodamine settings for visualizing the antibody against cleaved caspase3 (excitation 528-553; emission 600-660). Treatments were repeated in three independent experiments with two coverslips per treatment in each experiment. Each data point represents the mean of 500-600 cells, counted in 40-50 different fields per coverslip. The percent of apoptotic cells was calculated based on the number of condensed/fragmented nuclei divided by the total number of nuclei. Comparisons between groups were analyzed using a student's *t*-test.

**Progenitor Cultures**

Cultures were done essentially as described (Ghosh and Greenberg, 1995; Gloster et al., 1999; Menard et al., 2002; Slack et al., 1998). Briefly, E14-E15 Sprague-Dawley rat embryos were collected in ice-cold Hank's buffered salt solution (HBSS, Sigma). Cortices were dissected out and digested for 15 min at 37°C in 0.1% DNase (Sigma) in HBSS. After digestion, the tissue was washed 3 times in ice-cold HBSS and then triturated with a plastic pipette into small clusters of cells that were plated in plating media consisting of Neurobasal medium (Gibco) supplemented with B27 (Gibco), L-glutamine (500 μM), and L-glutamate (25 μM) and 20ng/mL of bFGF (BD Biosciences, Bedford, MA). Cells were then counted and plated at 0.75 x 105 cells/well in twelve well plates with 1 ml plating media, each well containing one 10 mm glass coverslip (Assistent) that had been preincubated overnight in 40 μg/ ml poly-D-lysine (Fischer) and 2.5 μg/ml laminin (Fischer), rinsed 3 times in water, and then incubated in plating medium. After 4 days in vitro (DIV), half the plating media was replaced with feeding medium (plating medium minus L-glutamate). Cultures were maintained in a humidified 37°C incubator with a 5% CO2 atmosphere. For lentiviral infection of cultures 1μl of virus at a titer 1 X 106 pfu was added to the culture at plating.

**Lentivirus generation**

Five different cDNA sequences spanning the *cpg15* core domain were synthesized, fused to a loop region, then annealed to their antisense sequences and cloned separately into pSilencer1.0-U6 plasmid (Ambion) downstream of the U6 promoter. To

test the effectiveness of the *cpg15*-shRNAs in reducing CPG15 levels, HEK293T cells were separately co-transfected with each one of the pSilencer-*cpg15*-shRNA plasmids together with the pIRES-EGFP-CPG15-FLAG plasmid at a 40:1 ratio using Lipofectamine 2000 (Invitrogen). CPG15-IRES-EGFP mRNA knockdown was determined by reduced expression of EGFP. The most effective small hairpin sequence (GGGCTTTTCAGACTGTTTG) was then amplified with its upstream U6 promoter by PCR and subcloned into the pFUGW lentivirus transfer vector (Lois et al., 2002). To clone the scrambled shRNA construct, the small hairpin sequence was modified to GGGCTTGACTTACTGTTTG (the inverted sequence is underlined) and cloned into the pFUGW lentivirus transfer vector as described above. The pFUGW lentivirus transfer vector alone was used for the production of the EGFP control lentivirus. The CPG15-FLAG-IRES-EGFP cDNA was subcloned into pFUIGW downstream of the ubiquitin promoter. For GFP cell labeling, lentivirus particles were generated from the pFUGW transfer vector, in which GFP is downstream of the ubiquitin-C promoter (Lois et al., 2002). For the CPG2-GFP fusion protein, the cpg2 coding sequence was subcloned into pFUGW downstream of and in frame with the GFP coding sequence. The shRNA sequences with the U6 promoter were amplified from the pSilencer1.0 plasmid by PCR and subcloned into pFUGW immediately upstream of the ubiquitin-C promoter. The clathrin A1 light chain-GFP fusion sequence from EGFP-C1 (Blanpied et al., 2002) was subcloned into pFUGW downstream of the human ubiquitin-C promoter. Lentivirus particles were produced by cotransfecting the transfer vector, the HIV-1 packaging vector Δ8.9, and the VSVG envelope glycoprotein vector into 293T cells with Lipofectamine 2000 (Invitrogen). Culture medium was collected 48 to 60 hr after transfection, filtered,

and centrifuged at 25,000 RPM for 90 min at 4°C. Supernatants were discarded, and the virus pellets were rehydrated overnight in PBS. Viral particles were resuspended, aliquoted, and frozen at −80°C. Viral titers were determined by application of serial dilutions to 293T cells and quantifications of GFP expression 72 hr later. Typical titers were 1-5 × 106 pfu/μl.

### *In Utero* Injections

Rats were anesthetized with a ketamine/xylazine mixture (50mg/10mg/kg ) ip and hair on the belly was removed using pet trimmers. The surface of the skin was cleaned with 70% EtOH and betadine scrub three times each and a midline incision from the xyphoid process to the pubis was made. The incision along the midline peritoneum was completed using scissors (taking care not to incise the underlying bowel) to expose the abdominal contents. Embryos were elevated one to three at a time out of the peritoneal cavity using sterile cotton swabs and transilluminated with a fiber optic light to visualize the cerebral ventricles. Embryos were kept moist at all times by applying warmed lactated ringers or sterile PBS. Using a customized Hamilton syringe (No. 75; 5μl) and needle (1", PT4) viral solution containing 0.025% fast green dye to help with visualization was injected into the lateral ventricles of the embryos. Once injections were completed the uterus was placed back into the peritoneal cavity and lavaged with lactated ringers (or sterile PBS) until it is clear of all blood. The abdominal musculature is then sutured using 4-0 silk sutures on a curved needle. Finally the skin is closed using a Clay Adams 9mm autoclips, 0.5cm apart. The animal is then placed on its back in the cage onto a homeothermic blanket, or under a heat lamp until it exhibits some mild

recovery. 0.05mg/kg, of buprenophine is injected subcutaneously prior to recovery from anesthesia.

**Immunocytochemistry of Cultured Progenitors**

For immunocytochemical detection of nestin (1:2000; Chemicon, Temecula, CA) and neurofilament M (1:3000; Chemicon), cells are fixed for 20 min with 4% paraformaldehyde. Cultures are then washed with PBS and permeabilized for 5 min in 0.3% Triton-X100 in PBS, and then blocked for 1hr with in 0.1% Triton-X100 in PBS containing 10% goat serum. Cells are then incubated at 4°C overnight with primary antibodies in 0.1% Triton PBS containing 5% goat serum. After three washes with PBS, cells are incubated at room temperature for 60 min with Alexa-647 conjugated goat anti-rabbit (1:2000, Molecular Probes ), Alexa-555 conjugated goat anti-mouse (1:2000; Molecular Probes), prepared in 0.1% Triton PBS containing 5% goat serum. Samples were washed three times with PBS and then counterstained for 10 min with Hoechst 33258 (Sigma, St. Louis, MO) before examination by fluorescence microscopy. For quantitation, 5 random images of each treatment (per experiment) were captured and processed, and counted.

**Immunohistochemistry**

Histological and immunohistochemical analysis of embryonic brains. Animals were euthanized at E18, E19 or E22: 3, 4 or 7 d, respectively, after viral injection. Brains were removed and submerged in 4% paraformaldehyde in PBS overnight at 4 °C, transferred to 30% sucrose in PBS at 4 °C until they sank, and then frozen and sectioned

at 20  m using a cryostat. Every sixth section was Nissl stained and used to match sections from different brains. Sections at equivalent levels on the anterior-posterior axis were then processed for TUNEL. Cryosections were blocked in 10% goat serum, 0.3% Triton X-100 for 1 h. Primary antibodies against nestin (1:200, Chemicon, mouse monoclonal) and neurofilament (1:200, Chemicon, rabbit polyclonal) were diluted in blocking solution. Sections were counterstained with Hoechst 33342 in PBS for 10 min to highlight nuclear DNA. Fluorescent secondary antibodies Alexa-555-conjugated goat anti-mouse (1:200, Molecular Probes) and Alexa-647-conjugated anti-rabbit (1:200, Molecular Probes) were used for visualization as described above.

**Tunel Staining**

TUNEL staining on frozen brain sections was performed as described by the manufacturer using the Roche In Situ Cell Death Detection Kit, TMR red. The total number of TUNEL-positive cells present in the neocortex and the diencephalon of uninfected (three each at E18 and E22), EGFP-infected (three at E22) and *cpg15* shRNA–infected (four at E22) brains was quantified. Six to twelve sections were analyzed from each brain. Statistical significance was determined by Student's t-test.

**Measuring Proliferation**

To label proliferating cells in the cortex of E18 embryos, BrdU was injected intraperitoneally into the mother at 50 mg kg-1 body weight. BrdU and Ki-67 or p-H3 double labeling was done as previously described for BrdU and Ki6733. Primary antibodies used were BrdU (1:200; Harlan, rat monoclonal), Ki-67 (1:500; Novocastra,

rabbit polyclonal), and p-H3 (1:1,000; Upstate, rabbit polyclonal). BrdU-labeled and p-H3–labeled cells were counted from matching sections and percentages were compared by unpaired Student's t-tests. For cell cycle exit studies, a similar approach was used: Ki-67 expression was scored in 50 BrdU-positive cells from each of five randomly chosen fields of view from at least three sections per brain (two brains per each experimental group). Statistical significance was determined by Student's t-test.

