Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
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Oral examination: 27.11.2003

TrkB and gene expression

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Acknowledgements

I wish to thank all those people who have supported and encouraged me during my time at the EMBL. I particular I would like to thank my Thesis advisor, Liliana Minichiello, for offering me the opportunity to work in a scientific environment of excellent quality and for teaching me a scientific approach for biological question.

I acknowledge the members of my thesis committee, Claus Nerlov, Iain Mattaj, Klaus Unsicker, for their criticism, support and encouragement.

I thank the members of Liliana Minichiello's laboratory, who provided a pleasant and warm environment around me: Diego Medina, Carla Sciarretta, Markus Muller, Susanne Pedersen, and Che Serguera. I am grateful to all the members of Claus Nerlov's laboratory for the scientific discussions during our lab-meetings.

Furthermore, I acknowledge the people of the transgenic service and the people of the animal care for their professional assistance.

Finally, the EMBL for providing a unique scientific and cultural experience.

Publications during the PhD period

1. Postigo A,***Calella AM**[*], Fritzsch B[*], Knipper M[*], Katz D, Eilers A, Schimmang T, Lewin GR, Klein R, Minichiello L.
   Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons.
   Genes Dev. 2002 Mar 1;16(5):633-45.
   *These authors contributed equally to this work

2. Minichiello L, **Calella AM**, Medina DL, Bonhoeffer T, Klein R, Korte M.
   Mechanism of TrkB-mediated hippocampal long-term potentiation.
   Neuron. 2002 Sep 26;36(1):121-37.

3. MedinaDL, Sciarretta C, **Calella AM**, Minichiello L.
   Removal of BDNF/TrkB signaling during embryogenesis causes mistiming of cortical neuronal and glial migration.
   (Submitted)

4. Publication of the work presented in this thesis is in preparation

Abbreviations

aa	amino acids
ATP	adenosine triphosphate
ATF4	activating transcription factor 4
BAD	bcl-xl/bcl-2 associated death promoter
bcl-2	B-cell lymphoma/leukemia 2
□-gal	□-galactosidase
bHLH	basic helix loop helix
bp	base pair
BSA	bovine serum albumine
CaMKII	calcium/calmodulin-dependent kinase II
CaMKIV	calcium/calmodulin-dependent kinase IV
CBP	CREB binding protein
C/EBP	CCAAT enhancer-binding protein
c-fos	cellular FBJ osteosarcoma
CNTF	ciliary neurotrophic factor
CNS	central nervous system
CREB	cAMP-response-element binding protein
Csk	control of src kinase
C-terminus	carboxy-end of a protein
Da	dalton
DAG	diacyl glycerol
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethlypyrocarbonate
DIV	day in vitro
DMSO	dimethylsulphoxide
DNA/cDNA	deoxyribonucleic acid/complementary DNA
dNTPs	deoxynucleotide triphosphate
DOC	sodium deoxycholate
DTT	dithiotreitol
E	embryonic stage
EDTA	ethylendiaminetetraacetic acid
Egr1	early growth response-1
Egr2	early growth response-2
EGTA	Ethylene-bis(oxyethylene-nitrilo)tetraacetic acid
ERK	extracellular signal-regulated protein kinase
EST	expressed sequence tag
FRS2	fibroblast growth factor receptor substrate 2
GABA	□□amino-n-butyric acid
Grb2	growth factor receptor bound protein 2
GST	glutathione S-transferase
HEB	human bHLH
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethansulphonic acid)
hr	hours
IPTG	isopropyl-•-thiogalactoside
IP3	inositol(1,4,5)triphosphate
LZ	leucine zipper
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEM	modified Eagle's medium
mGif	mouse GDNF inducible factor

MOPS	3-(N-morpholino)-propanesulfonic acid
Neuro2A	mouse neuroblastoma cell lines
Ngn	neurogenin
NHS	normal horse serum
N-terminus	amino-end of a protein
ON	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	phosphate-buffered paraformaldehyde
PH	pleckstrin homology domain
PIP2	phosphatidylinositol 4,5 bisphosphate
PKB/Akt	protein kinase B
PKC	protein kinase C
pRb	retinoblastoma tumor suppressor
rAPS	rat homolog of APS (adaptor protein with PH and SH2 domains)
RNA/mRNA	ribonucleic acid/messenger RNA
rpm	revolutions per minute
Rsk	p90 ribosomal S6 kinase
RT-PCR	reverse transcription polymerase chain reaction
SDS sodium	dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	src homology domain
SH2-B	adaptor protein with SH2 binding domain
Shc	SH2 contaning molecule
SHP-2	src homology phosphatase
SOS	mammalian homolog of son of sevenless
SSC	sodium cloride/sodium citrate
TBP	TATA binding protein
TBS	Tris-buffered saline solution
TFIIB	transcription factor IIB
TNT	*in vitro* coupled transcription and translation
Tris	Tris-(hydroxymethyl)-aminomethane
UV	ultraviolet

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Abstract

The neurotrophins are a family of secreted proteins that potently regulate diverse neuronal responses. Family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5(NT4/5).
Neurotrophins bind the Trks receptor family (TrkA, B, C). NGF is the preferred ligand for TrkA, BDNF and NT4/5 are preferred for TrkB, and NT3 for TrkC, although NT3 also binds with less affinity to TrkA and TrkB.
During my PhD I have focused my interest in understanding how neurotrophins regulate gene expression and, in particular, how BDNF does this through its high affinity receptor TrkB during neurogenesis. In order to answer this question, I planned to adopt the following approaches. First, to analyze global changes in gene expression after TrkB/BDNF activation, using microarray technology. Second, once a set of regulated genes was identified, to characterize the regulation of these genes at the promoter level, in order to understand which common elements are important for their regulation.
The high-density oligonucleotide array of Affymetrix was performed using mRNAs that were obtained from cortical neurons of wild type mouse embryos (E15.5), and of mouse embryos possessing TrkB receptors mutated at either tyrosine 515 (trkB/shc point signaling mutants), or tyrosine 816 (trkB/plc-γ point signaling mutants), or at both sites. In all cases the primary neurons were either unstimulated or stimulated with BDNF. The sensitivity of the Affymetrix system allowed me to identify a set of transcription factors that showed a higher fold induction compared to the others class of genes. This group consisted of: egr1, egr2, c-fos and mGIF/Tieg1. These genes were found to be differentially regulated in the signaling point mutant mice. Although the promoter of mGif/TIEG1 is not yet characterized and also the function of this gene is not completely clear, egr1, egr2 and c-fos are well characterized, and, several data suggest that these genes share cis acting 5' regulatory elements. To better understand which elements and transcription factors are important for BDNF-dependent gene expression I choose the c-fos promoter as a model. Using luciferase reporter gene constructs transfected in E15.5 cortical neurons isolated from wild-type and signaling point mutant mice, I discovered that the pathways activated through the shc site promoted higher activation of c-fos promoter than pathways activated through the plc-γ site, and the two sites are both required for BDNF-dependent activation of c-fos promoter. Additionally experiments using c-fos promoter constructs, mutated at single or multiple elements, revealed that the c/ebp binding site together with the E-box are fundamental for the activation of c-fos downstream BDNF/TrkB. This result suggested that C/EBPs and bHLH transcription factors might collaborate to induce the activation of the promoter downstream of BDNF. I have demonstrated, both in vivo and in vitro, that Mash1 and NeuroD are the members of bHLH family that bind C/EBP transcription factors at different domains. That interaction is BDNF independent, and the complex is constitutively present on the c-fos promoter. The BDNF regulation of gene expression is through the post-translation modification of that complex. In fact BDNF stimulation induces an increase in C/EBPβ phosphorylation on Thr188 (ERK1/2-dependent). The phosphorylation by ERK1/2 could explain the transcriptional activation of the C/EBP-Mash1-NeuroD complex downstream BDNF/TrkB. These studies identify a novel neurotrophins-regulated signaling cascade that mediates the gene expression during neurogenesis.

1

INTRODUCTION

The diverse functions of the vertebrate nervous system, which range from sensory perception and motor coordination to motivation and memory, depend on precise connections formed between distinct types of nerve cells. The formation of these cell types and the connections are made between them occur during embryonic and postnatal development.

During the past decade there have been many striking advances in our understanding of the molecular basis of neuronal development. These advances include the identification of proteins that determine how nerve cells acquire their identities, extend axons to target cells, and form synaptic connections, and have provided insight into how synaptic connections are modified by experience.

It is clear that development of the mammalian nervous system is determined by a complex interplay between intrinsic genetic mechanism and extrinsic cues such as growth factors. Within the central nervous system, this interplay has perhaps been best studied in the developing cortex.

After a brief introduction about the development of the entire nervous system, we focus specifically on the development of the cortex, and on three classes of molecules that play a role in this process: bHLH transcription factors, C/EBP transcription factors and neurotrophins. I will also describe the c-fos promoter that I used as a model to understand the regulation of gene expression downstream BDNF/TrkB, and the mGif/TIEG1 transcription factor, that we discovered to be downstream BDNF/TrkB signaling.

1.1 The nervous system arises from the ectoderm

The nervous system begins to develop at a relatively late stage in embryogenesis. Prior to its formation, three main cell layers have been generated. The endoderm, the innermost layer, give rise to the gut, esophagous, stomach, pancreas, lungs, and liver; the mesoderm, the middle layer, give rise to blood, adipose tissue, connective tissue, muscle, and the vascular system; and the ectoderm, the outermost layer, give rise to the major tissue of the central and peripheral nervous systems (Kandel et al.).

Ectodermal cells form a sheet along the dorsal midline of the embryo at the gastrula stage. As this ectodermal sheet begins to acquire neural properties it forms the neural

tube, a columnar epithelium. Ectodermal cells that fail to follow the neural program of differentiation give rise instead to the epidermis of the skin.

Soon after the neural tube has formed it begins to fold into a tubular structure through a process called neurulation. The caudal region of the neural tube gives rise to the spinal cord, and the rostral region becomes the brain.

During these early stages cell proliferation is not uniform along the length of the neural tube, and as a result, specialized regions of the mature central nervous system begin to form. The proliferation of cells in the rostral part of the neural tube initially forms three brain vesicles: the forebrain (or prosencephalon), the midbrain (or mesencephalon), and the hindbrain (or rhombencephalon). Later in development, two of the three primary embryonic vesicles subdivide (table1). The forebrain vesicle gives rise to the telencephalon and diencephalon, and the hindbrain vesicle gives rise to the metencephalon and myelencephalon. These subdivisions, together with the spinal cord, comprise the six regions of the mature nervous system.

Table 1 The main subdivisions of the embryonic central nervous system and mature adult forms(Kandel, Schwartz et al.)

Three-vesicle stage	Five-vesicle stage	Major mature derivatives
1. Forebrain	1a.Telencephalon	1.cerebral cortex, basal ganglia, hippocampus, amygdala, olfactory bulb
	1b.Diencephalon	2.Thalamus, hypothalamus, subthalamus, epithalamus, retina, optic nerves and tracts
2. Midbrain	2. Mesencephalon	3.Midbrain
3. Hindbrain	3a.Metencephalon	4.pons and cerebellum
	3b.Myelencephalon	5.Medulla
4.Cadual part of neural tube	4.Cadual part of neural tube	6. Spinal cord

1.2 Cortical development

The telencephalon is subdivided into the ventral telencephalon, which develops into basal ganglia, and the dorsal telencephalon, which gives rise to the hippocampus, the olfactory cortex, and the neocortex (Marin and Rubenstein, 2001; Parnavelas, 2000)(figure1).

The neocortex is generated from neural stem cells of the CNS. These cells are defined by their ability to self-renew, and have the potential to become neurons, astrocytes, and oligodendrocytes.

During development of the neocortex, positional information is helping to specify the cell fate of progenitor cells. Once a progenitor goes through its final round of cell division, it begins to differentiate while simultaneously migrating from the ventricular zone to its final destination. Differentiation of neural precursor into neurons, astrocytes, and oligodentrocytes takes place in overlapping, but temporally distinct waves, giving rise sequentially to the neurogenic phase, the astroglial phase, and the oligodendroglial phase (Sauvageot and Stiles, 2002). Neuronal differentiation in the mammalian neocortex peaks approximately two-thirds of the way through gestation, corresponding E15.5 in the mouse. Astrocyte differentiation occurs next, peaking at birth. Oligodendrocyte differentiation follows later still, occurring during the first month of murine life.

Neuronal migration in the cerebral cortex begins when the first cohort of postmitotic neurons leaves the germinal zone (VZ) to form the primordial plexiform layer, or preplate, at the surface of the cerebral vesicles (figure2A-C) (Nadarajah and Parnavelas, 2002). This early-generated zone is then split by the arrival of accumulating cortical plate (CP) neurons into the superficial marginal zone (MZ) and the deeper subplate. Layers II-VI of the cerebral cortex are generated in an “inside-out” sequence (figure 2D), such that neurons that are generated early on reside in the deepest layers, whereas last-born cells migrate past the existing layers to form the superficial layers (Rakic, 1988). Consequently, the MZ and subplate contain the earliest-generated neurons of the cerebral cortex. Those in the MZ (layer I) differentiate into Cajal-Retzius cells and other types of neuron that have not yet been fully characterized. The subplate is separated from the VZ by the intermediate zone (IZ), a layer that will eventually contain the afferent and efferent axons of the cortex (white matter).

As the CP emerges, another layer of proliferating cells appears between the VZ and IZ, the so-called subventricular zone (SVZ). This germinal zone contains cells, produced in the VZ, that give rise mainly to glia. The SVZ expands greatly in late gestation and in early postnatal life as the VZ disappears.

Similarly (though later in development), neocortical astrocytes are born in the germinal zone of the neocortex and then migrate locally within this structure. In contrast, two other neocortical cell types, GABAergic interneurons and oligodendrocytes, arise from the germinal zone of the ventral telencephalon and make long journeys, following tangential migratory routes, to their final destinations in the neocortex (Corbin et al., 2001; Parnavelas, 2000)(figure 1)

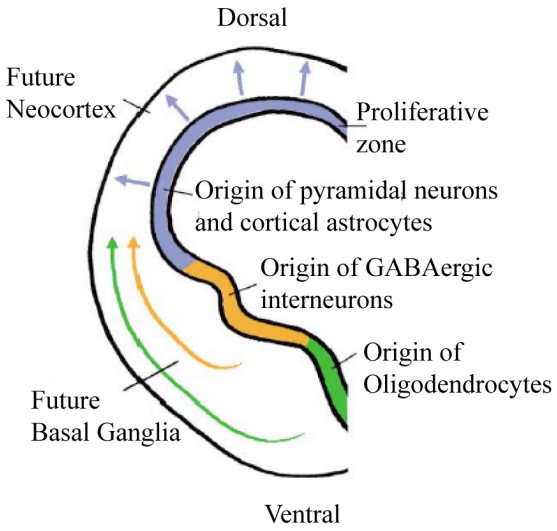


Figure 1 Diagram of the developing telencephalon illustrating the origin of the neural cell types that make up the neocortex (modified from (Ross et al., 2003)). Pyramidal glutamatergic neurons and cortical astrocytes arise from the proliferative layer of the dorsal telencephalon (blue). GABAergic inhibitory interneurons are born in the proliferative zone of the ganglionic eminences (orange). Oligodendrocytes arise from a discrete pool of progenitors in the most ventral region of the telencephalon (green). Not shown are cell types that originate in the ventral telencephalon and remain in the basal ganglia, such as striatal astrocytes and cholinergic neurons.

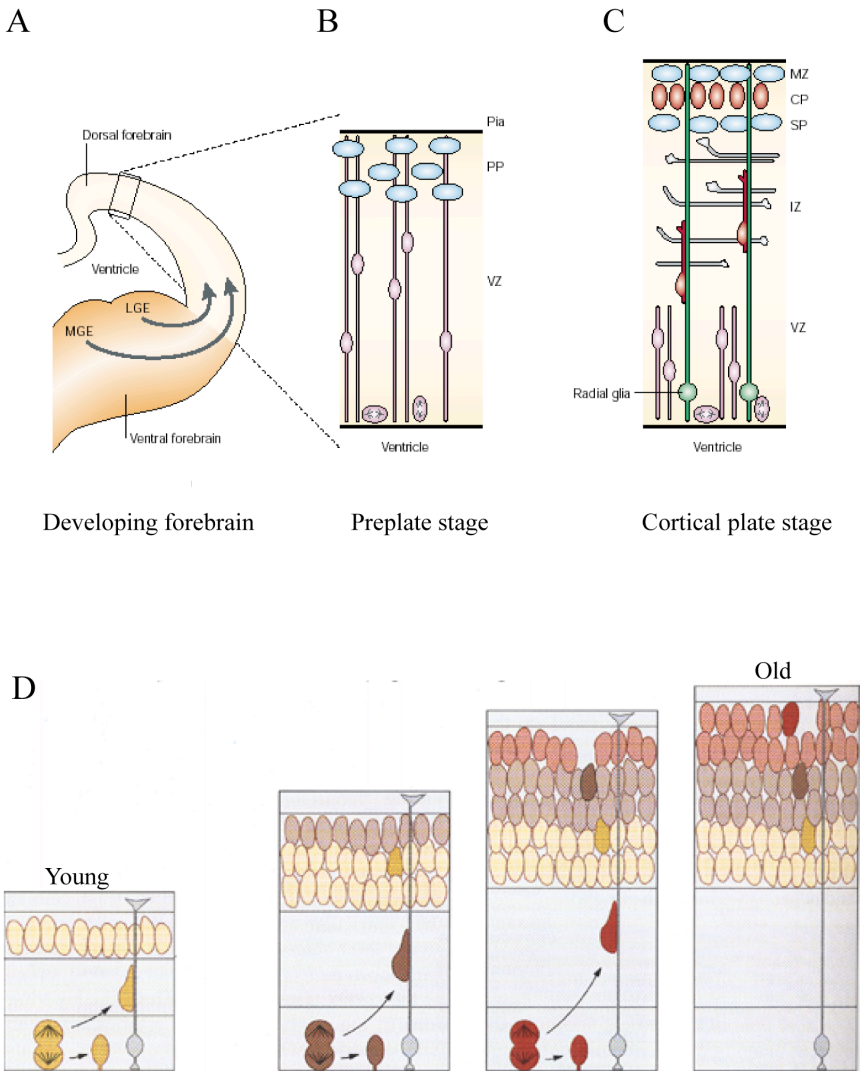


Figure 2 Neocortical development (modified from (Nadarajah and Parnavelas, 2002).
(A) Schematic diagram of a section through the developing rodent forebrain.
(B-C) Illustrations of the different stages of neocortical development. The lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) of the ventral forebrain generate the neurons of the basal ganglia and the cortical interneurons; the latter follow tangential migratory routes to the cortex (A; arrows). In the dorsal forebrain (A; boxed area), neuronal migration begins when first cohort of postmitotic neurons moves out of the ventricular zone (VZ) to form the preplate (PP) (B). Subsequent cohorts of neurons (pyramidal cells) migrate, aided by radial glia, through the intermediate zone (IZ) to split the PP into the outer marginal zone (MZ) and inner subplate (SP)(C). CP, cortical plate.
(D) Cortical cells obey an inside-first outside-last program of neurogenesis.
Neurons born within the ventricular zone at early stages migrate to the deepest layers of the cortical plate. Neurons generated at later stages migrate past the earlier-generated neurons to form the more superficial layers of the cortex.