**Figure 3-1. *cpg15* mRNA expression is biphasic**

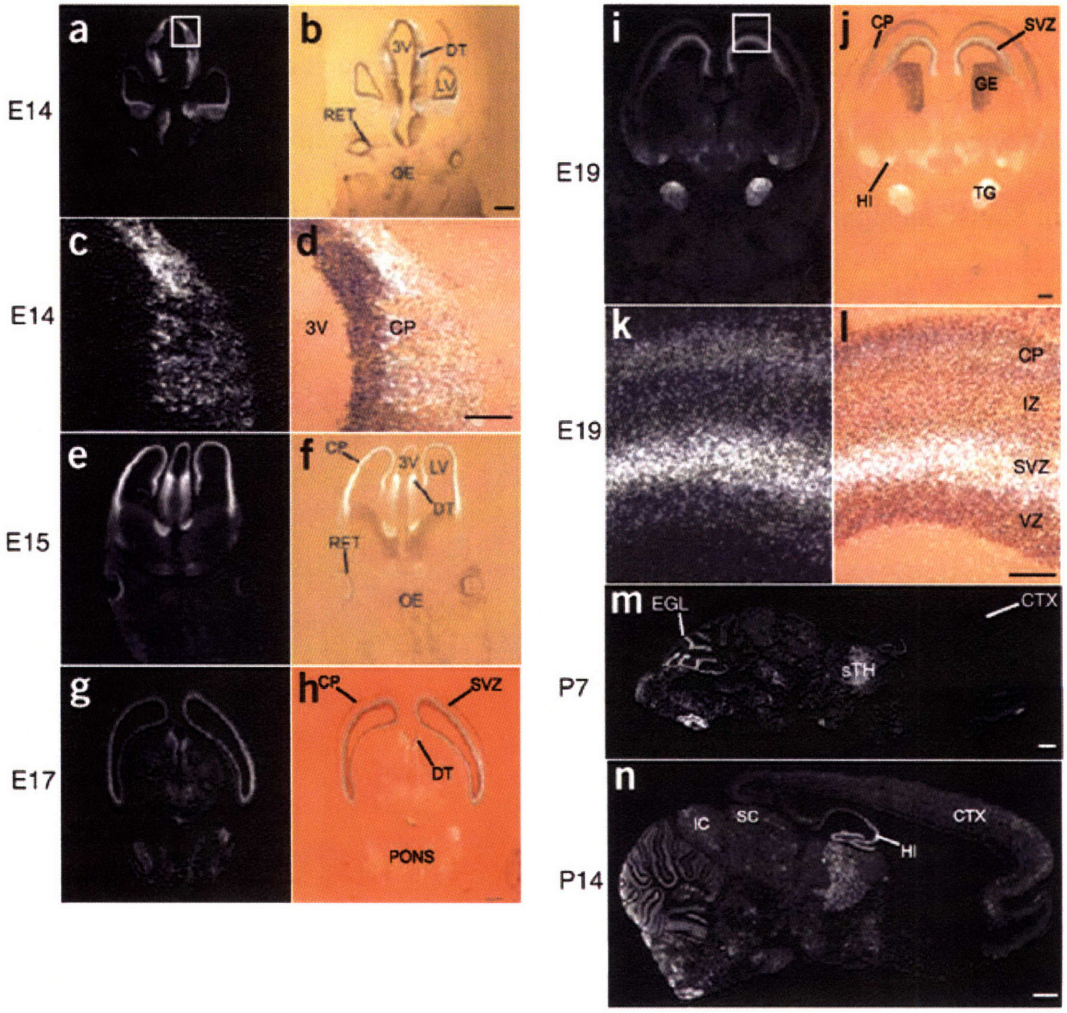


**Figure 3-2 CPG15 is predominantly expressed in a soluble secreted form.**

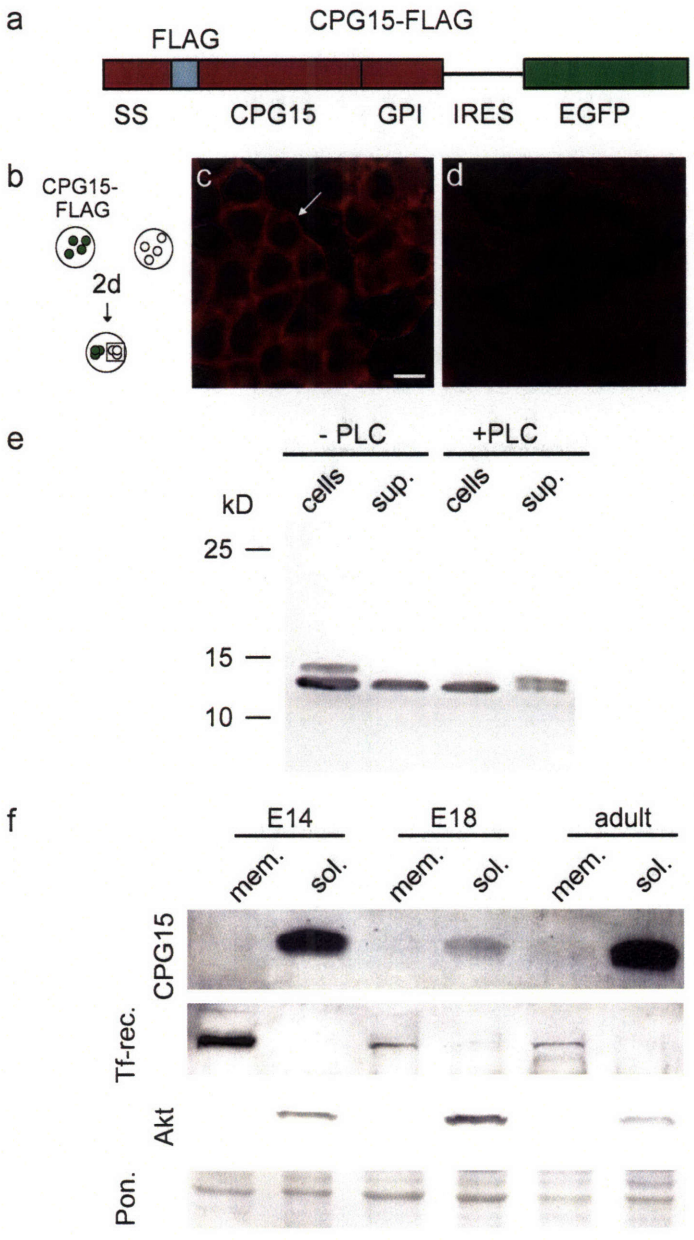


**Figure 3-3. Soluble CPG15 rescues cortical neurons from apoptosis induced growth factor deprivation.**

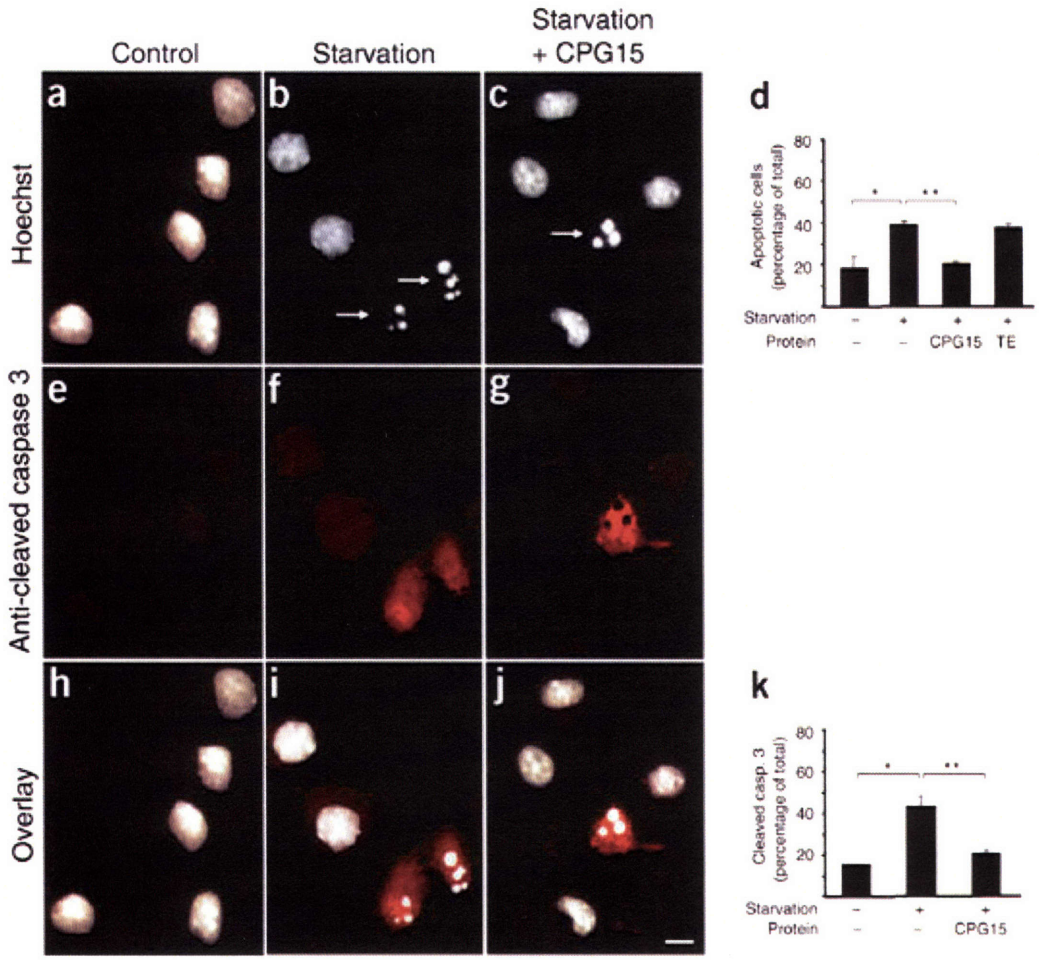


**Figure 3-4. A lentivirus-delivered *cpg15* small hairpin RNA (shRNA) knocks down CPG15 expression.**

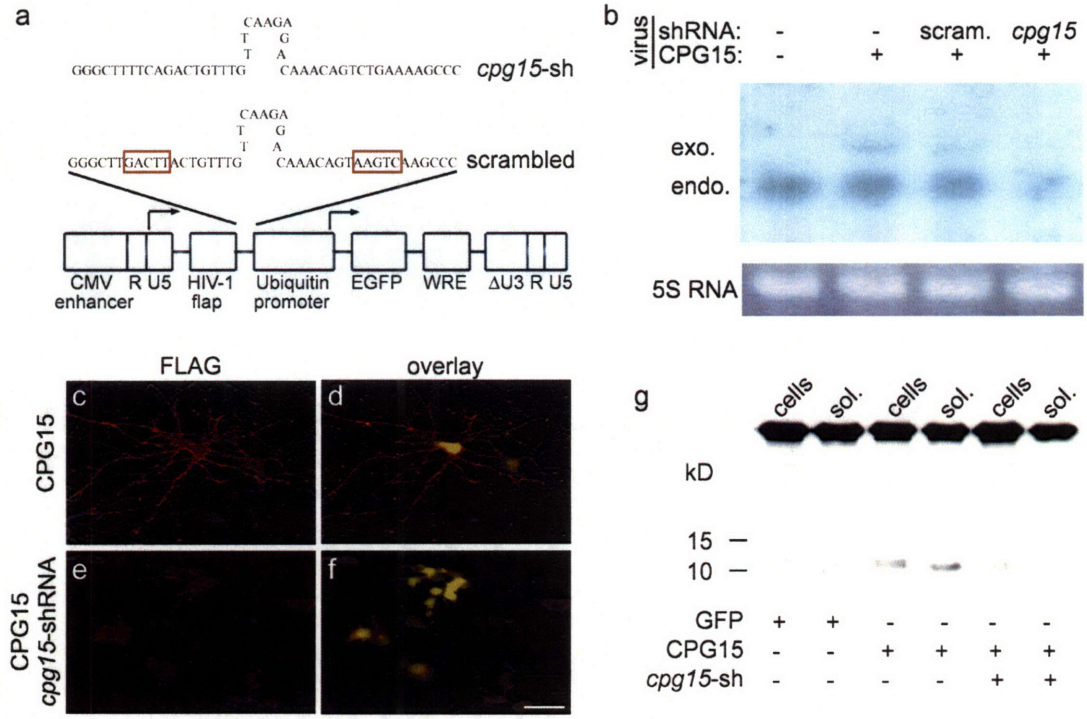