1.3 Basic Helix-Loop-Helix factors in cortical development

Recent insights into the molecular mechanisms regulating cortical development, have shown that proliferation, specification, and differentiation of cortical progenitor cells is controlled, to a large degree, by transcription factors with basic helix-loop-helix (bHLH) motifs. The bHLH transcription factors (named in reference to the structural motif that mediates their DNA binding and dimerization functions) (Murre et al., 1989) are a large family with around 125 members encoded in the human genome (Ledent et al., 2002). A small set of bHLH factors have been shown to have important roles during corticogenesis (figure 3). This set of bHLHs can be divided in 2 groups based on their function during cortical development: either inhibiting or promoting progenitors differentiation. Together they have been implicated in the four principal stages of cortical development: the proliferation of cortical progenitors, and subsequent the sequential formation of neurons, astrocytes, and oligodendrocytes.

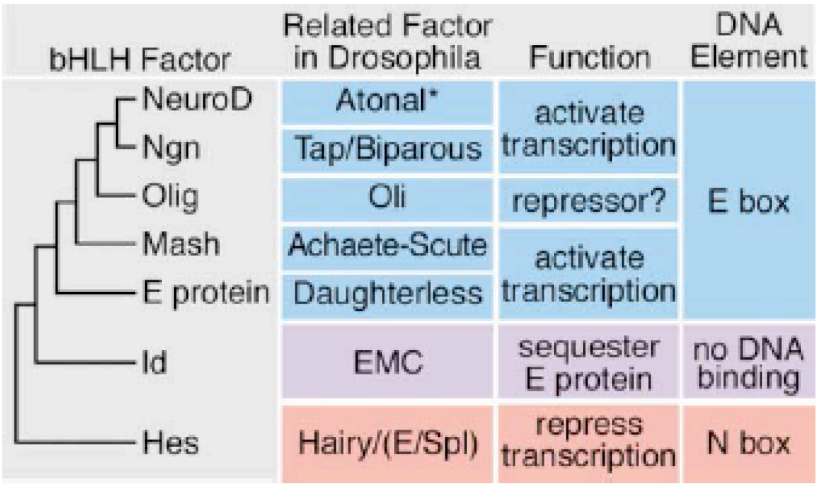


Figure 3 bHLH factors involved in cortical development (modified from (Ross et al., 2003)).
*Atonal is the Drosophila homolog of Math1, not NeuroD

1.3.1 Maintenance of neural progenitors

Two classes of inhibitory bHLH proteins, Hes and Id factors, employ multiple strategies to maintain cortical progenitors in a proliferative state, and to regulate the timing of their differentiation.

The important role of Hes factors during neural development was first revealed in Drosophila, where the Hes homologs, the Enhancer of split (E (spl)) family, were shown to be negative regulators of neuronal differentiation (Knust et al., 1987).

In mammals, it has been shown that Hes1 and Hes5 have key roles in telencephalic development (Ohtsuka et al., 1999). Both Hes1 and Hes5 are expressed in the ventricular zone throughout the telencephalon where they sustain progenitors in an undifferentiated, proliferative state and inhibit their differentiation into neurons. To achieve this goal, Hes factors use two mechanisms. First, they form homodimers and heterodimers with closely related family members, and bind to DNA elements called N boxes (CACNAG) to repress the expression of target genes, such as Mash1, that are required for neuronal differentiation (Chen et al., 1997; Davis and Turner, 2001) (figure 4A). The transcriptional repression activity of Hes proteins is mediated by their interaction with transcriptional corepressors of the Groucho/transducin-like enhancer of split (Gro/TLE) family. Paroush et al. have used a yeast two-hybrid system, to identify proteins interacting with hairy, a basic-helix-loop-helix (bHLH) protein that represses transcription during Drosophila embryonic segmentation. They find that the Gro protein binds specifically to hairy and also to hairy-related bHLH proteins encoded by deadpan and the Enhancer of split complex. The C-terminal WRPW motif present in all these bHLH proteins is essential for this interaction. They demonstrate that these associations reflect in vivo maternal requirements for Gro during neurogenesis, segmentation, and sex determination, three processes regulated by the above bHLH proteins, and they propose that Gro is a transcriptional corepressor recruited to specific target promoters by hairy-related bHLH proteins (Paroush et al., 1994). The histone deacetylase activity associated with this repressor complex modifies chromatin structure, making DNA inaccessible to transcription (reviewed in (Naar et al., 2001).

The second mechanism by which Hes factors inhibit progenitor differentiation is through a physical interaction with proneuronal bHLH proteins, resulting in a functional

antagonism of the activity of proneuronal bHLH proteins (figure 4B) (Alifragis et al., 1997; Sasai et al., 1992). Much of our understanding of Hes function comes from the appreciation that Hes1 and Hes5 are key target genes that are transactivated in response to Notch signaling (reviewed in (Justice and Jan, 2002). Accordingly, disruption of Notch/Hes signaling through loss of Notch, Hes1, or Hes5 results in similar CNS phenotypes, including premature neuronal differentiation in vitro and decreased proliferative ability in vivo (de la Pompa et al., 1997; Ishibashi et al., 1994). Moreover, the ability of Notch to inhibit neuronal differentiation is dependent on Hes1 and Hes5. In cortical progenitors from $Hes1^{-/-}$;$Hes5^{-/-}$ mice, constitutively active Notch signaling is unable to block neurogenesis (Ohtsuka et al., 1999). Ohtsuka et al. found, by retrovirally misexpressing the constitutively active form of Notch (caNotch) in neural precursor cells prepared from wild-type, Hes1-null, Hes5-null and Hes1-Hes5 double-null mouse embryos, that caNotch, which induced the endogenous Hes1 and Hes5 expression, inhibited neuronal differentiation in the wild-type, Hes1-null and Hes5-null background, but not in the Hes1-Hes5 double-null background. These findings indicate that Hes1 and Hes5 are critical Notch effectors that act to promote the maintenance of cortical progenitors by blocking their differentiation into neurons.

Notch/Hes signaling may function during cortical development to maintain a balance between the number of developing neurons and the number of remaining progenitors by a phenomenon known as lateral inhibition (reviewed in (Beatus and Lendahl, 1998). This process provides a mechanism by which a progenitor gives rise to nonequivalent descendents. Newly formed neurons up regulate the Notch ligand, Delta, as they differentiate, and Delta activates Notch/Hes signaling in adjacent cells to block progenitor differentiation. Lateral inhibition thus allows the differentiation of some cortical progenitors into neurons, while preventing simultaneous neurogenesis of all cortical progenitors. As newly differentiating neurons migrate out of the ventricular zone, lateral inhibition in the ventricular zone diminishes, thereby allowing a new set of cortical progenitors to begin neuronal differentiation.

The second class of inhibitory bHLH proteins is the Id factors. They are expressed in the ventricular zone of the telencephalon (Jen et al., 1997), and inhibit gene transcription by sequestration (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990).

Specifically, Ids form dimers with E proteins (E12, E47, E2-2, HEB) and thereby inhibit the activity of bHLH factors that require E proteins (including Mash1, NeuroD, Neurogenin and Olig families),(figure 4C). Id factors lack a DNA binding region and as consequence, Id/E proteins heterodimers do not bind DNA. Ids inhibit the premature differentiation of cortical progenitors into neurons and oligodendrocytes, but not astrocytes. In mouse models lacking both Id1 and Id3, cortical progenitors exit the cell cycle prematurely and undergo accelerated neuronal differentiation in vivo (Lyden et al., 1999). Similarly, when oligodendrocyte precursors from Id2 knockout mice are cultured in vitro, they undergo precocious growth arrest and differentiation, yielding premature oligodendrocytes (Wang et al., 2001). Forced expression of Ids in cell culture blocked both neurogenesis and oligodendrocyte formation (Kondo and Raff, 2000; Toma et al., 2000; Wang et al., 2001). In addition to their role as temporally regulated inhibitors of neuronal and oligodendroglial differentiation, the Id family members, Id2 and Id4, have been shown to play a role in promoting cell proliferation by inhibiting the ability of retinoblastoma (Rb) family members to interfere with E2F-mediated transcription (Iavarone et al., 1994; Lasorella et al., 1996; Norton, 2000) (figure 4D). Transcription factors of the E2F family induce the transcription of genes that mediate cell cycle progression. Members of the Rb family interact physically with E2F transcription factors and block their activity. Id2 and Id4 function, in part, by releasing E2Fs from inhibition by Rb, such that E2Fs can activate genes that promote the proliferation of cortical progenitors. It is not clear how the activity of Ids is regulated. One possibility may be the control of Id entry into the nucleus. Since they lack a nuclear localization sequence, the localization of Ids depends upon their interaction with nuclear proteins such as E proteins. Recently it was found, for example, that Id2 relocalizes from the nucleus to the cytoplasm at the onset of oligodendrocyte differentiation (Wang et al., 2001).

It is through these various mechanisms, therefore, that Hes and Id family members are believed to function in maintaining neural progenitors in non differentiate state.

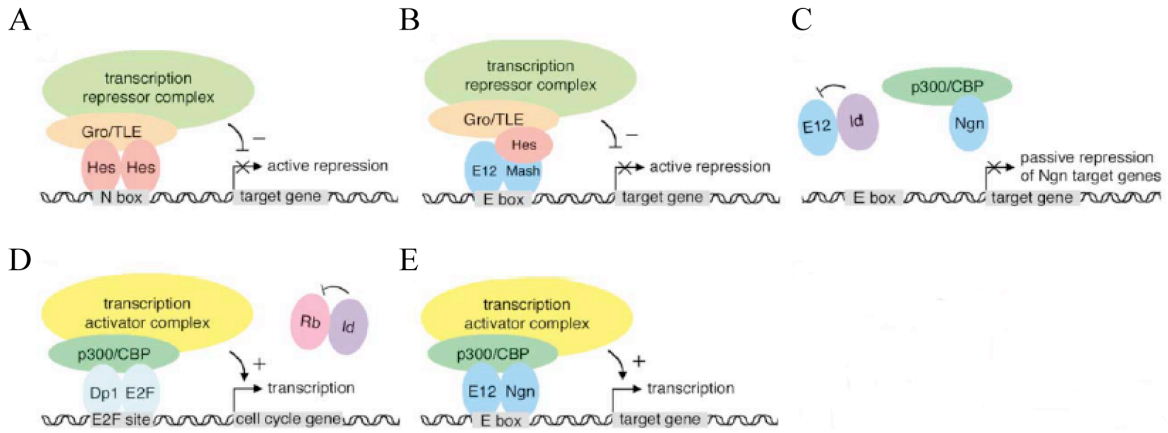


Figure 4 Mechanisms of bHLH factor activity (modified from (Ross et al., 2003)).
(A) Hes factors form homodimers and heterodimers within their class and bind to DNA elements called N boxes (CANNAG) to repress the expression of target genes. Transcriptional repression is mediated by the interaction of Hes proteins with corepressors, such as Groucho/TLE, which recruit large repressor complexes.
(B) Recent work in Drosophila suggest that Hes proteins may inhibit the transcription of genes at which proneural bHLH/e protein dimers are bound through direct interaction with proneural bHLH. Experiments are required to determine whether this mode of repression is conserved in mammals.
(C) Ids inhibit bHLH activity sequestering E proteins away from bHLH factors.
(D) Ids promote proliferation by inhibiting the ability of Rb to interfer with E2F-mediated transactivation of genes that are critical for cell cycle progression.
(E) bHLH dimerize with E proteins and bind to DNA elements called E boxes (CANNTG).

1.3.2 Formation of neurons

The initial phases of cortical development involve the rapid proliferation of cortical progenitors through symmetric division in which one cell gives rise to two identical proliferating daughter cells. At the time of neurogenesis, however, a subset of cortical progenitors becomes restricted to a neuronal lineage. It is likely that this restriction involves asymmetric division in which one cell is maintained as a multipotent cortical progenitor, while the other is fated to differentiate into a neuron within a few rounds of cell division. The transition from proliferation to neurogenesis involves a coordinate increase in proneural bHLH activity and a decrease in Hes and Id activity.

Neurogenesis is mediated by two broad categories of bHLH factors, proneural bHLH factors (Ngns and Mash1), which are involved in initiating neurogenesis, and neuronal differentiation bHLH factors (e.g. NeuroD), which are involved in mediating terminal differentiation. Proneuronal bHLH factors and neuronal differentiation bHLH factors are transcriptional transactivators. These proteins bind DNA as heterodimeric complexes

together with E proteins. The basic domain of these bHLH factors mediates interaction with DNA sequences that contain the core hexanucleotide motif CANNTG, known as the E-box (figure 4E) (Bertrand et al., 2002). Transactivation is mediated by interaction of bHLH heterodimers with coactivators, such as p300/CBP, which recruit a large complex that includes the basal transcriptional machinery.

Three proneural bHLH factors are known to be expressed in the telencephalon, Ngn1, Ngn2, and Mash1(Guillemot and Joyner, 1993; Sommer et al., 1996). During development, these bHLH factors are expressed at low levels while neural progenitors are being specified, and a transient increase in their expression occurs at the initiation of neurogenesis. Proneural bHLH factors are expressed in the ventricular zone, where progenitors begin to be differentiate, but are not expressed in the cortical plate, where fully differentiated neurons are situated. Ngns are expressed in the dorsal telencephalon, which gives rise to glutamatergic neurons, whereas Mash1 is predominantly expressed in the ventral telencephalon, which gives rise to GABAergic and cholinergic neurons, suggesting the possibility that different proneural genes are involved in the specification of different neuronal subtypes (Wilson and Rubenstein, 2000). An important role for proneural bHLH factors in the telencephalic development has been revealed by loss-of-function studies. In the Mash1 knockout mouse, the decrease in proneural bHLH activity causes a loss of progenitor cells in the ventral telencephalon, resulting in loss of GABAergic interneurons in the cortex (Casarosa et al., 1999). In the Ngn2 knockout mouse there is an up regulation of Mash1 found in the dorsal telencephalon, resulting in the differentiation of some progenitors into GABAergic neurons in this region (Fode et al., 2000). The signaling pathways that positively regulate the activity of proneural factors are unknown.

The transient expression of proneural bHLH factors induces a second, sustained wave of bHLH differentiation genes, which mediate the terminal differentiation of neurons (Kageyama and Nakanishi, 1997; Lee, 1997). These differentiation genes are members of the NeuroD/Nex family, and include NeuroD, NeuroD2, NeuroD related factor (NDRF), and Nex (Math2). Like proneural bHLH factors, bHLH differentiation proteins are E box binding transcriptional activators, whose expression begins in the immature neuron and is maintained during neuronal differentiation (Lee et al., 2000). Thus these factors are

expressed in the cortical plate, but not in the ventricular zone (Schwab et al., 1998). Although there is strong evidence from studies of knockout mice that the NeuroD/Nex family of genes is required for the differentiation of glutamatergic neurons in the hippocampus and cerebellum, no phenotypes has been reported in the neocortex for the single knockouts (NeuroD, NeuroD2, or Nex2) or the neuroD/nex1 double knockout (Miyata et al., 1999; Olson et al., 2001; Schwab et al., 2000). These findings suggest that members of the NeuroD family function in a largely redundant manner.

1.4 C/EBP transcription factors

1.4.1 C/EBPs: homology regions

The C/EBP proteins are a subfamily of the basic-region leucine zipper (BR-LZ) transcription factor family. In vertebrates, the family consist of six members namely C/EBP, C/EBP, C/EBP, C/EBP, C/EBP, and C/EBP (Akira et al., 1990; Antonson et al., 1996; Birkenmeier et al., 1989; Cao et al., 1991; Descombes et al., 1990; Poli et al., 1990; Ron and Habener, 1992; Thomassin et al., 1992; Xanthopoulos et al., 1989).

C/EBP proteins have also been found in invertebrates, such as Aplysia and Drosophila, although in these organisms only one C/EBP protein exists (Alberini et al., 1994; Montell et al., 1992).

The proteins of the C/EBP family share features in structure as well as function.

The structural similarities of the C/EBP family members are mostly in the C-terminal BR-LZ domains. The basic region contains the DNA binding specificity of the proteins (Agre et al., 1989; Suckow et al., 1993) but may also have other functions. The LZ is a -helical dimerization domain, and the various C/EBP factors are known to dimerise through this domain with other members of the family, as well as with BR-LZ factor ATF4 of the CREB family (Williams et al., 1991). Early experiments predicted that the only function of the LZ domain was to ensure dimerization of C/EBP factors: in fact, a zipper exchange mutant of C/EBP, containing the LZ domain of the yeast protein GCN4, showed DNA binding ability comparable with wild type C/EBP (Agre et al., 1989). Moreover, this Gz mutant was also able to substitute for the biological function

(embryonic survival) of Drosophila C/EBP (Rorth, 1994). Subsequent experiments have shown that dimerization is likely not the only function of the LZ domain of the C/EBP factors. C/EBP has been shown to display a much lower transactivational ability on the albumin promoter in HeLa cells (a human cervical carcinoma cell lines) compared to HepG2 cells (a rat hepatoma cell line), whereas the Gz mutant is equally active in both cell types. This suggests that the LZ may mediate the cell type specific activity of C/EBP (Nerlov and Ziff, 1994).

The N-terminus of C/EBP proteins is not as conserved between the different isoforms as the C-terminal BR-LZ domains, although there are areas of homology between the N-terminals of C/EBP, , and (Nerlov and Ziff, 1995). In these proteins the N-terminus contains the domains responsible for most, if not all, of the transcriptional activation of the proteins (Friedman and McKnight, 1990; Kowenz-Leutz and Leutz, 1999; Nerlov and Ziff, 1995). For C/EBP, (Nerlov and Ziff, 1995) three transactivation elements have been identified (termed TE-I 1-70aa, TE-II 71-96aa, TEIII 126-200 aa). TE-I and –II contain the main regions of homology between the N-terminal transactivation domains of C/EBP factors. Components of the basal transcription machinery (TBP and TFIIB) have been shown to interact with TE-I and –II via aminoacids conserved between the C/EBP, , . isoforms (Nerlov and Ziff, 1995). These conserved domains also mediate interactions between C/EBPs and pRB/p107 (Chen et al., 1996; Timchenko et al., 1999). The anti-mitotic ability of C/EBP has been proposed to require the N-terminus, as the short form of C/EBP is unable to induce growth arrest (Lin et al., 1993; Umek et al., 1991). C/EBP has long been known as an anti-mitotic protein (Umek et al., 1991). Several experiments have shown that C/EBP expression is able to commit cycling cells to growth arrest in cell cultures (Hendricks-Taylor and Darlington, 1995; Muller et al., 1999; Timchenko et al., 1996; Umek et al., 1991). In C/EBP, knock-out mice, the newborns display increased proliferation both in hepatocytes and in lungs (Flodby et al., 1996; Timchenko et al., 1997). Similar function has not been demonstrated so far for C/EBP.