**Figure 3-5. Endogenous CPG15 is required for cortical progenitor survival *in vitro*.**

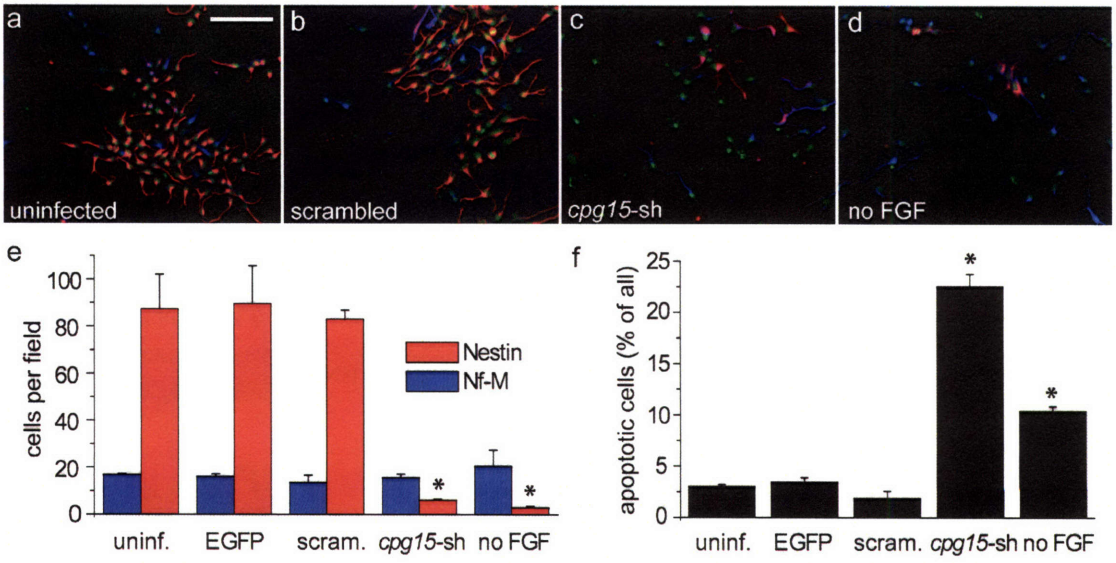


**Figure 3-6. *In vivo* knockdown of endogenous CPG15 causes shrinkage of the cortical plate and increased apoptosis of cortical neurons.**

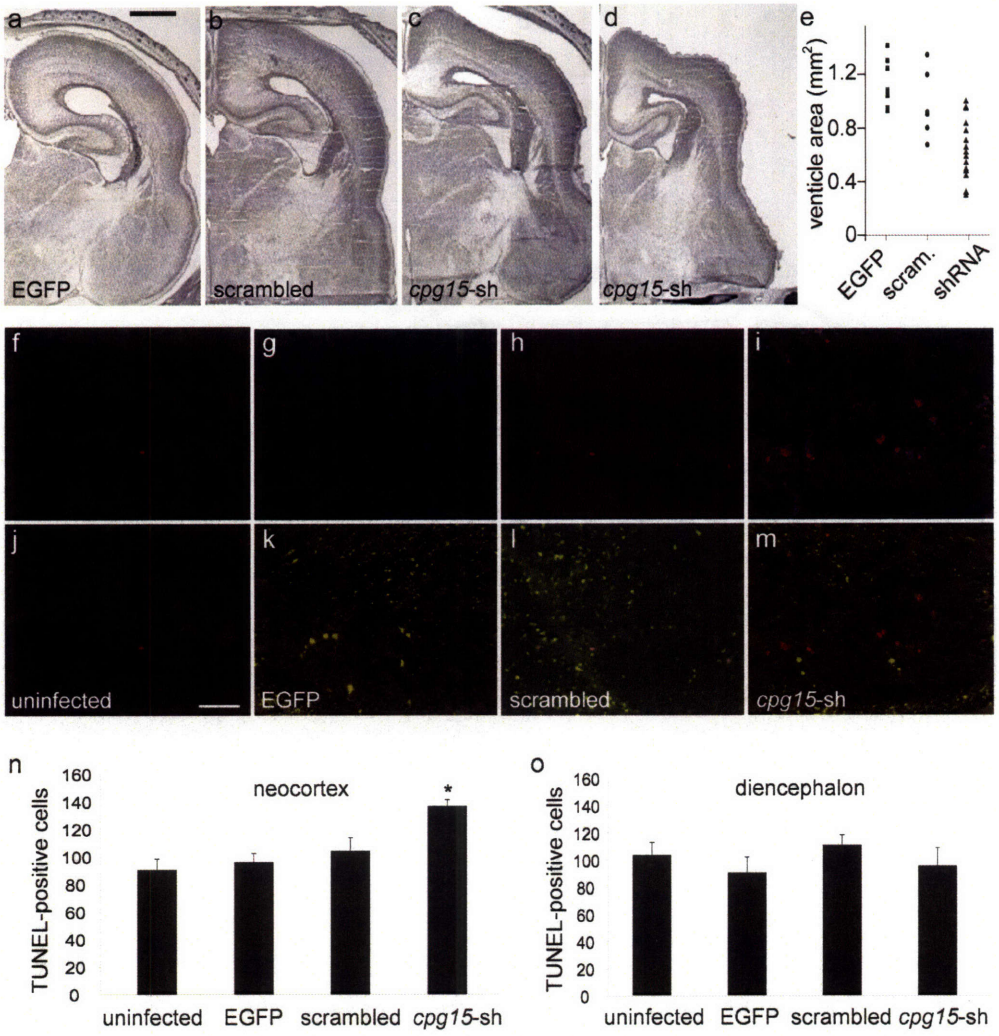


**Figure 3-7 *In vivo* CPG15 overexpression in the embryonic brain results in an expanded cortical plate and heterotopic cell masses in the ventricular zone.**