C/EBP and C/EBP exist as different isoforms. Two isoforms of C/EBP are genetated from its mRNA(Lin et al., 1993; Ossipow et al., 1993). The full length protein of 42kDa and the shorter form of 30kDa. The p30 form acts as a dominant

negative, however it is able to activate some promoters in transient transfections if expressed alone (Lin et al., 1993; Ossipow et al., 1993). Also C/EBP, is found in different forms encoded by the same mRNA. The full-length 35kDa protein, also termed LAP (liver-enriched transcriptional-activator protein), encodes for the conserved activation domain found in other C/EBP proteins, as well as two regulatory domains, RD1 and RD2, which confer DNA binding inhibition in a cell type specific manner (Williams et al., 1995). The truncated form 20 kDa, LIP (liver-enriched transcriptional-inhibitory protein), possess only the DNA-binding and leucine zipper domains (Descombes and Schibler, 1991; Poli et al., 1990). Heterodimerization of the truncated isoform with full length C/EBP, (LAP) attenuates transcriptional activity suggesting a dominant negative mechanism of regulation (Descombes and Schibler, 1991). There is also the form termed LAP* that cointains a conserved N-terminal extension termed CR1. CR1 has been shown to mediate interaction with a vertebrate homologue of the yeast SWI/SNF complex (Kowenz-Leutz and Leutz, 1999). C/EBP, is known to cooperate with Myb to induce transcription of the granulocyte-specific mim-1 gene, Kowenz-Leutz and Leutz show that the N terminus of the full-length C/EBP isoform, which is essential for induction of the mim-1 gene in chromatin, interacts specifically with the SWI/SNF complex. Grafting this domain onto Myb generates a chimeric activator that recruits SWI/SNF and induces mim-1 transcription in the absence of C/EBP. Pedersen et al. (Pedersen et al., 2001) demonstrated also, in adipocyte differentiation, that the C/EBP transactivation element III (TE-III;(Nerlov and Ziff, 1994) is required for adipose conversion, and this region interacts with the SWI/SNF chromatin remodeling complex during adipogenesis.

1.4.2 C/EBPs in neuronal cells

The vertebrate C/EBP transcription factors are expressed in many tissues and have specific functions in a number of specialized cells including granulocytes, adipocytes, hepatocytes, granulose cells, macrophages, mammary epithelium, keratinocytes, neurons and astrocytes (Birkenmeier et al., 1989; Christenson et al., 1999; Kuo et al., 1990; Seagroves et al., 1998; Zhang et al., 1997). Generally, C/EBP, , , and are expressed in differentiated tissue, while C/EBP and have a more ubiquitous expression pattern

(Lekstrom-Himes and Xanthopoulos, 1998). The C/EBP, , , are expressed in the adult nervous system, and in the embryonic telencephalon from E12 to E18, likely in both progenitors and neurons (Menard et al., 2002). C/EBP is widely expressed in the hindbrain, midbrain and forebrain (Sterneck and Johnson, 1998). Astrocytes were reported to express C/EBP in vitro upon exposure to glutamate, norepinephine, or certain neuropeptides (Cardinaux and Magistretti, 1996). In addition, distinct neuronal populations express C/EBP such as cerebellar purkinje cells and pyramidal and granule cells, and possibly inhibitory neurons in the hippocampal formation (Kuo et al., 1990).

The function of the C/EBPs in the mammalian nervous system is not completely clear. In in vitro studies C/EBP has been shown to induce the differentiation of mouse Neuro2A cells through the activation of the phosphatidylinositol 3-kinase pathway. C/EBP also seems to regulate C/EBP gene expression in the same neuroblastoma cells, and this in turn would play a role in the neuronal differentiation of these cells (Cortes-Canteli et al., 2002). Expression of C/EBP has also been reported in the PC12 cell line (Metz and Ziff, 1991), which undergoes differentiation to neuron-like cells in response to nerve growth factor. In transactivation assays, NGF stimulates C/EBP activity and mutation of the phosphorylation site for MAPK in C/EBP (Thr188) affects its capacity to transactivate in a promoter-specific way (Sterneck and Johnson, 1998). Recently the MEK-C/EBP pathway has been shown to have an essential role during growth factor regulated cortical neurogenesis (Menard et al., 2002). Inhibition of either MEK (by using dominant negative) or the C/EBP family of transcription factors (by using a recombinant adenovirus expressing an acidic form of C/EBP (A-C/EBP) that strongly and selectively binds to all C/EBP family members and inhibits their binding to DNA (Greenwel et al., 2000; Krylov et al., 1997) inhibits neurogenesis, while expression of a C/EBP mutant that is a phosphorylation-mimic at a MEK-Rsk site enhances neurogenesis. Conversely inhibition of C/EBP-dependent transcription enhances CNTF-mediated generation of astrocyte from the same progenitor cells

In the invertebrate Aplysia, it has been shown that the transcription factor Aplysia CCAAT enhancer-binding protein (ApC/EBP) plays an essential role in the consolidation of stable long term synaptic plasticity (Alberini et al., 1994). More recent studies in mammals have suggested an implication of C/EBP and C/EBP in long term synaptic

plasticity and memory consolidation in rat hippocampus (Yukawa et al., 1998). Taubenfeld et al., blocked C/EBP by injecting antisense oligodeoxynucleotides into hippocampus of rats, and showed that consolidation but not reconsolidation of inhibitory avoidance memory requires the expression of the transcription factor C/EBP in the hippocampus (Taubenfeld et al., 2001). Sterneck et al demonstrated a selectively enhanced contextual fear response in mice carrying a targeted genomic mutation in C/EBP (Sterneck et al., 1998). To examine the role of C/EBP-related transcription factors in long-term synaptic plasticity and memory storage, Chen et al. have used the tetracycline-regulated system and expressed in the forebrain of mice a broad dominant-negative inhibitor of C/EBP (EGFP-AZIP), which preferentially interacts with several inhibiting isoforms of C/EBP. EGFP-AZIP also reduces the expression of ATF4, a distant member of the C/EBP family of transcription factors that is homologous to the Aplysia memory suppressor gene ApCREB-2. Consistent with the removal of inhibitory constraints on transcription, they find an increase in the pattern of gene transcripts in the hippocampus of EGFP-AZIP transgenic mice and both a reversibly enhanced hippocampal-based spatial memory and LTP. They suggest that several proteins within the C/EBP family including ATF4 (CREB-2) act to constrain long-term synaptic changes and memory formation. Relief of this inhibition lowers the threshold for hippocampal-dependent long-term synaptic potentiation and memory storage in mice (Chen et al., 2003).

Although insights into the functions of C/EBP have been gained from these studies, to better understand the role of C/EBP family members in the central nervous system, it may be necessary to knock-out two or three members at the same time in order to observe the effect without compensation from the other members.

1.5 Neurotrophins signaling

The neurotrophins are a family of secreted proteins that potently regulate diverse neuronal responses. Family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5(NT4/5).

Neurotrophins bind two different classes of transmembrane receptor proteins, the Trks and the neurotrophin receptor p75.

The Trk family of receptor tyrosine kinases derives its name from the oncogene that resulted in its discovery (Barbacid et al., 1991). This oncogene was isolated in gene transfer assays from a carcinoma and, when cloned, was found to consist of the first seven of eight exons of nonmuscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase. Consequently, the proto-oncogene was named tropomyosin-related kinase (trk) and is now commonly referred to as trkA. The trkB and trkC genes were subsequently identified because of their high homology to trkA (Barbacid, 1994; Kaplan et al., 1991; Klein et al., 1991).

The neurotrophins bind the Trks receptor in a specific way. NGF is the preferred ligand for TrkA, BDNF and NT4/5 are preferred for TrkB, and NT3 for TrkC. NT3 is also able to bind with less affinity TrkA and TrkB. Whereas the tyrosine kinase domains are highly related (80% amino acid identity), the extracellular domain are more divergent (figure 5)

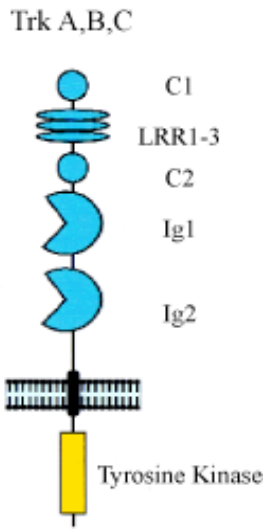


Figure 5 The Trk receptors.
The Trk receptors are transmembrane glycoproteins of ~140 kD. They are tyrosine kinases with an extracellular ligand-binding domain containing multiple repeats of leucine-rich motifs (LRR1-3), two cysteine clusters (C1, C2), two immunoglobulin-like domains (Ig1, Ig2), and a single transmembrane domain. Binding specificity of the Trk receptors is mostly determined by the second Ig-like domain, whereby each Trk receptor binds the corresponding ligand through a distinct specific sequence.

1.5.1 Signal transduction through the Trk receptors

Binding of the neurotrophins to the Trk receptors leads to receptor tyrosine phosphorylation. Ligand-induced dimerization (Jing et al., 1992) results in the phosphorylation of specific tyrosine residues, located in the so called activation loop of the tyrosine kinase domain. Phosphorylation of these residues leads to an open conformation of the receptor, resulting in its trans-phosphorylation, as well as allowing the access of substrates to the kinase. Phosphotyrosine residues on Trk receptors act as docking sites for adapter molecules (figure 6). Two specific phosphorylated tyrosine residues, located in the juxtamembrane domain (Y490 in TrkA, Y515 in TrkB, Y516 in TrkC) and in C-terminus (Y785 in TrkA; Y816 in TrkB), serve as docking sites for adapter molecules (Obermeier et al., 1994; Stephens et al., 1994).

Phosphorylated Y490 (or Y515, and Y516), binds and phosphorylates the adaptor protein Shc via its PTB (phosphotyrosine binding) domain (Dikic et al., 1995). The recent identification of Shc analogs raises the question of recruitment of different Shcs that may be specific for TrkA, TrkB, and TrkC.

Elimination of the shc-site in the trkB (trkB$^{SHC/SHC}$) gene revealed distinct responses of this receptor to BDNF and NT4/5 (Minichiello et al., 1998). NT4/5-dependent sensory neurons were mostly lost in the trkB$^{SHC/SHC}$ mutant mice, whereas BDNF-dependent neurons were only modestly affected. Comparative analysis of trkB$^{SHC/SHC}$ and trkC$^{SHC/SHC}$ mice reveal distinct requirements for the shc-site in TrkB and TrkC signaling in sensory neurons in vivo (Postigo et al., 2002)*. Both receptors promote long-term survival of sensory neurons in an shc site independent manner, however, target innervations of sensory neurons was lost in trkB$^{SHC/SHC}$ mice, whereas target innervations and neuronal function were maintained in trkC$^{SHC/SHC}$ mice. The juxtamembrane tyrosine is also the docking site for another membrane anchored adaptor protein, FRS2 (Kouhara et al., 1997), which is tyrosine phosphorylated in response to NGF and BDNF (Easton et al., 1999).

*Postigo, A., Calella, A. M., Fritzsch, B., Knipper, M., Katz, D., Eilers, A., Schimmang, T., Lewin, G. R., Klein, R., and Minichiello, L. (2002). Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons. Genes Dev *16*, 633-645.

Paper in collaboration during my PhD studies. My contribution was at the level of biochemical analysis in primary cortical neuron, and counts of vestibular neurons.

Phosphorylation of Y785 (or Y816) induces the binding (via SH2 domain) and phosphorylation of phospholipase C-γ (PLC-γ). Furthermore, phosphorylated Y785 also leads to the association of TrkA with CHK (csk homologous kinase), which appears to be involved in NGF-promoted neurite outgrowth via MAPK (Yamashita et al., 1999).

Three main signaling cascades are activated by the Trk receptors and their substrates. First, the activation of the Ras/Raf/MEK/MAPK pathway results from the formation of a variety of complexes of adapter molecules. Phosphorylated Shc leads to the activation of the Ras/Raf/MEK/MAPK pathway (Nakamura et al., 1996). Phospho Shc binds to the Grb2–SOS complex, which activates Ras, and MAP kinase is activated through Raf- and MEK. Similarly, phosphorylated FRS2 recruits a complex of the tyrosine phosphatase SHP-2/Grb2/SOS and activates Ras/Raf/MEK/MAPK (Kouhara et al., 1997) .

Other docking molecules seem to form complexes with Grb2/SOS, such as rAPS and SH2-B (Qian et al., 1998). They were identified in developing neurons and may be involved in neuronal differentiation. This complexity of activation allows a sustained activation of the MAPK pathway in response to neurotrophins, as well as a fine-tuning of the responses.

Second, IRS (insulin receptor substrates IRS-1 and IRS-2) and Gab-1 (Grb-associated binder-1) are adapter proteins that mediate the association and activation of PI3K (Holgado-Madruga et al., 1997), which does not directly interact with the Trk receptors. PI3K is especially implicated in neuronal survival through the activation of PKB/AKT kinase (Crowder and Freeman, 1998), resulting in the phosphorylation and inactivation of the proapoptotic protein BAD of the Bcl-2 family (Datta et al., 1997). PKB/AKT also leads to the phosphorylation of the Forkhead transcription factor, which controls the expression of genes involved in cell death, such as Fas ligand (Brunet et al., 1999). Third, the association of PLCγ with Trk regulates intracellular Ca2+ levels and protein kinase C activity through cleavage of the substrate PIP2 to DAG and IP3. Recent studies have contributed to our understanding of the physiological functions of the PLCγ pathway. This include the stimulation of neurotrophin-mediated neurotrophin release from cells (Canossa et al., 1997), and the regulation of synaptic plasticity (Minichiello et al., 2002)*. The importance of the PLCγ pathway for synaptic plasticity was revealed in vivo by mutating the recruitment site, Y816, to phenylalanine. Mice homozygous for the

Y816F mutation (trkB$^{PLC/PLC}$ mutants) showed deficiencies in the induction of both the early and late phases of hippocampal CA1 long-term potentiation (LTP). Results are similar to those observed in animals in which a floxed trkB allele was deleted in the postnatal forebrain using Cre recombinase expressed under the control of the CaMKII promoter (Minichiello et al., 1999). Surprisingly, while the MAPK pathways have been implicated in late phase hippocampal LTP, BDNF-dependent phosphorylation of MAPK and the distribution of phospho-MAPK appear to be unaffected in cortical neurons of trkB$^{PLC/PLC}$ mutants. In contrast, phosphorylation of CREB, CaMKII, and CaMKIV are severely impaired in these neurons. Interestingly, expression of the zinc finger transcription factor Egr-1 (Krox24, Zif268), which is a downstream target of both Ras-MAPK and CREB signaling and has been shown to be important for hippocampal LTP, is markedly reduced in the hippocampus of trkB$^{PLC/PLC}$ mutants.

1.5.2 Neurotrophins and cortical progenitors

Members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are probable candidates for influencing the cell lineage of neural stem cell. This statement is based on the finding that neurotrophins and their receptors, TrkA, TrkB, TrkC, or p75, are expressed in the ventricular and subventricular zones at the onset of cortical neurogenesis, and in cultured neural stem cells (Lachyankar et al., 1997);(Fukumitsu et al., 1998);(Benoit et al., 2001). Several reports have described the effects of neurotrophins on neural stem cells in vitro. NGF accelerated the proliferation of neural progenitor cells in conjunction with basic fibroblast growth factor-2 (FGF-2) (Cattaneo and McKay, 1990). BDNF enhanced neuronal differentiation of neurosphere forming neural stem cells (Ahmed et al., 1995), and promoted the survival of cortical neurons (Ghosh et al., 1994) and neurons generated from the forebrain subependymal zone (Kirschenbaum and Goldman, 1995).

*Minichiello, L., Calella, A. M., Medina, D. L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron *36*, 121-137.
Paper in collaboration during my PhD studies. My contribution was at the level of biochemical and immunocytochemical analysis of primary cortical neuron, and immunohistochemistry.

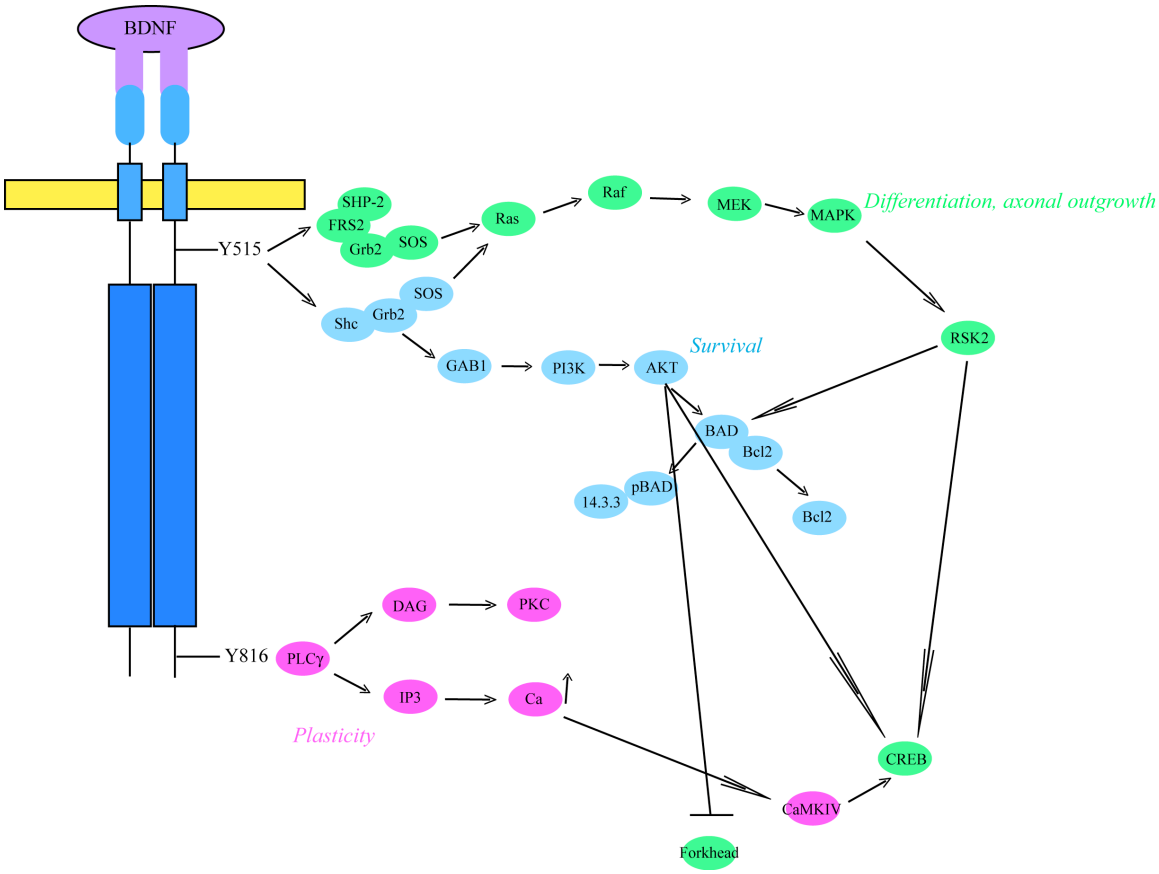


Figure 6 Signaling through the Trk receptors: the main pathways.
Neurotrophin binding to Trk receptors triggers dimerization leading to the activation of different signaling pathways through recruitment of various adapter molecules. The main binding sites for Trk substrates are two tyrosine residues that, on activation of the Trk receptors, become phosphorylated. Two complexes of adapter molecules bind to the tyrosine residue located in the juxtamembrane region of the Trk receptor, the Shc/Grb2/SOS and the FRS2/SHP-2/Grb2/SOS complex. Ras/Raf/MEK/MAPK induces the differentiation of neurons and neurite growth. The PKB/AKT pathway mediates the survival functions of the neurotrophins. The phosphorylated tyrosine in the C terminus recruits PLC□ which catalyses the cleavage of the substrate PIP2 to DAG and IP3, with DAG inducing activation of PKC and IP3 leading to release of calcium from internal stores.