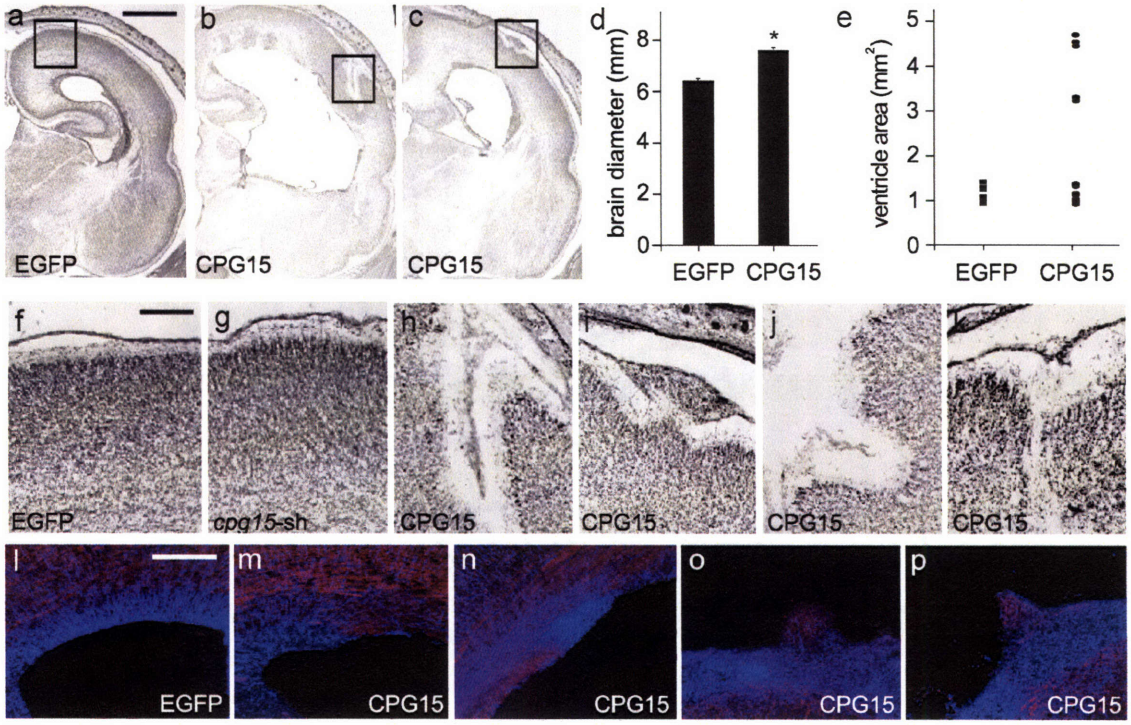
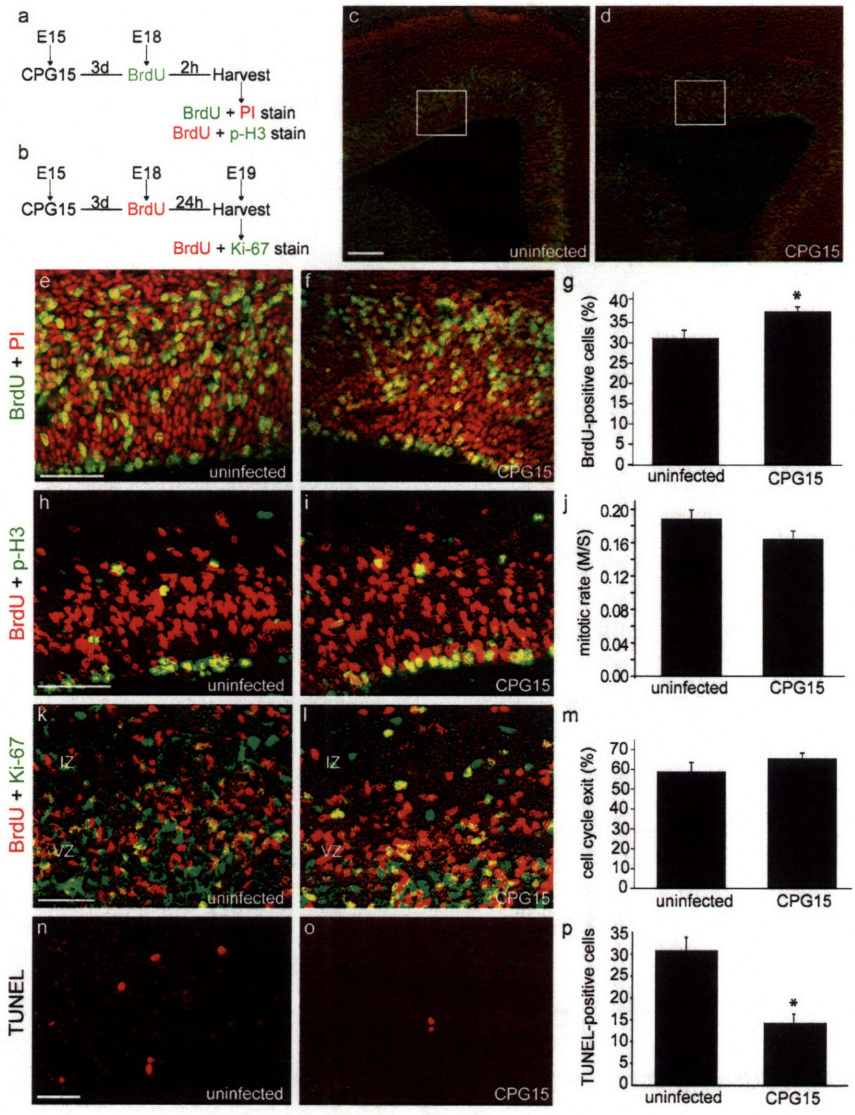


**Figure 3-8. CPG15 overexpression reduces apoptosis in the progenitor pool but does not affect mitotic index or cell cycle exit.**



## Figure Legends

**Figure 3-1. *cpg15* mRNA expression is biphasic.**

(a–n) *cpg15* in situ hybridizations on (a–l) coronal sections through the telencephalon during prenatal development, and (m,n) on saggital sections through postnatal brains (ages designated at left). Dark-field photomicrographs of embryonic times are shown in left column; on the right are bright-field photomicrographs of the same sections counterstained with toluidine blue and overlaid with their dark-field views. Marked are the third ventricle (3V), dorsal thalamus (DT), lateral ventricle (LV), retina (RET), olfactory epithelium (OE), cortical plate (CP), subventricular zone (SVZ), ganglionic eminence (GE), trigeminal ganglion (TG), hippocampus (HI), neocortex (CTX), sensory thalamus (sTH), external granular layer (EGL), inferior colliculus (IC) and superior colliculus (SC). Scale bars: a,b,e–j, 0.5 mm; c,d,k,l, 150 μm; m,n, 1 mm.

**Figure 3-2. CPG15 is predominantly expressed in a soluble secreted form.**

(**a**) Schematic of *CPG15-FLAG-IRES-EGFP* construct. The Flag peptide sequence (gray) was inserted between the secretion signal (SS) and the CPG15 core domain. Tagged, full-length CPG15 was then cloned upstream of an internal ribosome entry site (IRES) and EGFP. (**b**) HEK293T cells transfected with *CPG15-FLAG-IRES-EGFP* (green) were cocultured with untransfected cells (white) for 2 d. (**c**) Untransfected cells (outlined by square in **b**) show CPG15 immunoreactivity (white arrow). Scale bar, 10 μm. (**d**) HEK293T cells cocultured with cells transfected with a Flag-tagged control protein show no membrane staining. (**e**) Western blots of cell extracts and supernatants from *CPG15-*

*FLAG-IRES-EGFP* transfected HEK293T cells either untreated or treated with phospholipase C (PLC). (**f**) Western blots of membrane and soluble fractions from E14 and E18 brains, and adult cortices, probed with antibody against CPG15. Staining for the transferrin receptor (Tf-rec.) and the enzyme Akt serve as membrane- and soluble-fraction controls, respectively. Ponceau staining (Pon.) serves as a loading control.

**Figure 3-3. Soluble CPG15 rescues cortical neurons from apoptosis induced by growth factor deprivation (starvation).**

(a,b) Starvation increased the number of fragmented nuclei seen with Hoechst staining (marked by arrows). (c) CPG15 addition prevented this increase. (d) Quantification of a–c. Starvation significantly increased the percentage of apoptotic neurons (* $P < 0.002$). CPG15 application prevented this increase (** $P < 0.003$). (e,f) Starvation induces apoptosis, as seen by increased numbers of neurons immunopositive for cleaved caspase 3. (g) Purified CPG15 prevented the starvation-induced increase in cleaved caspase 3 immunoreactivity. (h–j) Overlay of a and e, b and f, and c and g shows that cells with fragmented nuclei scored by Hoechst staining contain activated caspase 3. (k) Quantification of e–g. Starvation significantly increased the percentage of cleaved caspase 3–immunopositive neurons (* $P < 0.001$). CPG15 application prevented this increase (** $P < 0.002$). Scale bar, 10 μm.

**Figure 3-4. A lentivirus-delivered *cpg15* small hairpin RNA (shRNA) knocks down CPG15 expression.**

(a) Schematic representation of short hairpin sequences and lentivirus vector used for CPG15 knockdown. The shRNA or scrambled shRNA sequences connected to the U6 promoter were inserted upstream of the ubiquitin-C promoter driving EGFP expression (see Methods for details). Red boxes mark the inverted region in the scrambled shRNA. (b–g) In cultured cortical neurons, lentivirus-delivered *cpg15* shRNA, but not the scrambled version, markedly reduces *cpg15* mRNA assayed by northern blotting (b) and reduces protein expression as assayed by immunocytochemistry (c–f) or by western blotting (g). Neurons were infected with CPG15-FLAG-IRES-EGFP lentivirus, alone (b, lane 2; c,d; and g, lanes 3–4), together with a scrambled *cpg15* shRNA control lentivirus (b, lane 3) or together with the *cpg15* shRNA lentivirus (b, lane 4; e,f; and g, lanes 5–6). CPG15 immunostaining is red, and EGFP (green) marks infected cells. In g, Sup., supernatant; *cpg15*-sh, *cpg15* shRNA. Scale bar, 25 μm.

**Figure 3-5. Endogenous CPG15 is required for cortical progenitor survival *in vitro*.** (**a**–**d**) Cortical progenitor cultures stained with the progenitor marker nestin (red), the neuronal marker neurofilament-M (Nf-M) (blue) and Hoechst nuclear staining (pseudocolored green). (**e**) Quantification of **a**–**d** and an additional EGFP lentivirus control. Comparison of total number of cells per field staining positive for nestin or Nf-M ($^{*}$ $P < 0.001$). Infection with the *cpg15* shRNA lentivirus (*cpg15*-sh), but not the scrambled control, leads to a marked decrease in the number of neural progenitors. (**f**) Quantification of apoptotic cells in **a**–**d** and an additional EGFP lentivirus control. Knockdown of CPG15 is accompanied by a significant increase in number of apoptotic cells ($^{*}$ $P < 0.001$). Scale bar, 100 μm.

**Figure 3-6. In vivo knockdown of endogenous CPG15 causes shrinkage of the cortical plate and increased apoptosis of cortical neurons.**

(a–e) In vivo knockdown of endogenous CPG15 results in cortical plate shrinkage. Coronal hemi-sections from (a) brains infected with EGFP lentivirus, (b) scrambled *cpg15*-shRNA or (c,d) *cpg15*-shRNA lentivirus. Low to high infection levels shown from left to right. (e) Scatter plot summarizing ventricular area of infected brains. Each symbol represents one hemisphere. (f–i) TUNEL staining of control and *cpg15*-shRNA lentivirus–infected brains. (j–m) Overlay of TUNEL with EGFP staining shows similar infection levels with control and *cpg15*-shRNA viruses. (n,o) Quantification of TUNEL-positive cells in the neocortex (n) or diencephalon (o) of control and infected brains (* $P < 0.003$). Scale bars: a, 1 mm; j, 50 μm.

**Figure 3-7. *In vivo* CPG15 overexpression in the embryonic brain results in an expanded cortical plate and heterotopic cell masses in the ventricular zone.** (**a**–**e**) CPG15 lentivirus–infected brains are larger in diameter and ventricular area. (**a**–**c**) Nissl stained coronal sections from EGFP and CPG15 lentivirus–infected brains. (**d**) Measurements of brain diameter comparing control and CPG15-overexpressing brains (* $P < 0.02$). (**e**) Scatter plot summarizing ventricular area of the same brains. Each symbol represents one hemisphere. (**f**–**k**) Nissl-stained coronal sections of brains infected with the indicated viruses. Boxes in (**a**–**c**) are shown at higher magnification in **f**, **h** and **i**, respectively. (**l**–**p**) Double labeling for nestin in blue and neurofilament in red on EGFP

lentivirus–injected (**l**) and CPG15 lentivirus–injected brains (**m–p**). Scale bars: **a**, 1 mm; **f**, 50 μm; **l**, 100 μm.

**Figure 3-8. CPG15 overexpression reduces apoptosis in the progenitor pool but does not affect mitotic index or cell cycle exit.**

(a) Schematic of BrdU labeling experiments shown in c–j. Embryos were injected with virus at E15 and then harvested at E18 2 h after BrdU injection. (b) Schematic of cell cycle exit experiments shown in k–m. Embryos were injected with virus at E15 then harvested at E19 24 h after BrdU injection. (c,d) Low-magnification view of BrdU labeling (green) and propidium iodide nuclear staining (red) in uninfected (c) and CPG15 lentivirus–infected (d) brains. (e,f) Areas outlined in white in c,d, respectively shown at higher magnification. (g) Quantification of BrdU staining shows a significant increase in the percentage of BrdU-labeled cells in CPG15 lentivirus–infected brains (* $P = 0.008$) as compared to uninfected controls. (h,i) Double labeling for BrdU (red) and the mitosis marker p-H3 (green). (j) Quantification of mitotic rates (ratio of S-phase BrdU-labeled cells to M-phase p-H3–labeled cells) showed no significant difference between CPG15 lentivirus–infected and uninfected brains. (k,l) Double labeling for BrdU (red) and the progenitor cell marker Ki-67 (green). (m) Quantification showed no significant difference in the rate of cell cycle exit between CPG15 lentivirus–infected and control brains. (n,o) TUNEL staining in the ventricular zone of uninfected (n) and CPG15 lentivirus–infected (o) brains. (p) Quantification of TUNEL-positive cells shows a significant decrease in ventricular zone apoptosis in CPG15 lentivirus–infected brains as compared to uninfected controls (* $P < 0.001$). Scale bars: c, 100 μm; e,h,k,n, 50 μm.

## Chapter 4

### Conclusion

*cpg15* was first isolated in a screen for putative plasticity effector genes that are regulated by activity. (Nedivi et al., 1993). It was later observed that *cpg15* is expressed in an early activity independent manner during early development that precedes the activity dependent pattern of expression that lead to its discovery (Corriveau et al., 1999; Lee and Nedivi, 2002). CPG15 has previously been shown to function non-cell autonomously to regulate growth of dendritic and axonal arbors and to promote synaptic maturation (Cantallops et al., 2000; Nedivi et al., 1998). In this thesis I have shown that the small secreted molecule *cpg15* appears to have evolved as a pleiotropic factor that can control very different functions depending on the cellular context in which it is expressed. In the adult barrel cortex the spatiotemporal pattern of *cpg15* expression is regulated by activity that has been shown to lead to alterations in sensory receptive fields. Currently *cpg15* is part of a small handful of downstream effector genes that have been shown to be regulated by activity that is capable of producing plasticity in the adult barrel cortex. While the precise mechanism of *cpg15* function in the barrel cortex has not yet been defined, it would seem plausible that the expression of *cpg15* in the context of receptive field plasticity could be related to its involvement in small scale synaptic changes, or even structural rearrangement of neurons that would function to alter cortical responses to peripheral manipulations (Cantallops et al., 2000; Lee et al., 2005; Nedivi et al., 1998).

During embryonic cortical development *cpg15* expression is concomitant with periods of rapid cell proliferation and apoptosis, and during circuit formation and target selection, consistent with a role for early CPG15 at a survival factor. Manipulating the

levels of CPG15 in the developing telencephalon had a profound effect on the size and shape of the neocortical plate. Decreasing endogenous CPG15 levels caused an increase in apoptosis of cortical progenitors and shrinkage of the cortical plate while, overexpression of CPG15 lead to morphological changes that were consistent with mice with mutations in cell death genes like Bax, Bcl-X (Bcl2l1), Apaf1, Casp9 and Casp3 that are prominently involved in the cell death cascade. During CPG15 overexpression and in these cell death mutants, there is an expansion of the cortical plate and a subsequent generation of superfluous neurons in the developing telencephalon. Our *in vitro* studies have shown that CPG15 prevents apoptosis by inhibiting activation of Caspase 3, however it is still unknown how far upstream, and through what receptor pathway CPG15 may be functioning.

In creating a complete and functional cortex there is a complex interplay between cell proliferation, cell cycle exit, and apoptosis to regulate the size of the progenitor pool and ultimately the final number of neurons generated in the developing brain (Chenn and Walsh, 2003; de la Rosa and de Pablo, 2000; Huang and Reichardt, 2001; Rakic, 1995; Suh et al., 2001). The question remains as to why cortical progenitor cells would be created just so that just to be immediately killed off. One explanation is that cells with errors in DNA replication, or differentiation are signaled to die, or automatically activate their cell death program (Voyvodic, 1996). A second explanation is that cells may have some phenotype recognition mechanism where apoptosis would function to select for specific cell lineages, and the subsequent postnatal complement of cells (Blaschke et al., 1996). The third possibility is that cell death in the proliferative zone is not specific, but is instead the result of a limited supply of exogenous survival factors. Similar to the

model of trophic factor dependence thought to mediate the second wave of cell death that occurs during synapse formation. The pattern of CPG15 expression during cortical development, coupled with the observation that exogenous CPG15 appears to rescue additional progenitors from apoptosis, provides strong support for this hypothesis. In this model limiting amounts of soluble CPG15 would be dispersed throughout the ventricular and subventricular zone of the developing brain to ensure that the correct number of neural precursors will survive to move onto the next developmental stage. Additional factors would then ensure that the surviving progenitors would go through the correct number and type (asymmetric or symmetric) of cell divisions in order to create the proper number and type of cells in the brain (Chenn and Walsh, 1999). At later stages of development CPG15 may function to promote outgrowth and synaptic maturation of neurons as they go on to form mature cortical circuits. Finally CPG15 expressed in the adult brain in an activity dependent manner will function to modify the structure and/or synaptic properties or mature neurons in order to modify connections in response to changes in patterns of activity.

**Future Directions**

Our previous studies using *in utero* lentiviral injections demonstrated that CPG15 promotes survival of neurons and neural progenitors in the developing cerebral cortex. However even with viral injections at the earliest time technically feasible for us (E14-15, in rat), because of the lag from viral infection to transgene expression and protein accumulation, by the time cortical cells were exposed to exogenous CPG15 or to an anti-CPG15 short hairpin RNAi (*cpg15sh*-RNA) they were likely closer in age to E15-16. At

E16, endogenous CPG15 expression is already strong, and cortical progenitors are only a handful of divisions away from final mitosis. Technical limitations thus precluded any examination of CPG15 function during the earliest stages of neurogenesis.

To circumvent this problem we propose use lentiviral transgenesis to create transgenic mice overexpressing CPG15 or a *cpg15sh*-RNA. In the gain of function mutants, CPG15 expression will be driven by the pan-neuronal α-tubulin promoter that has been shown to be expressed in both differentiated and progenitor neural populations starting as early as E9.5 (Gloster et al., 1999). Since there are no tissue specific promoters for RNA expression, the *cpg15sh*-RNA in the loss of function mutants will be driven by a conventional U6 promoter. This will allow us to analyze the mutant founder embryos for defects in cortical development starting with formation of the preplate at E11-12 (Allendoerfer and Shatz, 1994), through formation of the cortical plate and the subventricular zone at approximately E13 and E16, respectively (Super et al., 1998). This approach will allow for highly efficient generation of transgenic animals (Lois et al., 2002) for studies of CPG15 function at times too early to manipulate using the *in utero* injections.