NT-3 has been suggested to regulate cell cycle exit and neuronal differentiation of cortical precursor cells (Ghosh and Greenberg, 1995), and promoted clonal expansion of oligodendroglial precursor cells (Barres et al., 1994). More recently, however, neurotrophins are also believed to participate in neuronal maturation processes, rather than in neuronal fate determination (Takahashi et al., 1999). In addition it has been demonstrated that in cortical progenitor cells BDNF and NT3, acting via TrkB and TrkC respectively, promote survival and neurogenesis through two distinct and separable

signaling pathways: the PI3-kinase pathway mediates survival, and the MEK pathway is important for neurogenesis (Barnabe-Heider and Miller, 2003).

1.6 Proto-oncogene fos: complex but versatile regulation

The selective modulation of gene expression in response to temporal, spatial, and environmental cues underlies a broad range of biological processes in the central nervous system. To a large extent, the regulation of gene expression is mediated by alterations in transcription rates that occur in response to the interaction of trans-acting transcription control proteins (transcription factors) with cis-acting DNA sequence elements.

The fos proto-oncogene (c-fos) has provided a useful experimental tool for the study of gene expression. In the majority of cell types, it is expressed at relatively low levels, but it can be rapidly and transiently induced by a broad range of stimuli.

c-fos is a basic-region leucine-zipper protein, and it is a member of a class of "immediate early" response genes (Almendral et al., 1988). A number of sequence elements within the 5'-flanking region of the gene supply targets for various inducing agents. The serum response element (SRE) has been described as a protein binding site required for the induction of c-fos expression by serum (Treisman, 1985). The SRE is composed of at least two indipendent transcriptional sub-elements: 1) a 20 base pair dyad symmetry element (DSE) positioned from residues –317 to –298 (Greenberg et al., 1987) and 2) a region comprising residues –303 to-281, the c-fos AP-1 site(Velcich and Ziff, 1990), with sequence homology to the consensus binding for the transcription factors AP-1 (Lee et al., 1987) and CREB (Montminy and Bilezikjian, 1987; Montminy et al., 1986). Both the DSE and FAP element respond to a number of extrinsic factors. The DSE mediates induction by serum, a number of polypeptide growth factors, 12-O-tetradacanoyl phorbol-13 acetate (TPA) and cAMP (Gilman, 1988; Gilman et al., 1986; Greenberg et al., 1987; Siegfried and Ziff, 1989; Stumpo et al., 1988). In addition, the DSE has been shown to be a target for autorepression (Konig et al., 1989; Sassone-Corsi et al., 1988a; Schonthal et al., 1989). Oligonucleotides containing FAP element are activated via TPA-, cAMP -, and EGF-induced pathways (Fisch et al., 1987; Fisch et al., 1989a; Fisch et al., 1989b; Siegfried and Ziff, 1990; Velcich and Ziff, 1990) The transcription factor named

serum response factor (SRF) binds to the sequence element ($CC(A/T)_6GG$), termed CArG box (Minty and Kedes, 1986) within the DSE (Rivera and Greenberg, 1990; Treisman, 1986; Treisman, 1987). That binding was found to be necessary, but not sufficient, for serum induction of the SRE (Norman et al., 1988; Treisman, 1987). In vivo footprinting analysis shows that SRF is constitutively bound to the SRE in both quiescent and growth factor stimulated cells (Herrera et al., 1989).This suggests that it is the transcriptional activation of a complex of SRF and its accessory proteins that is regulated rather than regulation of SRF DNA binding. The ternary complex factors (TCFs) are members of the ets family of transcription factors. The TCF family members Elk-1 (Hipskind et al., 1991), SAP-1 (Dalton and Treisman, 1994), and SAP-2/ERP/NET (Giovane et al., 1994; Lopez et al., 1994) have been found to have a role in regulating the SRE. TCFs cannot bind the SRE autonomously, but require protein-protein interactions with SRF in order to bind the SRE (Janknecht and Nordheim, 1992; Shaw et al., 1989; Treisman et al., 1992).The C-terminal of TCF (aa 352-399), contains several consensus mitogen activated protein kinase (MAPK) phosphorylation sites. The TCFs have been found to be targets for all three families of MAPKs: the extracellular signal-regulated kinases 1/2 (ERK1/2), the jun-N-terminal kinases/stress activated protein kinases (JNK/SAPK), and the p38 kinase (Treisman, 1996; Whitmarsh and Davis, 1996). TCFs are stimulated by phosphorylation of the C-terminal MAPK sites (Treisman, 1994; Treisman, 1996; Whitmarsh and Davis, 1996), this mechanism provide the transcriptional activation. It has been also shown that both p35-C/EBP and p20-C/EBP could interact with SRF in vivo and that the interaction between SRF and p35-C/EBP, but not between SRF and p20-C/EBP, is stimulated by activated Ras. The target for this Ras stimulation is Thr188 in a consensus MAPK site in C/EBP (Hanlon and Sealy, 1999).

In PC12 cells it has also been shown that E12 and C/EBP bind to the SRE at sites adjacent to the DSE (Metz and Ziff, 1991), but there is not much known about the possible role for the E-box and the C/EBP binding site in the context of c-fos promoter, one possibility could be a form of tissue specificity control.

Another regulatory element, in the c-fos promoter, is located between –57 to-63 in the human, mouse and chicken c-fos gene and contains the calcium and cyclic (cAMP) response element, it mediates rapid gene induction by elevated intracellular calcium and

cAMP (Fisch et al., 1989b; Sassone-Corsi et al., 1988b; Sheng et al., 1988). The induction of c-fos by calcium has been proposed to occur through phosphorylation of the cAMP response element binding protein (CREB) which binds to this site in vivo (Sheng et al., 1990)

1.7 mGif/TIEG1 transcription factor

The glial cell-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-β (TGFβ) superfamily reviewed in (Kingsley, 1994). Yajima et al. (Yajima et al., 1997) reported a transcription factor that is the first nuclear protein known to be induced by GDNF, thus designated murine GDNF inducible factor (mGif). The cDNA was cloned in the course of investigating transcription factor that bind to SP1 consensus sequences. Sequence analysis indicated that mGIF is homologous to the human TGFβ inducible early gene (TIEG1) and human early growth response gene-α (EGR-α). Subsequently also an other member of the family was cloned and called TIEG2 (Cook et al., 1998).

The human TIEG1 cDNA was originally isolated as a transcript that was rapidly induced by TGFβ in human osteoclasts (Subramaniam et al., 1995) instead EGR-α was cloned from prostate carcinoma cells (Blok et al., 1995). TIEG1 and EGR-α have identical aminoacid sequences except for 12 residues absent in the N-terminus of EGR-α. Further studies demonstrated that the accumulation of TIEG1 cDNA was also regulated by bone morphogenic protein-2 (BMP-2), epidermal growth factor (EGF), and estrogen (Subramaniam et al., 1995; Tau et al., 1998). Instead EGR-α was induced by EGF and repressed by androgens (Blok et al., 1995). The existence of three zinc finger domains in the carboxyl terminus of the predicted proteins led to the hypotesis that TIEG1/ EGR-α were inducible transcription factors with a possible role in mediating the effects of some growth factors on the target cells. In fact the carboxyl-terminal three zinc finger DNA binding domain allows TIEG1 to directly associate with GC-rich sequences of DNA in target gene promoters (Johnsen et al., 2002a; Tanabe et al., 2001; Yajima et al., 1997). The three zinc fingers are highly conserved between TIEG1 and the related transcription factor TIEG2, suggesting that they may regulate the same sequences in target gene

promoters (Cook et al., 1998). However, outside of the DNA binding domain, TIEG1 and TIEG2 bear little sequence homology. Yet both factors are negative regulators of gene transcription and contain an α-helical repression domain (Cook et al., 1999; Johnsen et al., 2002a; Yajima et al., 1997; Zhang et al., 2001). This domain is capable of repressing gene transcription when fused to a heterologous DNA binding domain and can directly interact with the transcriptional co-repressor mSin3A (Cook et al., 1999; Zhang et al., 2001)

The protein sequence of human TIEG1 is 88% identical to the murine mGif. The sequence of mouse TIEG1 (mTIEG1) is identical to that of mGif. However, previous studies of mGif expression and mTIEG1 revealed a different tissue distribution pattern (Fautsch et al., 1998; Yajima et al., 1997) that might be due to a different mouse strain used for the two analysis. So we can assume that mGif and TIEG/TIEG1 are the same transcription factor.

The mouse TIEG1 gene spans 7 kb and contains 4 exons that code for mTIEG1 (Fautsch et al., 1998). Exon 1a is 149 bp long and contains a 5' untraslated region that is 9 bp shorter than that seen in human TIEG1. Exon 2 is 230 bp long and is separated from exon3 by a small 94-bp intron. Exon 3 is 913 bp long and contains two of the three zinc-finger motifs. Exon 3 and 4 are separated by 450 bp intron. Exon 4 contains the third zinc-finger motif and the long 3' untraslated region. In the human TIEG1 gene, an additional exon was found 3' of the first TIEG1 exon. This exon, designated 1b was found to code for the 5' end of EGR-β (Blok et al., 1995). Comparison of the human EGR-β specific exon to the entire span of the mouse gene did not reveal the high sequence conservation seen with other exons from the TIEG gene (Fautsch et al., 1998). However, the mouse TIEG1 gene contains an exon 3' of the first TIEG1 exon (called 1c) that codes for the 5' end of an EST isolated from a cDNA library obtained from liver. But the meaning of this finding is not well known. Although little sequence similarity can be found between most of the introns of the mouse and human TIEG1 genes, an 87% identity can be found over a 650 bp region immediately 5' of human EGR-β specific exon 1b and sequence conservation exist in the region upstream of exon 1a. As with the human TIEG1 gene, no consensus TATA box sequences are found in the 5' upstream

region of the mouse gene. In addition the position of diverse SP1 binding sites, E-box, CTF/JunB, AP2 sites are conserved.

Yajima et al identify different post-translationally modified sites in mGif/TIEG1 that could be involved in signal transduction pathways modulating the expression of various genes. In particular they found 10 Pro-X-X-Pro sequences, which are putative src homology 3 (SH3) binding motifs (Yu et al., 1994) and 16 Ser/Thr-Pro sequences, which may be targets for proline-directed kinases. Among these Ser/Thr-Pro sequences, six are putative target sites for MAPK (Alvarez et al., 1991) two are for cyclin-dependent protein kinase (CDK) (Moodie et al., 1993) and one is for glycogen synthase kinase (GSK) (Pelech, 1995).

About the function of mGif/TIEG1, at beginning it was characterized as a negative-acting transcription factor that can be induced by GDNF in murine neuroblastoma cells (Yajima et al., 1997). After TIEG1 was shown to play a unique role in TGF/Smad signaling by down-regulating negative feedback through Smad7. By repressing Smad7 gene transcription, TIEG1 is able to enhance transcription of important TGF-regulated genes such as the cyclin-dependent protein kinase inhibitor p21 and the plasminogen activator inhibitor-1 (PAI-1) (Johnsen et al., 2002a). Johnsen et al (Johnsen et al., 2002b) described also the identification of an E3 ubiquitin ligase, Seven in Absentia homologue-1 (SIAH1), as a TIEG1-interacting protein. They showed that TIEG1 and SIAH1 interact through an amino-terminal domain of TIEG1. Co-expression of SIAH1 results in proteasomal degradation of TIEG1 but not of the related factor TIEG2. Importantly, co-expression of SIAH1 completely reversed repression of Smad7 promoter activity by TIEG1. Furthermore, overexpression of a dominant negative SIAH1 stabilizes TIEG1 and synergizes with TIEG1 to enhance TGF□/Smad-dependent transcriptional activation. These findings suggest a novel mechanism whereby the ability of TGF□ to modulate gene transcription may be regulated by proteasomal degradation of the downstream effector TIEG1 through the SIAH pathway. Overexpression of TIEG1 mimics TGF□ action in several cell types by modulating differentiation markers, decreasing proliferation, and inducing apoptosis (Chalaux et al., 1999; Hefferan et al., 2000; Ribeiro et al., 1999; Tachibana et al., 1997). Accumulating in vitro and clinical data in the literature suggests that TIEG1 serves a tumor suppressor role because overexpression

reduces proliferation and induces apoptosis(Chalaux et al., 1999; Hefferan et al., 2000; Ribeiro et al., 1999; Tachibana et al., 1997). Further support for this role arose from studies demonstrating a decrease in TIEG1 protein levels that coincides with the increasing stages of cancer in breast tumor biopsies (Subramaniam et al., 1998)

In vivo analysis revealed that mGif/TIEG1 mRNA in the adult mouse is present in heart, lung, brain, liver, kidney, and testis but not in spleen or skeletal muscle (Yajima et al., 1997). The distribution of mGif/TIEG1 mRNA was also examined in the adult mouse brain by in situ hibrydization and it was found in several region particularly in hippocampus, cerebral cortex, amygdala, and cerebellum, with weaker signals in striatum, substancia nigra, and thalamus (Yajima et al., 1997). The expression pattern reassemble for some aspects that from TGF-β and GDNF (Yajima et al., 1997). Also during embryogenesis at E16.5 mGif/TIEG1 is widely expressed in kidney and intestine, including the enteric nervous system, consistent with the fact that GDNF-null mutant mice lack kidneys and enteric neurons (Moore et al., 1996; Schuchardt et al., 1994; Schuchardt et al., 1996) and that GDNF mutations are associated with the human intestinal disorder Hirschsprung (Angrist et al., 1996; Ivanchuk et al., 1996). During embryogenesis mGif it also expressed in cerebral cortex and cerebellar primordial and the dorsal hedge of spinal cord.

It would be interesting to have mGif-null allele mice to better understand the function of this transcription factor in vivo.

1.8 The Thesis Project

Evidence suggest that neurotrophins, in particular NGF, BDNF and NT3, may play a role in regulating cortical progenitor cell biology, but their precise role for this biological function is still unclear, as are the signaling mechanisms.

During my PhD I have focused my interest in understanding which set of transcription factors mediate the functions of BDNF in cortical formation.

In order to answer this question, I adopted the following approaches. First, I analyzed global changes in gene expression after BDNF/TrkB activation in primary cortical neurons, using microarray technology. Second, once a set of regulated genes was

identified, I characterized the regulation of these genes at the promoter level, in order to understand which common elements are important for their regulation. This information has contributed to our understanding of which transcription factors mediate BDNF/TrkB-dependent gene expression in primary cortical neurons isolated at the peak of neurogenesis.

2

RESULTS

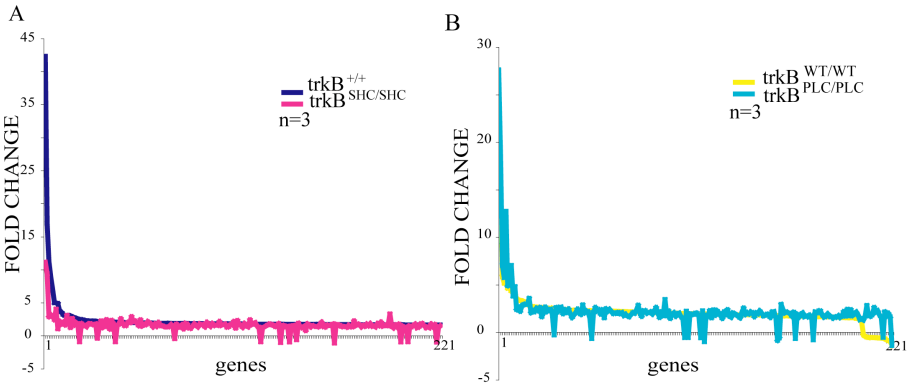
2.1 Differential regulation of immediate early genes induced by BDNF through the shc-site or the plc-γ site of TrkB receptor.

To examine the global changes in gene expression in response to BDNF/TrkB activation in cortical neurons at the peak of neurogenesis, we performed an Affymetrix analysis. The RNA used was obtained from cortical neurons isolated from E15.5 wild-type mouse embryos. The cells were cultured for 24 hours and then stimulated for 1 hour with 50 ng/ml of BDNF in the absence of serum. The mRNA was then used as a probe to screen oligonucleotide arrays (Affymetrix, Inc. UV74v2), which contained approximately 6000 annotated genes and 6000 EST sequences. The microarray analysis was repeated three times with three different mRNA preparations and gave very similar results. 1.6% of the genes present on the array showed at least two fold changes in expression and fell into diverse classes: transcription factors, metabolic enzymes, kinases and unknown genes.

Having identified a group of regulated immediate early genes (IEGs), we sought to assess the contribution of individual cytoplasmatic tyrosines of the TrkB receptor on the induction of these IEGs. For this purpose we analyzed mutants of TrkB carrying Y-F point mutations. From previous studies using PC12 cells it was established that the most important tyrosines, mediating TrkB signaling are: Y515, in the juxtamembrane region of the TrkB receptor, and Y816, in the C-terminal region. To investigate how the identified IEGs are regulated by the mutant TrkB receptors, we obtained mRNA from primary cortical neurons of E15.5 mouse embryos of $trkB^{SHC/SHC}$, $TrkB^{PLC/PLC}$, and their respective controls, $trkB^{+/+}$ or $trkB^{WT/WT}$ embryos, and screened an Affymetrix array. Moreover, we also tested mRNA from cortical neurons of embryos carrying a double point mutation (shc-site, plc-γ site) in the TrkB receptor.

As shown in the figure 7 (A-B) the selected genes, which were induced 2 fold in the control cells following BDNF stimulation, were down regulated in $trkB^{SHC/SHC}$ mutants and up regulated in the $trkB^{PLC/PLC}$ mutants compared to controls.

These results suggest that the pathway/s activated through the shc-site promote a higher activation of gene expression in response to BDNF than pathways activated through the plc-γ site.