## Appendix

### Preliminary Studies of CPG15 *In Vitro* and Modeling Putative CPG15 Function

Classical neurotrophins that promote neuronal survival also promote differentiation of neural progenitors. To investigate whether during early cortical development, in addition to promoting progenitor survival, CPG15 also functions to promote their differentiation, we overexpressed CPG15 with a lentivirus under the control of alpha-tubulin promoter in 6d neural progenitor cultures. During development, neural progenitors are influenced by mitogenic factors such as fibroblast growth factor (FGF) or epidermal growth factor (EGF) to progress through sequential commitment steps. A differential response to these factors may represent distinct progenitor populations that are at different points in their development. We therefore tested for the effect of CPG15 on neural progenitors in the presence of EGF, FGF, or both. CPG15 overexpression *in vitro* in the presence of EGF, lead to an increase in the mitotic index of neural progenitors as measured by phosphohistone-H3 staining (Figure 5-1a-g). In order to determine whether the increase in mitotic divisions was generating additional progenitors or postmitotic neurons, we stained the cultured cells for ther progenitor marker nestin and the neuronal marker Nf-M. We found that in the presence of EGF, CPG15 overexpression significantly increased the number of neurons generated in the 6d progenitor cultures (Figure 5-1h-n). The increase in mitotic index resulting in early generation of postmitotic neurons was not observed in brains injected *in utero* with the lentivirus containing the *cpg15* transgene. One possibility is that exposure to CPG15 in the *in vivo* experiment is too short or too weak for observing this additional role. Another possibility is that CPG15 is acting upon a very specific subpopulation of neural

progenitor cells during a discrete developmental period that is prior to exogenous CPG15 delivery. One model that we have of CPG15 function during early cortical development is that CPG15 may function early as a survival factor for radial glial progenitors and/or intermediate progenitors. As development progresses CPG15 could then go on to promote terminal neurogenic mitotic divisions of EGF responsive intermediate progenitors, or a combination of EGF and soluble CPG15 signaling may function to promote neurogenesis in neural progenitors in the SVZ (Figure 5-2).

**Figure 5-1. Long term overexpression of CPG15 *in vitro* promotes increased neurogenic terminal mitosis.**

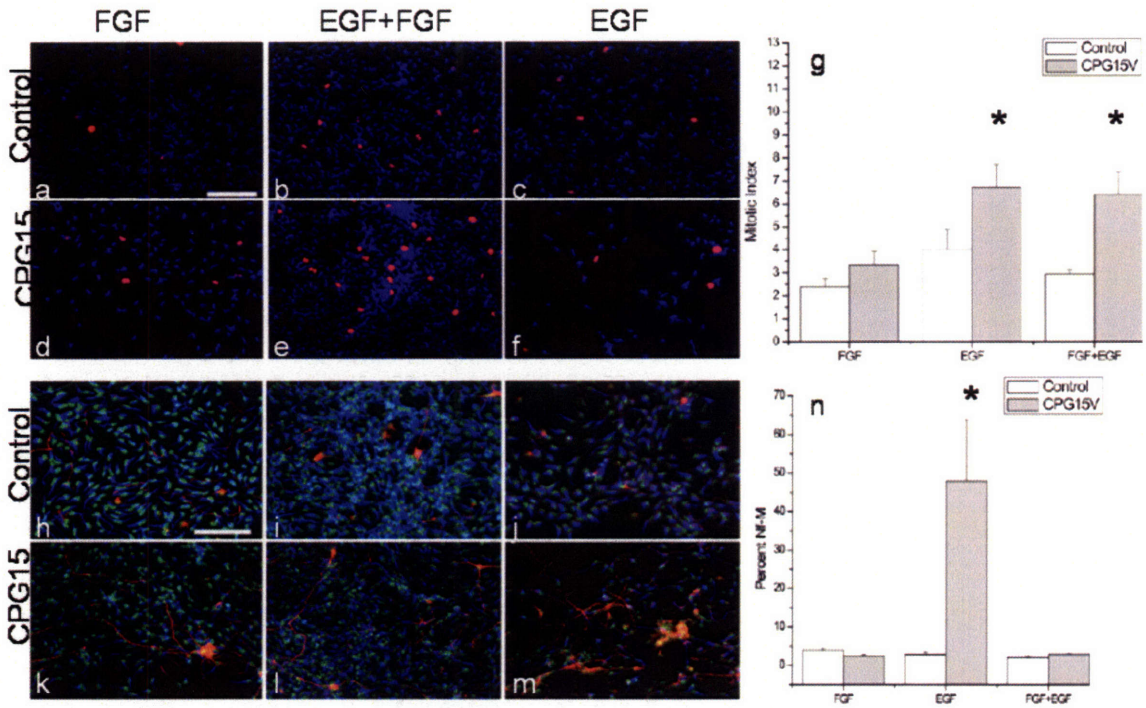
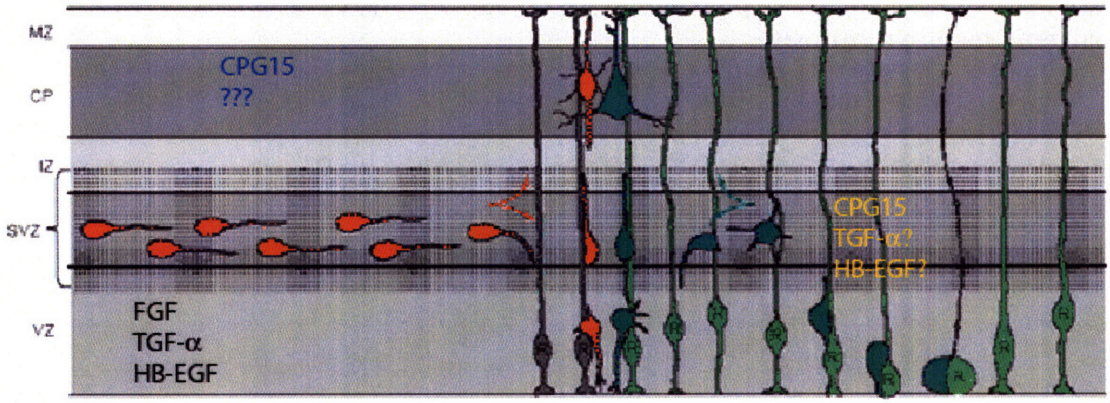


**Figure 5-2. A model for CPG15 function during early cortical development.**



## Figure Legends

**Figure 5-1. Long term overexpression of CPG15 *in vitro* promotes increased neurogenic terminal mitosis**
**(a-f)** Six day cortical progenitor cultures stained with mitotic cell marker phosphohistone-H3 (red) and dapi nuclear stain (blue). **(g)** Quantification of mitotic index of a-f comparing the number of phosphohistone-H3 positive cell per 100 cells. Lentiviral overexpression of CPG15 under the control of alpha-tubulin promoter leads to an increased mitotic index in progenitors cultured in the presence of the mitogen EGF (**h-m)** Six day cortical progenitor cultures stained with the progenitor marker nestin (blue), the neuronal marker neurofilament-M (red) and dapi nuclear stain (pseudocolored green). **(n)** Comparison of the percentage of Nf-M positive cells per field. Infection of progenitors with CPG15 lentivirus, leads to a marked increase in the percentage of Nf-M positive cells. Scale bar, 100μm (*$P < 0.05$).

**Figure 5-2. A model for CPG15 function during early cortical development.**
Soluble CPG15 expressed in the SVZ may function as a survival factor for VZ and/or SVZ neural precursor cells, and neuronal differentiation factor for a subset of neural precursors in the SVZ in combination with other factors such as EGF, or FGF. CPG15 expressed in the cortical plate, may then go on to promote outgrowth and synapse maturation in differentiated neurons.

# References


(1970). Embryonic vertebrate central nervous system: revised terminology. The Boulder Committee. Anat Rec *166*, 257-261.

Allen, C. B., Celikel, T., and Feldman, D. E. (2003). Long-term depression induced by sensory deprivation during cortical map plasticity in vivo. Nat Neurosci *6*, 291-299.

Allendoerfer, K. L., and Shatz, C. J. (1994). The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. Annu Rev Neurosci *17*, 185-218.

Aloe, L. (2004). Rita Levi-Montalcini: the discovery of nerve growth factor and modern neurobiology. Trends Cell Biol *14*, 395-399.

Armstrong-James, M., Diamond, M. E., and Ebner, F. F. (1994). An innocuous bias in whisker use in adult rats modifies receptive fields of barrel cortex neurons. J Neurosci *14*, 6978-6991.

Armstrong-James, M., and Fox, K. (1987). Spatiotemporal convergence and divergence in the rat S1 "barrel" cortex. J Comp Neurol *263*, 265-281.

Bai, J., Ramos, R. L., Ackman, J. B., Thomas, A. M., Lee, R. V., and LoTurco, J. J. (2003). RNAi reveals doublecortin is required for radial migration in rat neocortex. Nat Neurosci *6*, 1277-1283.

Barnabe-Heider, F., and Miller, F. D. (2003). Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci *23*, 5149-5160.

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992). Cell death in the oligodendrocyte lineage. J Neurobiol *23*, 1221-1230.

Barth, A. L., McKenna, M., Glazewski, S., Hill, P., Impey, S., Storm, D., and Fox, K. (2000). Upregulation of cAMP response element-mediated gene expression during experience-dependent plasticity in adult neocortex. J Neurosci *20*, 4206-4216.

**Bayer, S. A., Altman, J., Russo, R. J., Dai, X. F., and Simmons, J. A. (1991). Cell migration in the rat embryonic neocortex. J Comp Neurol *307*, 499-516.**

**Bisler, S., Schleicher, A., Gass, P., Stehle, J. H., Zilles, K., and Staiger, J. F. (2002). Expression of c-Fos, ICER, Krox-24 and JunB in the whisker-to-barrel pathway of rats: time course of induction upon whisker stimulation by tactile exploration of an enriched environment. J Chem Neuroanat *23*, 187-198.**

**Blaschke, A. J., Staley, K., and Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development *122*, 1165-1174.**

**Blaschke, A. J., Weiner, J. A., and Chun, J. (1998). Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. J Comp Neurol *396*, 39-50.**

**Brady, R., Zaidi, S. I., Mayer, C., and Katz, D. M. (1999). BDNF is a target-derived survival factor for arterial baroreceptor and chemoafferent primary sensory neurons. J Neurosci *19*, 2131-2142.**

**Brunstrom, J. E., Gray-Swain, M. R., Osborne, P. A., and Pearlman, A. L. (1997). Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. Neuron *18*, 505-517.**

**Cantallops, I., Haas, K., and Cline, H. T. (2000). Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo. Nat Neurosci *3*, 1004-1011.**

**Caviness, V. S., Jr., Takahashi, T., and Nowakowski, R. S. (1995). Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci *18*, 379-383.**

**Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell *94*, 727-737.**

**Chenn, A., and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell *82*, 631-641.**

**Chenn, A., and Walsh, C. A. (1999). Perspectives: neurobiology. Cranking it up a notch. Science *286*, 689-690.**

**Chenn, A., and Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science *297*, 365-369.**

**Chenn, A., and Walsh, C. A. (2003). Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. Cereb Cortex *13*, 599-606.**

**Clarke, P. G., and Clarke, S. (1995). Historic apoptosis. Nature *378*, 230.**

**Clarke, P. G., and Clarke, S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. Anat Embryol (Berl) *193*, 81-99.**

**Cohen, S., and Levi-Montalcini, R. (1956). A Nerve Growth-Stimulating Factor Isolated from Snake Venom. Proc Natl Acad Sci U S A *42*, 571-574.**

**Cohen-Cory, S. (1999). BDNF modulates, but does not mediate, activity-dependent branching and remodeling of optic axon arbors in vivo. J Neurosci *19*, 9996-10003.**

**Conover, J. C., and Yancopoulos, G. D. (1997). Neurotrophin regulation of the developing nervous system: analyses of knockout mice. Rev Neurosci *8*, 13-27.**

**Conradt, B., and Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.**

**Corbo, J. C., Deuel, T. A., Long, J. M., LaPorte, P., Tsai, E., Wynshaw-Boris, A., and Walsh, C. A. (2002). Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. J Neurosci *22*, 7548-7557.**

**Corriveau, R. A., Shatz, C. J., and Nedivi, E. (1999). Dynamic regulation of cpg15 during activity-dependent synaptic development in the mammalian visual system. J Neurosci *19*, 7999-8008.**

**de la Rosa, E. J., and de Pablo, F. (2000). Cell death in early neural development: beyond the neurotrophic theory. Trends Neurosci *23*, 454-458.**

**Diamond, M. E., Armstrong-James, M., and Ebner, F. F. (1993). Experience-dependent plasticity in adult rat barrel cortex. Proc Natl Acad Sci U S A *90*, 2082-2086.**

**Dono, R., Texido, G., Dussel, R., Ehmke, H., and Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. Embo J *17*, 4213-4225.**

**Ellis, H. M., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817-829.**

**Ernfors, P., Bengzon, J., Kokaia, Z., Persson, H., and Lindvall, O. (1991). Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis. Neuron *7*, 165-176.**

**Ernfors, P., Merlio, J. P., and Persson, H. (1992). Cells Expressing mRNA for Neurotrophins and their Receptors During Embryonic Rat Development. Eur J Neurosci *4*, 1140-1158.**

**Ernfors, P., Wetmore, C., Olson, L., and Persson, H. (1990). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron *5*, 511-526.**

**Fan, G., Copray, S., Huang, E. J., Jones, K., Yan, Q., Walro, J., Jaenisch, R., and Kucera, J. (2000). Formation of a full complement of cranial proprioceptors requires multiple neurotrophins. Dev Dyn *218*, 359-370.**

**Filipkowski, R. K., Rydz, M., Berdel, B., Morys, J., and Kaczmarek, L. (2000). Tactile experience induces c-fos expression in rat barrel cortex. Learn Mem *7*, 116-122.**

**Fox, K. (1994). The cortical component of experience-dependent synaptic plasticity in the rat barrel cortex. J Neurosci *14*, 7665-7679.**

**Fox, K. (2002). Anatomical pathways and molecular mechanisms for plasticity in the barrel cortex. Neuroscience *111*, 799-814.**

**Fujino, T., Lee, W. C., and Nedivi, E. (2003). Regulation of cpg15 by signaling pathways that mediate synaptic plasticity. Mol Cell Neurosci *24*, 538-554.**

**Fukumitsu, H., Furukawa, Y., Tsusaka, M., Kinukawa, H., Nitta, A., Nomoto, H., Mima, T., and Furukawa, S. (1998). Simultaneous expression of brain-derived neurotrophic factor and neurotrophin-3 in Cajal-Retzius, subplate and ventricular progenitor cells during early development stages of the rat cerebral cortex. Neuroscience *84*, 115-127.**

**Fundin, B. T., Silos-Santiago, I., Ernfors, P., Fagan, A. M., Aldskogius, H., DeChiara, T. M., Phillips, H. S., Barbacid, M., Yancopoulos, G. D., and Rice, F. L. (1997). Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. Dev Biol *190*, 94-116.**

**Gall, C. M., and Isackson, P. J. (1989). Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. Science *245*, 758-761.**

**Ghosh, A., and Greenberg, M. E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron *15*, 89-103.**

**Glazewski, S., Barth, A. L., Wallace, H., McKenna, M., Silva, A., and Fox, K. (1999). Impaired experience-dependent plasticity in barrel cortex of mice lacking the alpha and delta isoforms of CREB. Cereb Cortex *9*, 249-256.**

**Glazewski, S., Chen, C. M., Silva, A., and Fox, K. (1996). Requirement for alpha-CaMKII in experience-dependent plasticity of the barrel cortex. Science *272*, 421-423.**

**Glazewski, S., and Fox, K. (1996). Time course of experience-dependent synaptic potentiation and depression in barrel cortex of adolescent rats. J Neurophysiol *75*, 1714-1729.**

**Gloster, A., El-Bizri, H., Bamji, S. X., Rogers, D., and Miller, F. D. (1999). Early induction of Talpha1 alpha-tubulin transcription in neurons of the developing nervous system. J Comp Neurol *405*, 45-60.**

**Gotz, M. (2003). Doublecortin finds its place. Nat Neurosci *6*, 1245-1247.**

**Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., *et al.* (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell *94*, 339-352.**

**Hardingham, N., Glazewski, S., Pakhotin, P., Mizuno, K., Chapman, P. F., Giese, K. P., and Fox, K. (2003). Neocortical long-term potentiation and experience-dependent synaptic plasticity require alpha-calcium/calmodulin-dependent protein kinase II autophosphorylation. J Neurosci *23*, 4428-4436.**

**Haydar, T. F., Kuan, C. Y., Flavell, R. A., and Rakic, P. (1999). The role of cell death in regulating the size and shape of the mammalian forebrain. Cereb Cortex *9*, 621-626.**

**Henderson, C. E. (1996). Role of neurotrophic factors in neuronal development. Curr Opin Neurobiol *6*, 64-70.**

**Hevroni, D., Rattner, A., Bundman, M., Lederfein, D., Gabarah, A., Mangelus, M., Silverman, M. A., Kedar, H., Naor, C., Kornuc, M., *et al.* (1998). Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms. J Mol Neurosci *10*, 75-98.**

**Hogan, B. B., R, Constantini, F (1994). Manipulating the Mouse Embryo, 2 edn (New York, Cold Spring Harbor Laboratory).**

**Horch, H. W., Kruttgen, A., Portbury, S. D., and Katz, L. C. (1999). Destabilization of cortical dendrites and spines by BDNF. Neuron *23*, 353-364.**

**Huang, E. J., and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci *24*, 677-736.**

**Huang, W., Armstrong-James, M., Rema, V., Diamond, M. E., and Ebner, F. F. (1998). Contribution of supragranular layers to sensory processing and plasticity in adult rat barrel cortex. J Neurophysiol *80*, 3261-3271.**

**Huang, Z. J., Kirkwood, A., Pizzorusso, T., Porciatti, V., Morales, B., Bear, M. F., Maffei, L., and Tonegawa, S. (1999). BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. Cell *98*, 739-755.**

**Hummler, E., Cole, T. J., Blendy, J. A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F., and Schutz, G. (1994). Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc Natl Acad Sci U S A *91*, 5647-5651.**

**Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.**

**Kang, H., and Schuman, E. M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science *267*, 1658-1662.**

**Kernie, S. G., Liebl, D. J., and Parada, L. F. (2000). BDNF regulates eating behavior and locomotor activity in mice. Embo J *19*, 1290-1300.**

**Kerr, J. F. (1995). Neglected opportunities in apoptosis research. Trends Cell Biol *5*, 55-57.**

**Kerr, J. F., Harmon, B., and Searle, J. (1974). An electron-microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibers. J Cell Sci *14*, 571-585.**

**Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.**

**Kim, J. H., Liao, D., Lau, L. F., and Huganir, R. L. (1998). SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron *20*, 683-691.**