C

gene	*trkB*$^{+/+}$	*trkB*$^{SHC/SHC}$	*trkB*$^{WT/WT}$	*trkB*$^{PLC/PLC}$	*TrkB*$^{D/D}$
egr1	42.16	10.99	17.22	27.12	10
c-fos	16.81	9.24	13.87	18.31	5.4
egr2	11.03	2.77	4.72	12.25	4.5
mGif	8.73	3.06	4.73	5.03	2.6

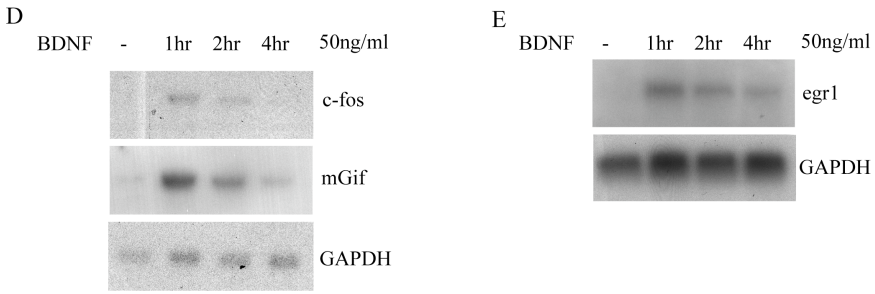


Figure 7 Identification of immediate early genes induced by BDNF at peak of neurogenesis by Affymetrix analysis.
(A-B) Genes up-regulated upon two fold induction in trkB$^{SHC/SHC}$ mutants compared to trkB$^{+/+}$ (A) and in the trkB$^{PLC/PLC}$ mutants compared to its own control trkB$^{WT/WT}$ (B). The mRNA for the Affimetrix analysis was obtained from E15.5 primary cortical neurons from the different mouse strains, the neurons were either left unstimulated or stimulated with BDNF for 1 hr. The fold change was averaged from three independent stimulated samples as compared to three independent unstimulated samples. n= number of samples for each group. The BDNF-induction of genes is reduced in trkB$^{SHC/SHC}$ mutant mice and increased in the trkB$^{PLC/PLC}$ compared to their controls.
(C) Fold inductions for the most highly regulated transcription factors in the different signaling point mutant mice.
(D-E) BDNF induces c-fos, mGif, and Egr1 mRNA. Total mRNA from wild type primary cortical neurons, either untreated or treated with BDNF at different time points, was analyzed on Northern blots using cDNA specific for c-fos, (D upper panel). Blots were stripped and reprobed with cDNA for mGif (D middle panel) or GAPDH (D lower panel) to evaluate loading. A new blot was used to check Egr1 expression (E upper panel), the blot was stripped and reprobed with cDNA for GAPDH (E lower panel) to evaluate loading. The experiment was repeated three times. Similar kinetics of transcription were observed for the different genes with peak expression occurring 1 hr after BDNF stimulation

2.2 A group of transcription factors show the same activation pattern

To identify genes with a similar expression pattern after BDNF induction, we used the GeneSpring software to analyze the Affymetrix data. Using a self-organizing map (SOM) the genes were clustered into 20 groups. Within the same group, using the correlation factor of 0.9, we found genes that shared a statistically similar expression pattern. Among these genes, the ones with the highest fold inductions were found to be the transcription factors: c-fos, Egr1, egr2, and mGif.

As shown in the figure 7C, these genes were found to be differentially regulated in the signaling point mutant mice, specifically they were down-regulated in trkB/shc point mutants, and up-regulated in the trkB/plc-γ point mutants. In the double mutants they were down regulated with the exception of egr2. The expression of this gene was down regulated in the absence of the shc-site, up regulated in the absence of plcγ-site, whereas in the absence of both sites its expression level did not change compared to knock in controls.

As shown in the figure 7(D-E), northern blot analysis confirmed that the mRNA induction of c-fos, Egr1 and mGif displayed similar kinetics, with a peak in expression occurring after 1 hour of BDNF stimulation.

Therefore, BDNF treatment of cortical neurons at the peak of neurogenesis results in the induction of c-fos, Egr1 and mGif expression; each showing the same kinetics of activation, as well as the same mode of regulation as revealed using the signaling point mutant mice.

2.3 Mutations at C/EBP and E-box sites on the c-fos promoter decrease its activity

Having identified c-fos, Egr1 and mGif as a group of transcription factors whose expression is similarly regulated by TrkB/BDNF during neurogenesis, we first looked at their promoters to determine if they shared common elements that could explain their similar regulation. Although the promoter of mGif/TIEG1 is not yet characterized, and the function of this gene is not completely clear (Yajima et al., 1997) (Fautsch et al., 1998), those of Egr1 and c-fos are well characterized, and several data suggest that these

genes share cis acting 5' regulatory elements (Janssen-Timmen et al., 1989; Sukhatme et al., 1988). These elements include SRE, which binds the SRF complex, a binding site for C/EBP, an E-box, which binds bHLH family members, and an AP1 site. Given the similarity of the promoters, we choose the c-fos promoter as a model (figure 8), to better understand which elements and transcription factors are important for BDNF-dependent gene expression. This question was addressed using E15.5 cortical neurons transfected with a wild-type c-fos promoter luciferase reporter plasmid, and examining the activation of this promoter following BDNF stimulation.

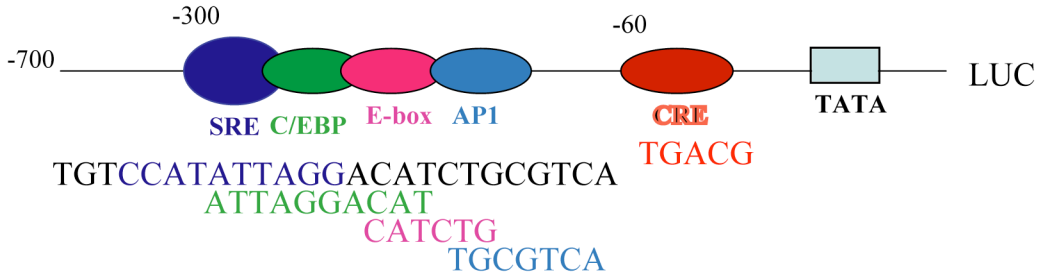


Figure 8 The c-fos promoter. Schematic representation of the proximal (-700 bp) c-fos promoter showing the location and sequence of the major responsive elements in different colors.

We initially performed a time course of luciferase activity following 50 ng/ml of BDNF stimulation in wild-type cortical neurons to determine the maximum luciferase activity, which was found to be between two and four hours (figure 9A). Given these results, we performed the subsequent experiments using two hours of BDNF induction.

At this point we wanted to know how the point mutations of tyrosine 515 and 816 in TrkB receptors affected the activation of the c-fos promoter. Using luciferase reporter gene constructs transfected in E15.5 cortical neurons isolated from wild-type and signaling point mutant mice, we discovered that the two sites (Y515, Y816) could compensate for each other, although the pathways activated through the shc-site promoted higher activation of c-fos promoter than pathways activated through the plcγ-site, and the two sites are both required for BDNF-dependent activation of the c-fos promoter ($trkB^{PLC/PLC}$ and $trkB^{D/D}$ $p<0.01$) (figure 9B).

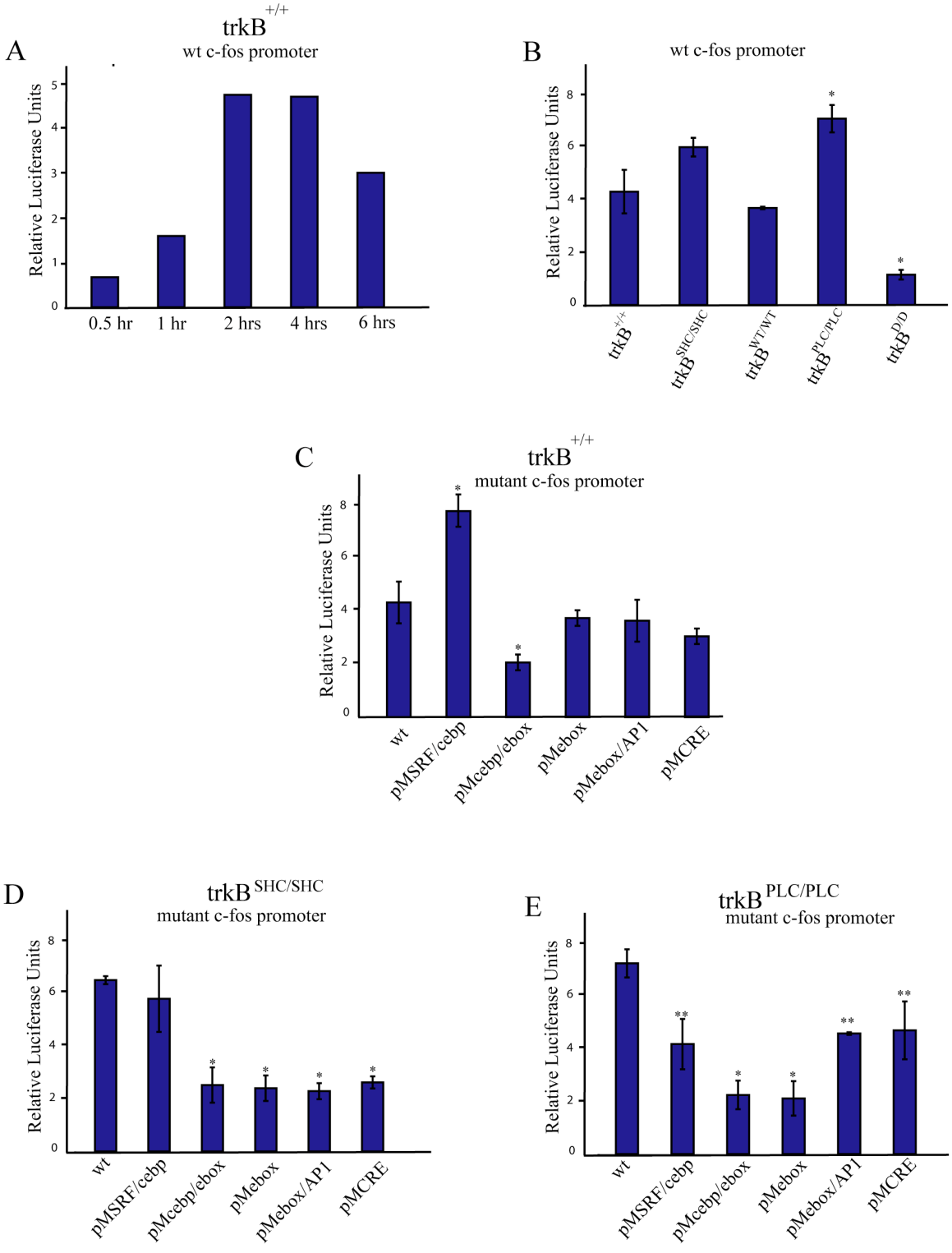


Figure 9. Analysis of transcriptional activation of the c-fos promoter after BDNF treatment of primary cortical neurons (next page).

(A)Time course of luciferase activity following BDNF stimulation of wild-type cortical neurons.
E15.5 plus 4DIV primary cortical neurons from wild type mice were transfected with 2.3 μg of c-fos promoter luciferase reporter plasmid and 3μg of pRSV-β-gal. Twenty-four hours after transfection, cells were left unstimulated or stimulated with 50ng/ml BDNF at different time points. Relative luciferase unit was defined as the ratio of β-galactosidase normalized luciferase activity from stimulated cells to the normalized luciferase activity from unstimulated cells. Maximal luciferase activity was between two and four hours.
(B) Point mutations of both tyrosines 515 and 816 in TrkB receptors reduce the activation of the c-fos promoter.
E15.5 plus 4DIV primary cortical neurons from controls or different trkB mutants were transfected as described in (A). Luciferase activity was measured after two hours of BDNF stimulation. Data are the average of three independent experiments. The results are indicate as the mean ± standard error *$p<0.01$. It is interesting and statistically significant that the pathways activated through the shc-site promoted higher activation of c-fos promoter than pathways activated through the plcγ-site. The two sites are required for BDNF-dependent activation of the c-fos promoter since with a double mutantions the promoter was minimally activated.
(C) The C/EBP binding site and E-box are fundamental for the activation of the c-fos promoter downstream BDNF/TrkB.
E15.5 plus 4DIV primary cortical neurons from wild-type mice were transfected with 2.3 μg of wild-type or mutated c-fos promoter luciferase reporter plasmids, and 3μg of pRSV-β-gal. Data are the average of three independent experiments, and results are indicated as the mean ± standard error *$p<0.005$. Cells transfected with a c-fos promoter mutated in both the C/EBP binding sites and E-box (pMcebp/ebox) showed a significant decrease in luciferase activity. Instead, cortical neurons transfected with a plasmid mutated in sre element together with the binding site for C/EBPs (pMSRF/cebp) revealed a significant increment in the activation of the promoter. No significant differences in luciferase activity were observed between the wild type c-fos promoter and the c-fos promoters mutated at the E-box alone (pMebox), E-box cassette and AP1 binding sites (pMebox\AP1) or CRE (pMCRE), in response to BDNF.
(D-E) Combining TrkB receptor mutation at the shc-site or at the plcγ-site with c-fos promoter elements mutations decrease dramatically the activity of c-fos promoter.
E15.5 plus 4DIV primary cortical neurons from $trkB^{SHC/SHC}$ (D) and $trkB^{PLC/PLC}$ (E) mutants were transfected as described in (C). Data are the average of three independent experiments, and the results are indicate as mean ± standard error *$p<0.001$, **$p<0.01$. In the presence of point mutant TrkB receptors all the sites on the c-fos promoter are important for its activation, except in the absence of both sre element and C/EBPs binding sites (pMSRF/cebp) in the $trkB^{SHC/SHC}$ mutants (D).

After examining the effect of mutations in the TrkB receptor on c-fos promoter activity, we analyzed the different elements in the promoter responsible for the activation downstream BDNF/TrkB. We used luciferase reporter gene constructs containing either the entire wild-type promoter region, up to nucleotide –700, or a series of mutations that progressively eliminated the known binding sites upstream of the transcriptional start site. These mutations included: (i) no sre element combined with the absence of the C/EBP binding site (pMSRF/cebp), (ii) absence of the C/EBP binding site and E-box (pMcebp/ebox), (iii) absence of E-box alone (pMebox), (iv) absence of E-box and AP1 site (pMebox/AP1), and (v) absence of CRE (pMCRE). Some of these mutants were provided from C.Nerlov (personal communication) and, the inserted mutations are indicated:

i. pMSRF/cebp: tgtccatcggaggacatctgcgtcagcaggtttccacggcc
ii. pMcebp/ebox: tgtccatattaggaacgctgcgtcagcaggtttccacggcc
iii. pMebox/AP1: tgtccatattaggacatccatggcagcaggtttccacggcc
iv. pMebox: tgtccatattaggaaatctgcgtcagcaggtttccacggcc
v. pMCRE: ccgtggttgagcccgtgatgtttacactcattcat

As shown in the figure 9C, in the presence of wild type receptors, the C/EBP binding site together with the E-box are fundamental for the activation of the c-fos promoter downstream BDNF/TrkB (pMcebp/ebox $p<0.005$). Instead, the absence of the sre element and the C/EBP binding site induced a higher activation of the promoter (pMSRF/cebp $p<0.005$). Possibly the absence of the SRF complex makes the promoter more accessible to other transcription factors.

To better understand if the activation of a putative complex binding the C/EBP binding site and E-box was downstream the shc-site or the plcγ-site, we transfected the same mutant constructs in E15.5 cortical neurons derived from point mutant mice. As shown in the figure 9D-E, in the absence of the E-box and C/EBP binding sites the c-fos promoter was no longer activated by BDNF in neurons from either $trkB^{SHC/SHC}$ ($p<0.001$) or $trkB^{PLC/PLC}$ ($p<0.001$) point mutant mice. From the same figure it is clear that when the receptor is mutated on either the shc-site or the plcγ-site, all the elements in the c-fos promoter become important for its activation (in $trkB^{SHC/SHC}$ pMebox, pMebox/AP1, pMCRE $p<0.001$, in $trkB^{PLC/PLC}$ pMSRF/cebp, pMebox/AP1, pMCRE $p<0.01$, pMebox

$p<0.001$), except in the absence of both the sre and C/EBP binding sites in the $trkB^{SHC/SHC}$ neurons, where there is no change in promoter activity in response to BDNF. Taken together these results suggest that wild-type TrkB receptor is capable of activating the c-fos promoter carrying mutations in its different elements, except in the absence of both the C/EBP binding site and the E-box. On the contrary, a receptor mutated on either the shc-site or plc-site is not able to activate the c-fos promoter carrying mutations in different elements.

2.4 Expression of C/EBP and , Mash1 and NeuroD peaks at the same time in cortical neurons in vitro

The promoter analysis of c-fos suggested that C/EBPs and bHLH transcription factors might collaborate to induce the activation of the c-fos promoter, or other promoters with similar features, downstream of BDNF/TrkB during the peak of cortical neurogenesis. So we examined cortical progenitors, isolated from E12-E13 embryos and cultured in the presence of FGF2 (Gloster et al., 1999; Slack et al., 1998; Toma et al., 2000), for the expression of C/EBP and , and bHLH transcription factors like Mash1 and NeuroD. At this stage and in these conditions, the majority of the cells are proliferating, nestin-positive progenitors, many of which exit the cell cycle and express the panneuronal markers III-tubulin, neuron-specific enolase (NSE), MAP-2, neurofilament-M, and NeuN.

As shown in figure 10, RT-PCR analysis demonstrated that C/EBP and C/EBP were already expressed in neurons after 2 DIV, and showed a peak in expression after 4DIV. Mash1, an early differention member of the bHLH family, showed the same expression pattern as C/EBPs, while NeuroD, a late differentiation factor, appeared only after 4 DIV, and remained highly expressed even after 6DIV. Thus, after 4DIV, we obtain a population of cortical neurons that express C/EBP, C/EBP, Mash1 and NeuroD.

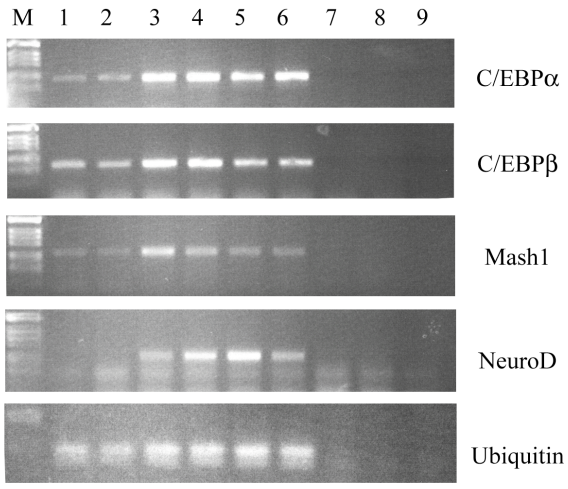


Figure 10. Expression analysis of C/EBP, α and β, Mash1 and NeuroD by RT-PCR in cortical neurons. Primary cortical progenitor cells were put in culture at E12.5 and harvested for total mRNA after 2 DIV, 4DIV and 6DIV. As a control cells were analyzed for ubiquitin levels of mRNA. Lanes 1 and 2 are two different preparations of total mRNA from E12.5 cells after 2DIV, lanes 3 and 4 comes from E12.5 cells after 4DIV, lanes 5 and 6 comes from E12.5 cells after 6DIV, lanes 7 and 8 are the same RNA of lines 5 and 6 without RT, lane 9 is water. Peak expression of C/EBPα, C/EBPβ, Mash1 and NeuroD occures after 4 DIV.

2.5 In vivo and in vitro interaction of C/EBPβ and δ with Mash1 and NeuroD

We next asked if C/EBPβ and δ interacted with Mash1 and NeuroD at the time of the peak of neurogenesis, and if this interaction was BDNF-dependent.

We performed biochemical analysis on primary cortical neurons, derived from wild-type animals, either left unstimulated and stimulated with BDNF for 1 hour. Immunoprecipitation assays showed that C/EBPβ binds Mash1 and NeuroD, and that binding is BDNF-independent (figure 11A-C). We also found that C/EBPδ binds NeuroD in a BDNF-independent manner (figure 11D), and, like C/EBPβ, is able to bind Mash1 (data not shown).

Because C/EBPβ and C/EBPδ have high homology in both the N-terminal and C-terminal regions, we wanted to identify the domains on C/EBPβ and δ responsible for these interactions. We performed an in vitro translation assay to label Mash1 or NeuroD, followed by pull-down analysis using GST fused to the N-terminal region of C/EBPβ,

the C-terminal region of C/EBP, or the C-terminal region of C/EBP. As shown in the figure 12A-B, NeuroD bound the C-terminal region of both C/EBP and , and not their N-terminal region. Conversely, Mash1 bound to the N-terminal region of C/EBP but no binding was revealed in the presence of the C-terminal region of C/EBP and . To verify the specificity of the binding we performed similar GST-pulldown experiments using the same amount of in vitro translated Myogenin (another member of bHLH family). As shown in figure 12C, there was no specific interaction between Myogenin and the different domains of C/EBP and . These results indicate that Mash1 interacts specifically with the N-terminal region of C/EBP, whereas NeuroD interacts with the C-terminal region of C/EBP and .

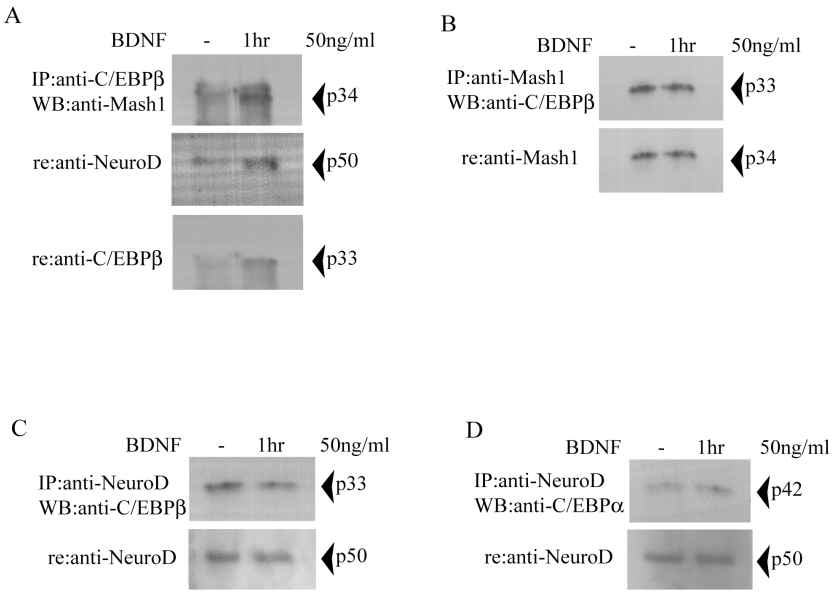


Figure 11 C/EBP and C/EBP bind Mash1 and NeuroD in BDNF-independent manner in vivo.
(A-C) BDNF-independent binding of C/EBP with Mash1 and NeuroD.
Cortical neurons derived from wild-type animals were either left unstimulated or stimulated with BDNF for 1 hour. Nuclear extracts were immunoprecipitated with anti-C/EBP antibody followed by immunoblotting with anti-Mash1 antibody. The same blot was reprobed with anti-NeuroD and then with anti-C/EBP to visualize protein level (A). Nuclear extracts were immunoprecipitated with anti-Mash1 antibody followed by immunoblotting with anti-C/EBP. The blot was reprobed with anti-Mash1 to visualize protein levels (B). Nuclear extracts were immunoprecipitated with anti-NeuroD antibody followed by immunoblotting with anti-C/EBP. The blot was reprobed with anti-NeuroD to visualize protein levels(C).
(D) BDNF-independent binding of C/EBP with NeuroD.
Nuclear extracts were immunoprecipitated with anti-NeuroD antibody followed by immunoblotting with anti-C/EBP. The blot was reprobed with anti-NeuroD to visualize protein levels.

Since the C-terminal region of C/EBPs includes both a basic region and a leucine zipper, we asked where the interaction with NeuroD occurs.

We used different mutants of C/EBPα (provided by C. Nerlov) (Porse et al., 2001) (figure 12D) containing mutated residues in the basic region that, based on molecular models of the C/EBPα protein derived from the crystal structures of the related c-Fos, c-Jun, and GCN4 proteins, are predicted to reside either on the non-DNA binding face of the basic region α-helix (BRM1, -2, and –3), or beyond the residues involved in DNA contact in known bZIP structures, but which are highly and specifically conserved in C/EBP proteins (BRM5) (figure 12D). Immunoprecipitation analysis, in the presence of labeled NeuroD and unlabeled wild-type C/EBPα or BRM mutants, revealed that BRM1 and BRM3, but not BRM2 and BRM5, were impaired in their ability to bind NeuroD (figure 12E-F).

Moreover, replacement of the C/EBPα leucine zipper with the GCN4 leucine zipper had no effect on the binding with NeuroD (figure 12E-F).

Taken together, our data indicate that Mash1 and NeuroD interact with C/EBPα and β in a BDNF-independent manner, and that these interactions involve different domains of C/EBPs: the N-terminal region for Mash1, and the C-terminal region for NeuroD. Furthermore, our analysis reveals that the binding of NeuroD is impaired by specific mutations in the non-DNA binding face of the basic region α-helix of C/EBPα.

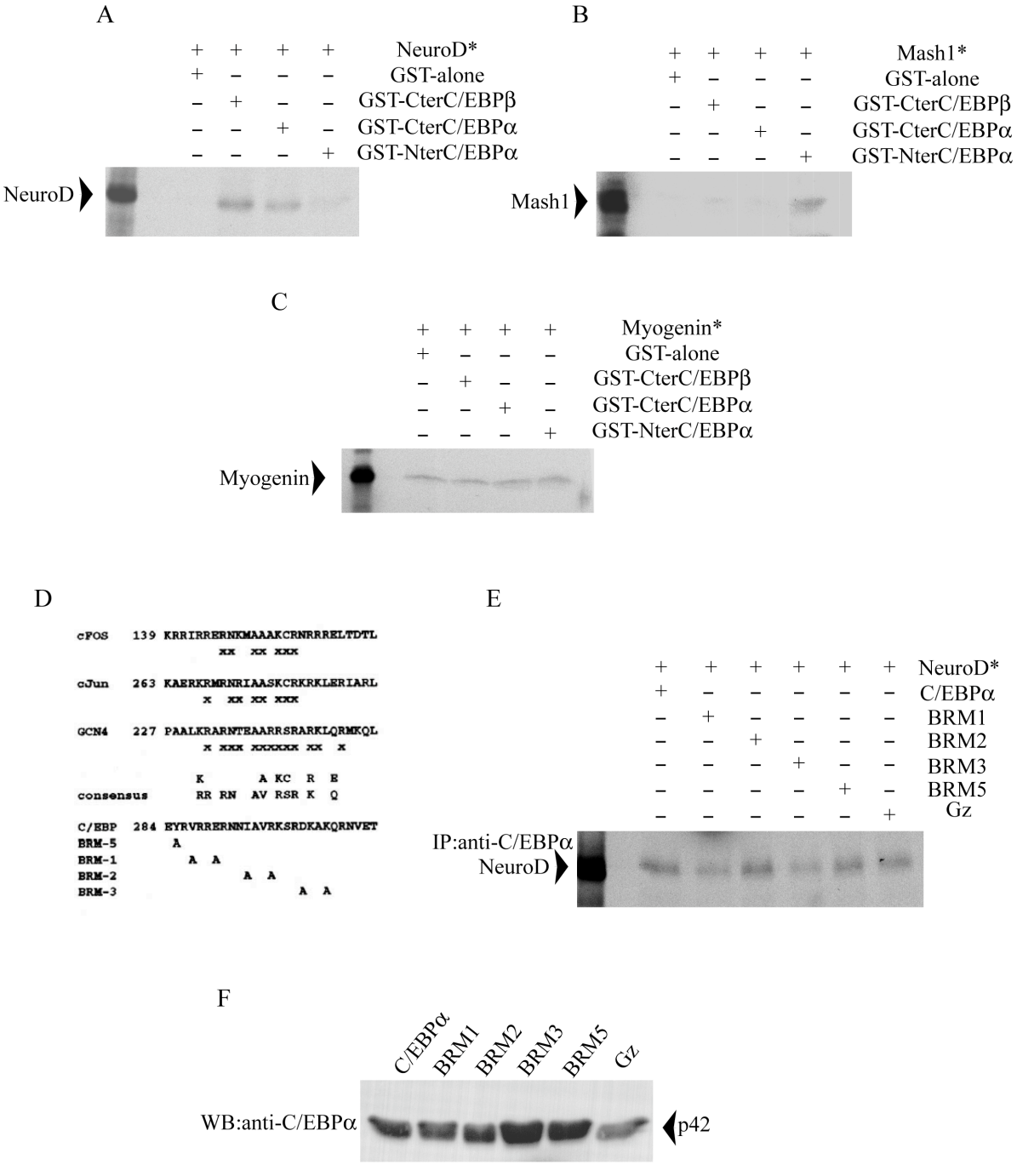


Figure 12 Mash1 and NeuroD interact with different domains of C/EBPs
(next page)

(A) Interaction of NeuroD with C-terminal region of C/EBP and . In vitro translated NeuroD (5 l; 500000 cpm) was subjected to a pull-down with the indicated GST fusion proteins. After washing, bound proteins were eluated and run on 12.5% SDS gel. Autoradiography revealed that NeuroD binds the C-terminal region of C/EBP and (GST-CterC/EBP, GST-CterC/EBP) and not the N-terminal region (GST-NterC/EBP).

(B) Interaction of Mash1 with N-terminal region of C/EBP.

Similar analysis as shown in (A) was carried out using the same amount of in vitro translated Mash1. Radioactive signals revealed that Mash1 binds the N-terminal region of C/EBP (GST-NterC/EBP) and not the C-terminal region of C/EBP and (GST-CterC/EBP, GST-CterC/EBP).

(C) Lack of interaction between Myogenin and diverse domains of C/EBP and .

Similar analysis as shown in (A) was carried out using the same amount of in vitro translated Myogenin as negative control.

(D) Alignment of the c-Fos, cJun, GCN4, and C/EBP basic regions. For the former three, the residues implicated in DNA binding by X-ray crystallography (Protein Data Bank accession no. 1FOS and 1YSA) are indicated below the sequence. Those positions where one or two amino acid residues account for all four proteins are shown as a consensus sequence. The positions of the basic region mutations introduced into C/EBP are indicated below the C/EBP sequence.

(E-F) Specific mutations in the basic region of C/EBPs affect the binding with NeuroD.

The same amount of in vitro translated NeuroD (5 l; 500000 cpm) was incubated with the indicated translated in vitro C/EBP proteins (wild-type and mutants). The mixtures were immunoprecipitated with anti-C/EBP antibody followed by autoradiography. The binding was impaired only in presence of BRM1 and BRM3 mutants. The similar amounts of unlabeled proteins were revealed by western blot using an antibody against anti-C/EBP (E).

2.6 C/EBPs, Mash1 and NeuroD are constitutively bound at c-fos promoter

Given that the interaction, between C/EBPβ, C/EBPδ, Mash1 and NeuroD appears to be BDNF independent, we wanted to determine if the preformed complexes were also present on the c-fos promoter independent of BDNF stimulation. UV-crosslinking assays using nuclear extracts from cortical neurons of wild-type mice, either left unstimulated or stimulated with BDNF for 1 hour, revealed the presence of Mash1 and NeuroD on the c-fos promoter in the absence of BDNF (figure 13 A).

The occupancy of the c-fos promoter by C/EBPβ and δ, Mash1 and NeuroD was further confirmed using chromatin immunoprecipitation. Soluble chromatin was prepared from entire forebrain of wild-type mice, and immunoprecipitated with the antibodies against C/EBPβ, C/EBPδ, Mash1 or NeuroD. We detected an association between all these proteins and the c-fos promoter. Amplified products were also detected in parallel immunoprecipitations performed in the presence of the antibody anti Histone-3 and its acetylated form (figure 13B), but no products were detected in the absence of antibody (figure 13B). These results suggest that Mash1, NeuroD, and either C/EBPβ or C/EBPδ are constitutively present on the c-fos promoter.

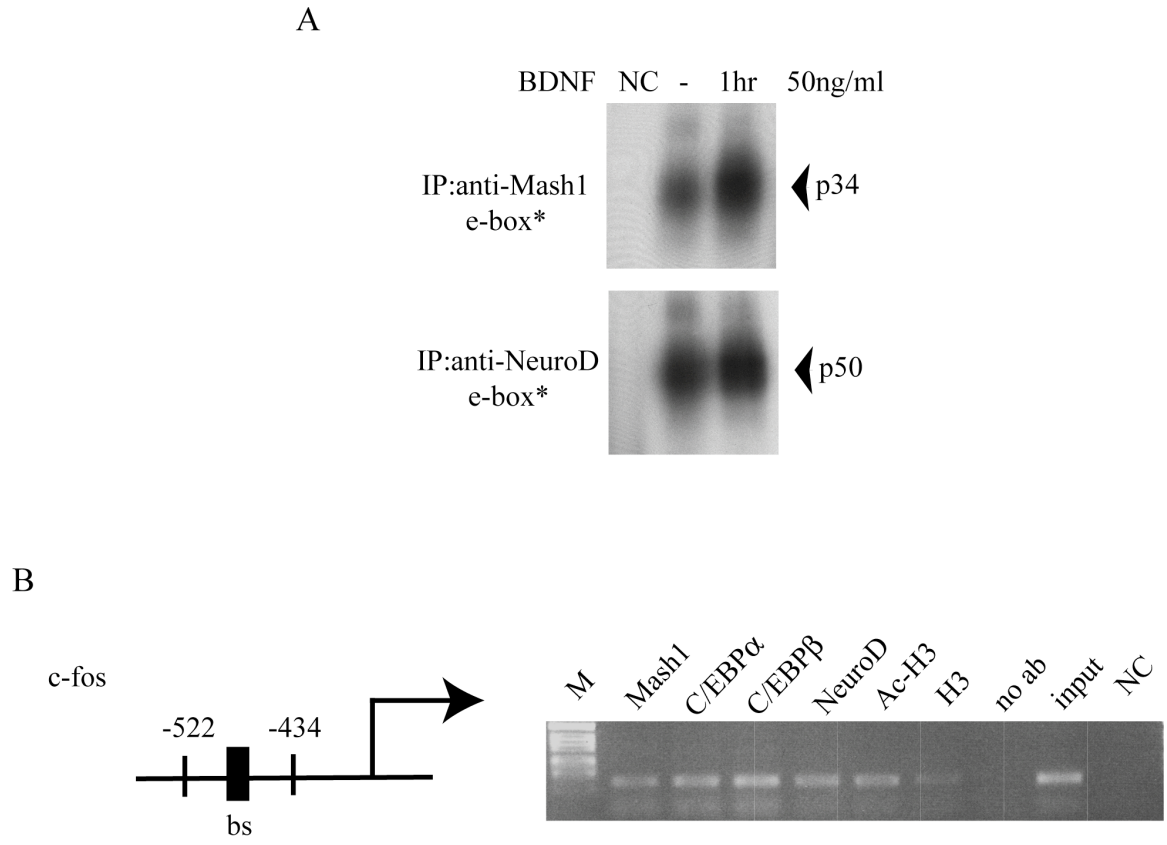


Figure 13 C/EBPs-Mash1, C/EBPs-NeuroD complexes are constitutively present on the c-fos promoter.
(A) In vivo detection of promoter occupancy by Mash1 and NeuroD using UV-crosslinking. Cortical neurons from wild-type mice were either left unstimulated or stimulated with BDNF for 1 hour. Nuclear extracts were incubated with a labeled oligo (2000000 cpm) containing the E-box motif in the contest of the c-fos promoter, followed by UV-crosslinking. Immunoprecipitations were performed with anti-Mash1 antibody and anti-NeuroD antibody. As a negative control (NE) the reaction was performed without nuclear extracts. Autoradiography revealed the presence of Mash1 and NeuroD on the c-fos promoter in a BDNF-independent manner.
(B) In vivo detection of promoter occupancy by C/EBPs, Mash1 and NeuroD using chromatin immunoprecipitation. Scheme for the c-fos promoter examined in this study. Bs represents the region containing the different binding sites, the small arrows indicate the primers used in PCR amplifications, and the large arrow represents published major transcription start site. Soluble chromatin was prepared from entire forebrain of wild-type mice and immunoprecipitated with the diverse antibodies against C/EBP□, C/EBP□, Mash1 and NeuroD. Control reactions lacking antibodies (NO Ab) and immunoprecipitation with anti-Histone3 (H3) and anti-acetyl-histone3 (Ac-H3) antibodies are also shown.
The final DNA extractions were amplified using pairs of primers that cover the region of the c-fos gene promoter as indicated. Input corresponds to PCR reactions containing 0.5% of the total amount of chromatin used in immunoprecipitation reactions, and the lane NC is PCR without template. The experiment was repeated three times, and gave very similar results.

2.7 BDNF induces the phosphorylation of Thr188 in C/EBPβ.

Given these results, it appears that BDNF may regulate gene expression through the post-translation modification of transcription factors. Support for this idea comes from the finding that ERK1/2 phosphorylate C/EBPβ on Thr188 (mouse sequence) to activate its transcriptional ability (Nakajima et al., 1993). Moreover, ERK1/2 is one of the major pathways activated by TrkB (Kaplan and Miller, 2000). We therefore asked whether C/EBPβ is phosphorylated at the ERK1/2 site in cortical neurons following BDNF treatment. As shown in figure 14A-E, after 15' of BDNF stimulation phosphorylated Thr188 form of C/EBPβ was observed throughout the cell bodies of cortical neurons in $trkB^{+/+}$ (A-B). Nuclear localization of the pC/EBPβ signal was demonstrated by double immunofluorescence staining with an anti-Thr188pC/EBPβ antibody and DAPI (figure 14G-I). Interestingly, we observed an absence of colocalization on pericentromeric heterochromatin.

Together, our data support the idea that the phosphorylation of C/EBPβ by ERK1/2 is responsible for the transcriptional activation of the C/EBP-Mash1and C/EBP-NeuroD complexes downstream BDNF/TrkB.