**Kim, U., and Ebner, F. F. (1999). Barrels and septa: separate circuits in rat barrels field cortex. J Comp Neurol *408*, 489-505.**

**Kingsbury, M. A., Rehen, S. K., Contos, J. J., Higgins, C. M., and Chun, J. (2003). Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. Nat Neurosci *6*, 1292-1299.**

**Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96-99.**

**Koralek, K. A., Jensen, K. F., and Killackey, H. P. (1988). Evidence for two complementary patterns of thalamic input to the rat somatosensory cortex. Brain Res *463*, 346-351.**

**Kuan, C. Y., Roth, K. A., Flavell, R. A., and Rakic, P. (2000). Mechanisms of programmed cell death in the developing brain. Trends Neurosci *23*, 291-297.**

**Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell *94*, 325-337.**

**Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature *384*, 368-372.**

**Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.**

**Lee, W. C., Huang, H., Feng, G., Sanes, J. R., Brown, E. N., So, P. T., and Nedivi, E. (2005). Dynamic Remodeling of Dendritic Arbors in GABAergic Interneurons of Adult Visual Cortex. PLoS Biol *4*, e29.**

**Lee, W. C., and Nedivi, E. (2002). Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of cpg15-like genes. J Neurosci *22*, 1807-1815.**

**Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*, 1154-1162.**

**Levine, E. S., Crozier, R. A., Black, I. B., and Plummer, M. R. (1998). Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci U S A *95*, 10235-10239.**

**Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.**

**Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.**

**Lu, S. M., and Lin, R. C. (1993). Thalamic afferents of the rat barrel cortex: a light- and electron-microscopic study using Phaseolus vulgaris leucoagglutinin as an anterograde tracer. Somatosens Mot Res *10*, 1-16.**

**Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M., and Yancopoulos, G. D. (1990). NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron *5*, 501-509.**

**McAllister, A. K., Katz, L. C., and Lo, D. C. (1999). Neurotrophins and synaptic plasticity. Annu Rev Neurosci *22*, 295-318.**

**McManus, M. T., and Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. Nat Rev Genet *3*, 737-747.**

**Menard, C., Hein, P., Paquin, A., Savelson, A., Yang, X. M., Lederfein, D., Barnabe-Heider, F., Mir, A. A., Sterneck, E., Peterson, A. C., *et al.* (2002). An essential role for a MEK-C/EBP pathway during growth factor-regulated cortical neurogenesis. Neuron *36*, 597-610.**

**Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998). Genetics of programmed cell death in C. elegans: past, present and future. Trends Genet *14*, 410-416.**

**Minichiello, L., Casagranda, F., Tatche, R. S., Stucky, C. L., Postigo, A., Lewin, G. R., Davies, A. M., and Klein, R. (1998). Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. Neuron *21*, 335-345.**

**Minichiello, L., and Klein, R. (1996). TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. Genes Dev *10*, 2849-2858.**

**Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., and et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science *267*, 1506-1510.**

**Naeve, G. S., Ramakrishnan, M., Kramer, R., Hevroni, D., Citri, Y., and Theill, L. E. (1997). Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. Proc Natl Acad Sci U S A *94*, 2648-2653.**

**Nedivi, E., Hevroni, D., Naot, D., Israeli, D., and Citri, Y. (1993). Numerous candidate plasticity-related genes revealed by differential cDNA cloning. Nature *363*, 718-722.**

**Nedivi, E., Javaherian, A., Cantallops, I., and Cline, H. T. (2001). Developmental regulation of CPG15 expression in Xenopus. J Comp Neurol *435*, 464-473.**

**Nedivi, E., Wu, G. Y., and Cline, H. T. (1998). Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. Science *281*, 1863-1866.**

**Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci *7*, 136-144.**

**Oppenheim, R. W. (1991). Cell death during development of the nervous system. Annu Rev Neurosci *14*, 453-501.**

**Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M., and Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A *95*, 5672-5677.**

**Pompeiano, M., Blaschke, A. J., Flavell, R. A., Srinivasan, A., and Chun, J. (2000). Decreased apoptosis in proliferative and postmitotic regions of the Caspase 3-deficient embryonic central nervous system. J Comp Neurol *423*, 1-12.**

**Poser, S., Impey, S., Xia, Z., and Storm, D. R. (2003). Brain-derived neurotrophic factor protection of cortical neurons from serum withdrawal-induced apoptosis is inhibited by cAMP. J Neurosci *23*, 4420-4427.**

**Rakic, P. (1995). A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. Trends Neurosci *18*, 383-388.**

**Rocamora, N., Welker, E., Pascual, M., and Soriano, E. (1996). Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. J Neurosci *16*, 4411-4419.**

**Roth, K. A., Kuan, C., Haydar, T. F., D'Sa-Eipper, C., Shindler, K. S., Zheng, T. S., Kuida, K., Flavell, R. A., and Rakic, P. (2000). Epistatic and independent functions**

of caspase-3 and Bcl-X(L) in developmental programmed cell death. Proc Natl Acad Sci U S A *97*, 466-471.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., *et al.* (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet *33*, 401-406.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem *166*, 368-379.

Shepherd, G. M., Pologruto, T. A., and Svoboda, K. (2003). Circuit analysis of experience-dependent plasticity in the developing rat barrel cortex. Neuron *38*, 277-289.

Shindler, K. S., Latham, C. B., and Roth, K. A. (1997). Bax deficiency prevents the increased cell death of immature neurons in bcl-x-deficient mice. J Neurosci *17*, 3112-3119.

Simons, D. J. (1978). Response properties of vibrissa units in rat SI somatosensory neocortex. J Neurophysiol *41*, 798-820.

Slack, R. S., El-Bizri, H., Wong, J., Belliveau, D. J., and Miller, F. D. (1998). A critical temporal requirement for the retinoblastoma protein family during neuronal determination. J Cell Biol *140*, 1497-1509.

Snider, W. D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell *77*, 627-638.

Staiger, J. F., Masanneck, C., Bisler, S., Schleicher, A., Zuschratter, W., and Zilles, K. (2002). Excitatory and inhibitory neurons express c-Fos in barrel-related columns after exploration of a novel environment. Neuroscience *109*, 687-699.

Stern, C. D. (2001). Initial patterning of the central nervous system: how many organizers? Nat Rev Neurosci *2*, 92-98.

**Stucky, C. L., DeChiara, T., Lindsay, R. M., Yancopoulos, G. D., and Koltzenburg, M. (1998). Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. J Neurosci *18*, 7040-7046.**

**Suh, J., Lu, N., Nicot, A., Tatsuno, I., and DiCicco-Bloom, E. (2001). PACAP is an anti-mitogenic signal in developing cerebral cortex. Nat Neurosci *4*, 123-124.**

**Super, H., Soriano, E., and Uylings, H. B. (1998). The functions of the preplate in development and evolution of the neocortex and hippocampus. Brain Res Brain Res Rev *27*, 40-64.**

**Takahashi, T., Nowakowski, R. S., and Caviness, V. S., Jr. (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. J Neurosci *16*, 6183-6196.**

**Takahashi, T., Nowakowski, R. S., and Caviness, V. S., Jr. (1997). The mathematics of neocortical neuronogenesis. Dev Neurosci *19*, 17-22.**

**Thomaidou, D., Mione, M. C., Cavanagh, J. F., and Parnavelas, J. G. (1997). Apoptosis and its relation to the cell cycle in the developing cerebral cortex. J Neurosci *17*, 1075-1085.**

**Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E., and Svoboda, K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature *420*, 788-794.**

**Trent, C., Tsuing, N., and Horvitz, H. R. (1983). Egg-laying defective mutants of the nematode Caenorhabditis elegans. Genetics *104*, 619-647.**

**Truman, J. W., Fahrbach, S. E., and Kimura, K. (1990). Hormones and programmed cell death: insights from invertebrate studies. Prog Brain Res *86*, 25-35.**

**Vaccarino, F. M., Schwartz, M. L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M., Doetschman, T., Coffin, J. D., Wyland, J. J., and Hung, Y. T. (1999). Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci *2*, 848.**

**Vaux, D. L., and Korsmeyer, S. J. (1999). Cell death in development. Cell *96*, 245-254.**

**Voyvodic, J. T. (1996). Cell death in cortical development: How much? Why? So what? Neuron *16*, 693-696.**

**White, F. A., Keller-Peck, C. R., Knudson, C. M., Korsmeyer, S. J., and Snider, W. D. (1998). Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. J Neurosci *18*, 1428-1439.**

**Wong-Riley, M. (1979). Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. Brain Res *171*, 11-28.**

**Woolsey, T. A., and Van der Loos, H. (1970). The structural organization of layer IV in the somatosensory region (SI) of mouse cerebral cortex. The description of a cortical field composed of discrete cytoarchitectonic units. Brain Res *17*, 205-242.**

**Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. Int Rev Cytol *68*, 251-306.**

**Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell *94*, 739-750.**

**Yuan, J., and Horvitz, H. R. (2004). A first insight into the molecular mechanisms of apoptosis. Cell *116*, S53-56, 51 p following S59.**

**Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell *75*, 641-652.**

**Yuan, J. Y., and Horvitz, H. R. (1990). The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. Dev Biol *138*, 33-41.**

**Zhang, J., Liu, X., Scherer, D. C., van Kaer, L., Wang, X., and Xu, M. (1998). Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. Proc Natl Acad Sci U S A *95*, 12480-12485.**

**Zhou, J., and Tang, X. C. (2002). Huperzine A attenuates apoptosis and mitochondria-dependent caspase-3 in rat cortical neurons. FEBS Lett *526*, 21-25.**

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