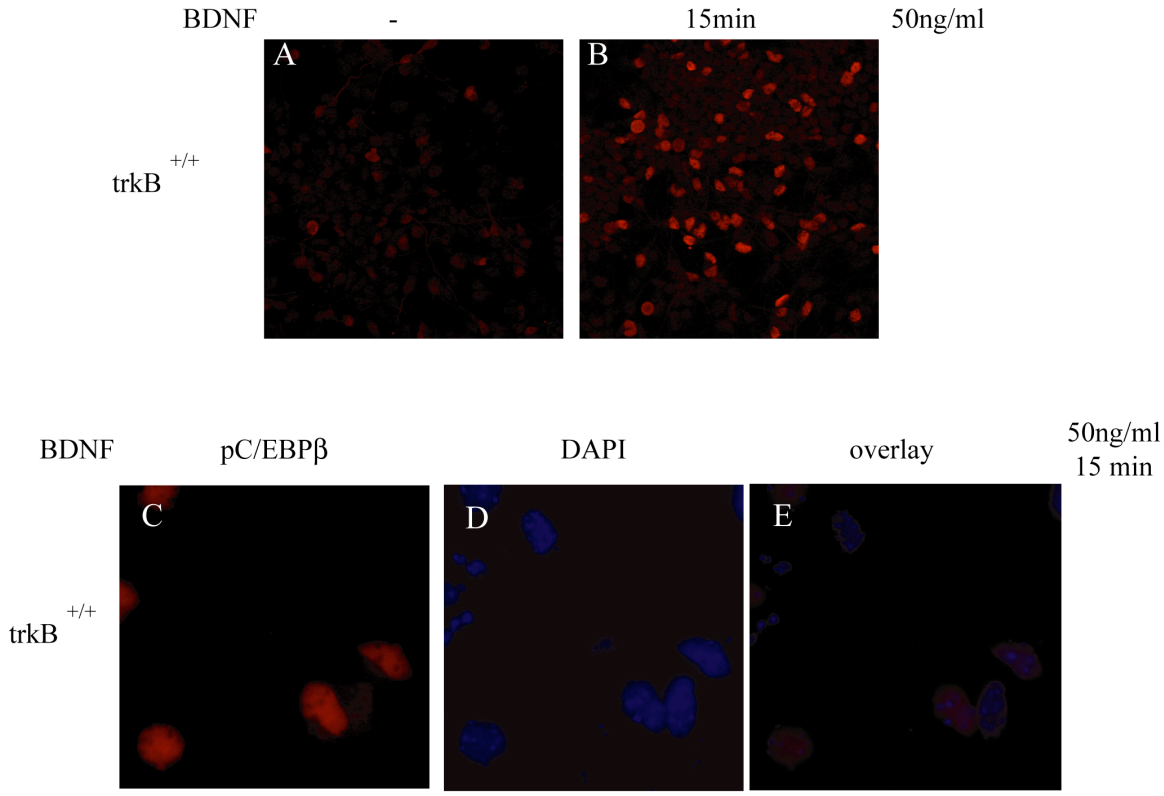


Figure 14 Analysis of activation and chromatin localization of C/EBP in cortical neurons.
(A-E) BDNF induces the phosphorylation of Thr188 of C/EBP.BDNF stimulation after 15' induced pC/EBP signal throughout the cell body of cortical neurons in $trkB^{+/+}$ (A-B).
(C-E) Chromatin localization of pC/EBP after BDNF stimulation. Primary cortical neurons from $trkB^{+/+}$ mice were stimulated for 15' with BDNF and subsequently double stained for pC/EBP (C) and DAPI (D). An overlay revealed absence of colocalization between pericentromeric heterochromatin and pC/EBP ( ) .

2.8 Generation of mGif/TIEG1 mutant mice

In order to determine the function of mGif/TIEG1, we decided to delete the gene in vivo using gene targeting. Since we did not know whether the deletion of this gene would be lethal we generated two different types of targeted alleles: a null allele and a conditional allele.

2.8.1 Isolation of the mGif/TIEG1 mouse genomic DNA

Microarray analyses revealed an up regulation of a cDNA homologous to exon 4 of mGIF. An EST containing this exon (IMAGE ID 876145) was obtained from RZPD. After amplification the clone was confirmed by sequencing and the plasmid (pT7T3D-Pac mod1) was digested with EcoRI and NotI to release a fragment of 1434 bp. This fragment was used to screen a genomic library by hybridization from IncyteGenomics. Four BAC (pBeloBAC11 vectors) clones were obtained. The four clones were digested with different enzymes and compared with mouse genomic DNA by southern blot analysis using the same probe that was used for the library screening. An EcoRI digestion identified a similar band of 12 kb in the genomic DNA and in the four clones. The CELERA confirmed the presence of two EcoRI sites at 12 kb that surrounded the mGif gene.

2.8.2 Targeting of mGif/TIEG1 gene via homologous recombination

2.8.2.1 Knock-out strategy

In order to inactivate the mGif/TIEG1 transcription factor, its third exon was deleted thereby eliminating a region containing two zinc fingers which are fundamental for its interaction with DNA (Fautsch et al., 1998). To achieve this, a targeting vector was constructed using part of the isolated mouse genomic DNA and ET-recombination strategy (Angrand et al., 1999). The final targeting vector contained: a long arm of 5978 bp, followed by a stop codon, an IRES-LacZ-polyA cassette, a blaP-Sve-neo-polyA cassette, flanked by loxP sites, and a short arm of 2732 bp (figure 15A).

IB10/C mouse embryonic stem (ES) cells were electroporated with a Xho linearized targeting vector, and cultured in medium containing selection agent (G418). Two

hundred clones, resistant to G418 were isolated and screened by southern blot analysis. After EcoRI digestion, four clones showed a mutant band of 8.9 kb and wild-type band of 12 kb, as revealed using probe 1, and a 4.7kb mutant band using probe 2 (figure 15B).
These results confirmed that in these four clones homologous recombination occurred, that is, the third exon, 913 bp long, and the neighboring intron of 450 bp, were replaced with a neomycin (neo) cassette, placed in the opposite transcriptional orientation, and an IRES followed by a LacZ cassette.

2.8.2.2 Conditional strategy

We flanked (floxed) the second and third exon of mGif/TIEG1 with two loxP sites by homologous recombination in ES cells. The targeting vector used contained a long arm of 6.9 kb followed by blaP-Sve-neo-polyA cassette, in the opposite orientation to the mGif/TIEG1 gene and flanked by FRT sites, and a short arm of 2.7 kb (figure 16A). The neo selection marker will be excised in vivo by crossing these mice with transgenic mice expressing the Flp recombinase (Minichiello et al., 1998). In this way the expression of mGif/TIEG1 should not be reduced due to the presence of the neo cassette.
IB10/C mouse embryonic stem (ES) cells were electroporated with an EcoRI linearized targeting vector, and cultivated in medium containing selection agent (G418). Two hundred clones, resistant to G418, were isolated and screened by southern blot analysis using the same two probes, as for the null-allele screening. After EcoRI digestion, two clones showed a mutant band of 14kb and wild-type band of 12kb using probe 1 and a, 14kb band, corresponding to a mutant allele, using probe 2 (figure 16B).
These results confirmed, that in these two clones homologous recombination had occurred and that the mGif/TIEG1 wild type allele was properly replaced with the targeted allele.

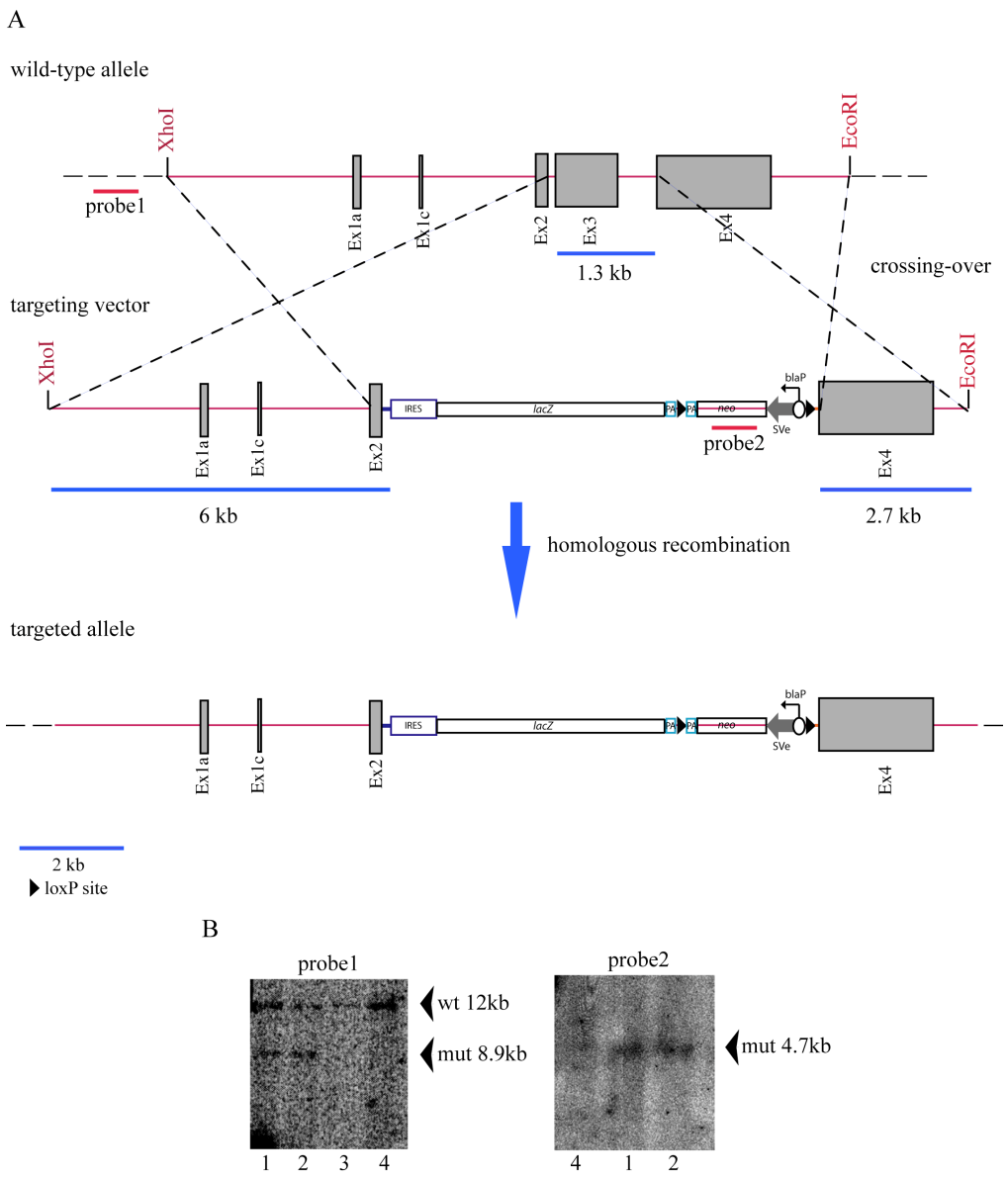


Figure 15 Targeting of the mGif/TIEG1 transcription factor for the null allele.
(A) Schematic drawings of the wild-type mouse mGif/TIEG1 locus (wild-type allele), of the targeting vector and of the mouse mGif/TIEG1 locus after genomic targeting (targeted allele). Grey vertical boxes represent the exons. A 1.3 kb genomic DNA fragment was deleted in the targeting. The 6 and 2.7 kb DNA fragments, corresponding to the long and short arm of the targeting vector, are located upstream and downstream, respectively, of the neo and LacZ cassettes. The transcription of the neo cassette is in the opposite orientation to the mGif/TIEG1 gene (arrows). Putative crossing-over between the wild-type mGif/TIEG1 allele and the targeting vector are indicated by crossed dashed lines. In the targeted allele, the DNA derived from the targeting vector (red) is distinguishable from the upstream and downstream genomic DNA (black dashed lines). Cleavage sites for restriction endonuclease, EcoRI and XhoI, are indicated. Probes used to screen the ES cell clones are indicated with blue lines; probe 1 maps in the genomic DNA sequence, upstream of the long arm, while probe 2 maps in the neo cassette.
(B) Southern blot analysis of genomic DNA from wild-type and two recombinant ES cell clones. The recombinant ES clones, indicated with numbers 1 and 2, show specific bands of 8.9 kb with the probe 1, and 4.7 kb with the probe 2. The wild-type clones are indicated with numbers 3 and 4.

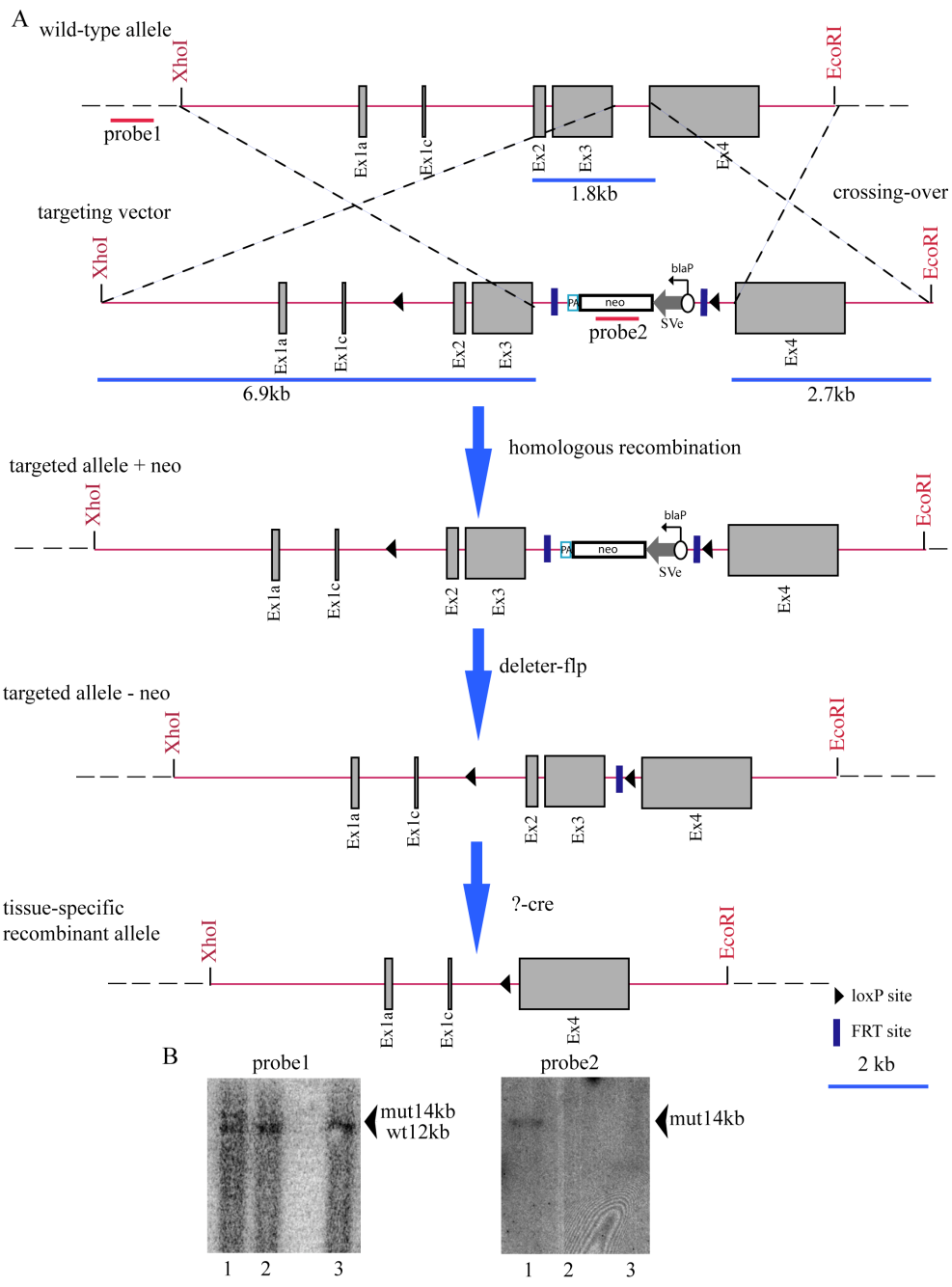


Figure 16 Targeting of the mGif/TIEG1 transcription factor for the conditional allele.
(A) Schematic drawings of : the wild-type mouse mGif/TIEG1 locus (wild-type allele), the targeting vector, the mouse mGif/TIEG1 locus after genomic targeting (targeted allele +neo), the mouse mGif/TIEG1 locus after deletion of the neo cassette in vivo using deleter-flp mice (targeted allele -neo), the mouse mGif/TIEG1 locus after deletion of the second and the third exon using specific CRE mice (tissue specific recombinant allele). Grey vertical boxes represent the exons. A 1.8 kb genomic DNA fragment was flanked by loxP sites in the targeting. The 6.9 and 2.7 kb DNA fragments, corresponding to the long and short arm of the targeting vector, are located upstream and downstream, respectively, of the neo cassette. The transcription of the neo cassette is in the opposite orientation to the mGif/TIEG1 gene (arrows), and it is flanked by FRT sites. Putative crossing-over between the wild-type mGif/TIEG1 allele and the targeting vector are indicated by crossed dashed lines.(next page)

2.8.3 Generation of mutant mice for the mGif/TIEG1 transcription factor

Two of the targeted ES clones, for each construct, were microinjected into C57BI/6J blastocysts and transferred into the uteri of pseudopregnant CD1 recipient mothers. For the null allele fifteen chimeric mice were obtained, displaying partially agouti coat colour, derived from the IB10/C ES cells, and partially black coat colour, derived from the C57BI/6J blastocysts. For the conditional allele eight chimeric mice were obtained, three of which were completely (100%) agouti in colour. Breeding of chimeras with black wild type C57BI/6J mice, revealed the capacity of the chimeras to trasmit the targeted allele to the progeny. This was easily established by looking at the coat colour of the offspring, since only agouti pups have the potential to be either wild-type or heterozygous for the mGif/TIEG1 mutation. The screening of the agouti mice is on going.

In the targeted alleles and in the tissue specific recombinant allele, the DNA derived from the targeting vector (red) is distinguishable from the upstream and downstream genomic DNA (black dashed lines). Cleavage sites for restriction endonuclease, EcoRI and XhoI, are indicated. Probes used to screen the ES cell clones are indicated with blue lines; probe 1 maps in the genomic DNA sequence, upstream of the long arm, while probe 2 maps in the neo cassette.
(B) Southern blot analysis of genomic DNA from wild-type and recombinant ES cell clones. The recombinant ES clone, indicated with number 1, shows specific bands of 14 kb with both probe 1 and probe 2. The wild-type clones are indicated with numbers 2 and 3.

3

DISCUSSION

Four major conclusions can be drawn from the data presented in this thesis. First, these studies demostrate that BDNF, through TrkB receptor, induces the expression of a group of transcription factors with similar kinetics in cortical neurons isolated at the peak of neurogenesis. Second, using the c-fos promoter as a model, our data suggest that C/EBPs and bHLH transcription factors collaborate to induce the activation of c-fos downstream of BDNF. Third, in vivo and in vitro experiments indicate that bHLH proteins, Mash1 and NeuroD, bind C/EBP transcription factors at different domains. Finally, our studies suggest that the formation of C/EBP-Mash1 and C/EBP-NeuroD complexes and their occupancy of the c-fos promoter, are BDNF independent, but that BDNF regulates gene expression by inducing post-translational modification of these complexes. In fact BDNF stimulation was found to induce an increase in C/EBP phosphorylation on Thr188, which is ERK1/2-dependent, suggesting that activation of ERK1/2 could be one of the possible mechanisms by which C/EBP-Mash1 and C/EBP-NeuroD complexes are transcriptionally activated downstream BDNF/TrkB.

Together these data reveal novel nuclear targets of neurotrophin signaling cascades that mediate gene expression in response to BDNF/TrkB activation in cortical neurons isolated at the peak of neurogenesis.

3.1 A new role for BDNF during neurogenesis

Previous work has clearly demonstrated that intrinsic genetic mechanisms play an essential role in regulating precursor cell survival, proliferation, and subsequent differentiation into neurons or glia. These intrinsic programs, however, are heavily influenced by environmental determinants such as growth factors (Shen et al., 1998), (Lillien, 1998)

At least two members of the neurotrophin family, BDNF and neurotrophin-3 (NT-3), along with their preferred tyrosine kinase receptors, TrkB and TrkC respectively, are expressed in the cortical ventricular/subventricular zones at the onset of cortical neurogenesis (Fukumitsu et al., 1998; Maisonpierre et al., 1990) and recent evidence suggest that they function in regulating cortical progenitor cell biology. BDNF and NT3 are believed to promote survival and neurogenesis of cortical progenitor cells through

two distinct and separable signaling pathways: the PI3-kinase pathway mediates survival, while the MEK pathway is important for neurogenesis (Barnabe-Heider and Miller, 2003). Neurotrophins have also been shown to play an essential role in postmitotic cortical neurons by regulating the survival, growth, phenotype, and ultimately, connectivity of these cells (Kaplan and Miller, 1997; Kaplan and Miller, 2000). It is still unclear, however, which transcription factors are activated by neurotrophins during neurogenesis, and are responsible for inducing the gene expression necessary to mediate these cellular changes. Data presented here provide some insight, by revealing that, with respect to the activation of the c-fos promoter, one of the neurotrophin family members, BDNF, acting through its high affinity receptor tyrosine kinase TrkB, requires transcription factors known to be directly involved in neuronal differentiation processes.
The importance of this mechanism in vivo during neurogenesis remains unclear. In fact, in contrast to the peripheral nervous system, in which single neurotrophins play essential roles in regulating the biology of specific neuronal populations, such as NGF for sympathetic neurons (for review, see(Klein, 1994; Snider, 1994), multiple neurotrophins appear to play redundant and overlapping roles in neurons of the central neurvous system. It is likely that this functional overlap between neurotrophins, and potentially also with other receptor tyrosine kinase ligands, such as FGF2, explains the relatively modest phenotypes observed in the developing cortex of single neurotrophin knock-out animals (Jones et al., 1994; Kahn et al., 1999; Ringstedt et al., 1998). Support this idea is derived from studies of animals lacking both TrkB and TrkC, who display numerous CNS abnormalities (Alcantara et al., 1997; Lotto et al., 2001; Martinez et al., 1998; Minichiello and Klein, 1996; Ringstedt et al., 1998; Xu et al., 2000).

3.2 The shc-site and plcγ-site of the TrkB receptor activate the same set of transcription factors in primary cortical neurons isolated at the peak of neurogenesis

We have examined mutant mice carrying signaling point mutations in the TrkB receptor using two approaches, microarray and promoter analysis, and discovered that the shc-site and plcγ-site mediate the majority of signaling following receptor activation, and that in

the absence of both sites gene expression is minimally activated by BDNF. Signalling pathways downstream of the shc-site and the plcγ-site also appear to activate the same set of transcription factors in primary cortical neurons isolated at the peak of neurogenesis. Thus, the two sites could compensate for each other, although there is a higher activation of gene expression in the plcγ-point mutant mice compared to the shc-point mutant mice. This could be explained by the possibility that the plcγ-site mediates the induction of not only positive regulators of gene expression, but also negative regulators, depending upon the presence of other extrinsic factors and/or the developmental or functional status of the cell. Therefore, in the absence of the plcγ-site the balance between activation and inhibition of gene expression downstream BDNF/TrkB may be disturbed, thus we observe an overall increase in gene expression. This type of negative regulation has been demonstrated for other signaling pathways. SOCS proteins (suppressors of cytokine signaling) for example, are cytokine-inducible proteins and in turn downregulate the same cytokine- activated JAK/STAT signaling pathway (Starr et al., 1997). An additional reason for the increased activation of gene expression in the plcγ-mutants may be through TCF. We found that the activation of TCF downstream BDNF/TrkB is solely dependent on signaling from the shc-site. Therefore the shc-site mediates an additional pathway leading to gene expression that does not exist downstream the plcγ-site. One way to test these hypotheses would be to identify other as yet unknown, proteins that are downstream of these two sites of TrkB, and whose functions could explain the difference in gene expression observed in the two signaling point mutant mice.

Having identified c-fos, Egr1 and mGif as a group of transcription factors whose expression in cortical neurons is similarly regulated by BDNF/TrkB at the time of neurogenesis, it is reasonable to assume that these genes play a role during neurogenesis. Although all these genes have previously been shown to be express in the central nervous system their function during cortical development is unclear. A role for c-fos in the differentiation of other cells types, however, has been described. Stable expression of c-fos in mice, for example, is found in developing bones and teeth, hematopoietic cells, germ cells, and neurons of the CNS. In the knockout model (Johnson et al., 1992; Wang et al., 1992) mice displayed growth-retardation, osteopetrosis with deficiencies in bone remodeling and tooth eruption, and as idiret effect an altered hematopoiesis. In addition,

it has been shown that fos mutant mice that develop osteopetrosis have a block in the differentiation of hematopoietic cells into bone-resorbing osteoclasts, which normally occurs through an intrinsic programme (Grigoriadis et al., 1994). Although it is clear, from both in vitro and in vivo studies, that c-fos is important for differentiation of osteoclasts, in the CNS the only described role for c-fos has been in the regulation of cellular mechanisms that mediate neuronal excitability and survival (Zhang et al., 2002). In Drosophila, Sanyal et al. recently analyzed the cellular function and regulation of the immediate early transcription factor AP-1, a heterodimer of the basic leucine zipper proteins FOS and JUN, and observed that AP-1 functions upstream of CREB to control synaptic plasticity by regulating synaptic strength and synapse number (Sanyal et al., 2002). Similar to c-fos, Egr1 has also been reported to play a role in the CNS. Inactivation of Erg1 in the central nervous system revealed its requirement for the long term, but not short term synaptic plasticity in the dentate gyrus (Jones et al., 2001). The results presented in this thesis extend our knowledge by revealing the presence of c-fos, Egr1 and mGif downstream BDNF/TrkB in primary cortical neurons isolated at the peak of neurogenesis.

3.3 Are C/EBPs determination or differentiation factors for cortical progenitors?

The C/EBP family of transcription factors have been shown to function as regulators of cellular differentiation in a number of different cell types, including hepatocytes, mammary epithelial cells, ovarian luteal cells, keratinocytes, neuronal cells and intestinal epithelial cells (Chandrasekaran and Gordon, 1993; Cortes-Canteli et al., 2002; Lekstrom-Himes and Xanthopoulos, 1998; Robinson et al., 1998; Seagroves et al., 1998; Sterneck et al., 1997; Zhu et al., 1999). Their role in mediating cell differentiation have been best characterized, moreover, during the processes of hematopoiesis and adipogenesis. Four members of the family (□, □, □and □) are expressed in myeloid cells and show a unique expression profile during in vitro myeloid-cell differentiation (Morosetti et al., 1997; Natsuka et al., 1992; Scott et al., 1992; Yamanaka et al., 1997b). For example, the expression of C/EBP□ is constant in the bone marrow-derived cells and

decreased in polymorphonuclear neutrophils (Bjerregaard et al., 2003). Conversely, the myeloid-restricted member, C/EBP, is preferentially expressed during granulocytic differentiation (Morosetti et al., 1997; Yamanaka et al., 1997b), whereas C/EBP is up-regulated during macrophage differentiation (Natsuka et al., 1992). The most compelling evidence that C/EBPs are crucial for myeloid-cell differentiation and maturation has come from studies on knockout mice. C/EBP deficient mice fail to undergo myeloid differentiation beyond the myeloblast stage, and therefore, lack neutrophil granulocytes, and eosinophils (Zhang et al., 1997). C/EBP deficient mice, on the other hand, fail to produce atypical neutrophils,(Yamanaka et al., 1997a). Finally, the phenotype of C/EBP deficient mice indicates a potential role for this gene in the activation of macrophages as well as in Th1 immune responses (Poli, 1998; Screpanti et al., 1995; Tanaka et al., 1995).

The importance of the C/EBP family in adipocyte differentiation has been confirmed using a number of approaches. First, ectopic expression of C/EBP and C/EBP in 3T3-L1 cells is able to initiate the differentiation programme in the absence of adipogenic hormones, whereas overexpression of C/EBP accelerates the process triggered by these agents (Freytag et al., 1994; Lin and Lane, 1994; Yeh et al., 1995). Second, expression of antisense C/EBP RNA in 3T3-L1 cells blocks differentiation (Lin and Lane, 1992). Third, embryonic fibroblasts lacking both C/EBP and C/EBP are unable to initiate the differentiation programme in response to hormonal stimulation (Tanaka et al., 1997). Finally, C/EBP deficient mice have dramatically reduced lipid accumulation in the adipose tissue (Wang et al., 1995).

During these processes, various members of the C/EBP family have been shown to act in concert with cell type-specific determination signals to promote or repress the transcription of genes essential for cell differentiation, and as a result have been classified as lineage instructive transcription factors (Freytag et al., 1994; Nerlov et al., 1998; Radomska et al., 1998). For example the induction of eosinophil lineage commitment by C/EBP appears to be caused by its ability to down-regulate the expression of the Friend of GATA (FOG) protein, allowing cooperative activation by C/EBP proteins and GATA-1 (Querfurth et al., 2000). Also the activation of chromatin-embedded myeloid genes in heterologous cell types, such as fibroblasts, has been accomplished by a combination of Myb and C/EBP transcription factors (Burk et al. 1993;Ness et al. 1993), and this has

been shown to involve the recruitment of the SWI/SNF chromatin-remodeling complex by the N-terminal CR1 region of C/EBP (Kowenz-Leutz and Leutz, 1999). Finally, Pedersen et al. have also shown that the ability of the C/EBP transcription factor to direct adipocyte differentiation of uncommitted fibroblast precursors and to activate SWI/SNF-dependent myeloid-specific genes both depend on the transactivation element III (TE-III), domain of C/EBP which binds the SWI/SNF chromatin remodeling complex. TE-III collaborates with C/EBP TBP/TFIIB interaction motifs during the induction of adipogenesis and adipocyte-specific gene expression (Pedersen et al., 2001). Again these results indicate that C/EBPscanact as lineage-instructive transcription factors through SWI/SNF-dependent modification of the chromatin structure of lineage-specific genes, followed by direct promoter activation via recruitment of the basal transcription–initiation complex. Together these findings suggest that C/EBP/ function as determination factors for multiple cell lineages.

In the current study we used the well-characterized c-fos promoter as a model to understand which transcription factors are important for BDNF-dependent gene expression. The c-fos promoter analysis we performed clearly demonstrates the importance of the E-box and C/EBP binding site for the activation of c-fos downstream BDNF, suggesting that C/EBPs and bHLH transcription factors might collaborate to induce the activation of this promoter downstream of BDNF/TrkB during neurogenesis. This notion is further supported by the identification of complexes that exist between bHLH and C/EBP transcription factors bound to the c-fos promoter.

Given these current findings it is not clear whether C/EBPs act as determination factors in conjunction with bHLH transcription factors in cortical progenitors to induce the formation of neurons versus glial cells. Menard et al. have recently shown that the inhibition of C/EBP activity biases cortical progenitor cells to remain as progenitors instead of giving rise to neurons. Conversely, inhibition of C/EBP-dependent transcription enhances CNTF mediated generation of astrocytes from the same progenitor cells (Menard et al., 2002). These results have led Menard et al. to propose that in cortical progenitors, C/EBPs act not as “determination” factors in the same sense as bHLHs like the neurogenins, but as differentiation factors, responsible for initiating the transcription of early neuronal genes in response to growth factor cues. In their model, progenitor cells

are partially biased to become neurons as a consequence of the repertoire of bHLHs that they express, and the growth factor-mediated activation of C/EBPs provides the final "push" to trigger expression of a neuronal phenotype through the transcription of genes, such as T1β-tubulin. Therefore, expression of the appropriate bHLHs would be a requisite for the neurogenic actions of the C/EBPs; such necessary cooperativity with cell specific determination factors, would allow C/EBPs to function as growth factor-regulated differentiation signals in a variety of different cell types. In this sense, with regard to their proneurogenic activities, the C/EBPs act neither as classic differentiation or fate signals, but as necessary cofactors for fate-biasing molecules such as the bHLHs. However, based on the data of Menard et al. it is possible that C/EBPs act as determination factors, since in their absence the authors report an absence of neurogenesis. In our study we show that C/EBPs bind bHLH transcription factors, and in this way may collaborate to determine the fate of cortical progenitors. More study is therefore necessary to determine the precise function of C/EBPs in the CNS, that is, whether they are required for cell determination or differentiation.

3.4 How might the C/EBPs collaborate with the neurogenic bHLHs to generate a postmitotic neuron?

We have now demostrated that in neurons, C/EBPβ and δ bind two members of two different classes of bHLH transcription factors, the early differentiation factor Mash1, and the late differentiation factor NeuroD. The identification of both Mash1-C/EBP and NeuroD-C/EBP complexes, in our cell culture, is probably due to the fact that they contain a mixed population of neurons at different stages of differentiation. Moreover, the RT-PCR data presented here also indicate that C/EBPβ, δ, Mash1 and NeuroD are all expressed in cultured cortical progenitors from E12 to E18, in which, both progenitors and neurons are likely to be present. In vivo it has been shown that Mash1 and NeuroD are expressed at different times and in different places of the telencephalon (Nieto et al., 2001), suggesting that the complexes we have identified occur in different cells. Taken together, we conclude that C/EBPs are expressed during neurogenesis, and are able to

cooperate with cell specific neurogenic bHLHs.

Do the C/EBPs also play a role in regulating terminal mitosis as progenitors become neurons? Although our data do not directly address this issue, C/EBPs have been implicated in growth arrest in nonneural cells, an effect that they mediate at least partially via the pRb family of proteins which are essential for cortical progenitor differentiation into neurons (Slack et al., 1996; Slack et al., 1998). Given these findings, Menard et al, have suggested that interactions between the C/EBPs and pRb family could provide an integral link between terminal mitosis and the induction of the neuronal phenotype in cortical progenitor cells. Based on our findings, we can suggest an additional possibility, that C/EBPs directly interact with different bHLH factors at different stages of differentiation: specific interactions at an early phase induce the differentiation process, whereas interactions with different bHLHs at a later phase activate the gene expression necessary for terminal cell differentiation. In fact we find that Mash1, an early differentiation factor, interacts with the N-terminal region of C/EBP, in which resides its anti-mitotic effect (Lin et al., 1993; Umek et al., 1991), whereas NeuroD, a late differentiation factor, interacts with the C-terminal region of C/EBP and and that interaction is impaired by mutations in highly conserved residues in the non-DNA binding face of the basic region. Porse et al have shown in mice that mutations in the basic region of C/EBP are defective in repressing E2F-dependent transcription, and fail to support adipocyte and granulocyte differentiation in vivo (Porse et al., 2001). If bHLH and E2F proteins bind C/EBP in the same regions, it is possible to have a competition for the C/EBP binding by these two proteins. C/EBP interaction with E2F represses the expression of E2F-dependent genes, which are genes known to stimulate cell cycle progression and/or inhibit differentiation. Conversely, C/EBP, binding to bHLH proteins, induces the expression of genes involved in cell differentiation. Therefore it is through multiple pathways that C/EBP likely promotes cell differentiation.

3.5 How does BDNF control the transcriptional activity of the complexes formed between bHLHs and C/EBPs?

From our studies it is clear that BDNF regulates neither the formation of Mash1-C/EBP

and NeuroD-C/EBP complexes, nor their translocation onto the promoter of genes that are regulated by BDNF. BDNF induction of the c-fos promoter, however, is low in the absence of the C/EBP binding site and E-box, suggesting that BDNF/TrkB signaling may influence the transcriptional activity of these complexes by inducing their post-translational modification.

For the bHLH transcription factors, little is known about the regulation of their transcriptional activity by post-translational modification. The activity of C/EBP, however, is known to be regulated through their direct phosphorylation of ERK1/2 at Thr188 (Nakajima et al., 1993; Williams et al., 1995) and by Rsk, which can itself be induced by ERK1/2, one of the major and well-characterized kinase pathway downstream of tyrosine kinase receptors (Davis, 1995). Thus, these receptors may signal to activate the C/EBPs via several parallel signaling cascades, one of which involves the Raf/RAS/MAPK pathway. In neurons, Menard et al have shown that phosphorylation of C/EBP at the Rsk site (Thr217) is sufficient to enhance neurogenesis. Through this same mechanism, both FGF2 and PDGF have been shown to activate C/EBPs in osteoblasts (Wadleigh and Herschman, 1999), and a MEK-ERK-Rsk- C/EBP pathway has recently been implicated in the control of hepatocyte apoptosis in response to TGF (Buck et al., 2001). Data presented here indicate that during neurogenesis BDNF can activate the C/EBP family via MEK-ERK1/2 phosphorylation of Thr188.

In conclusion, the present study implicates the ERK-C/EBP pathway as a novel growth factor regulated signaling cascade, that promotes the genesis of neurons from neural precursors in response to BDNF/TrkB activation. We propose that this pathway provides an intracellular mechanism for integrating and coupling diverse growth factor stimuli to the intrinsic cellular machinery, thereby ensuring that neurons are generated in appropriate numbers at the appropriate time during development.

3.6 Future directions

To verify the role of C/ EBPs in neurogenesis it would be necessary to develop a mouse model in which at least two C/EBP family members are eliminated from the CNS (to avoid redundancy) and examine the effects on neuronal differentiation.

The expression patterns of the members of this family often overlap, making it difficult to discern the specific regulatory functions of each protein. Mice carrying single gene deletions of C/EBP and do not display any phenotype in the CNS. Mice lacking C/EBP die shortly after birth due to hypoglycemia and an absence of stored glycogen in the liver (Wang et al., 1995). Mice deficient for C/EBP are viable but display immune defects, (Screpanti et al., 1995; Tanaka et al., 1995) and female sterility (Sterneck et al., 1997). To overcome these problems, and to address the role of C/EBPs in the CNS, it would be best to create a mouse model with a null deletion of C/EBP, and conditional deletion of C/EBP in the CNS. Once created these mice should be specifically examined for defects in the differentiation of GABAergic populations, which is predicted to require a C/EBP association with Mash1, and of principal neurons, which require NeuroD function. The results of such analyses would reveal whether C/EBPs are required for determining the fate or differentiation of these cell types.

3.7 mGif/TIEG1 transcription factor: new target for BDNF/TrkB

The function of mGif/TIEG1 has been studied only in vitro, so to better understand the function of this gene we decided to generate the null and conditional alleles of mGif/TIEG1 in mice. mGif/TIEG1 is widely expressed and is present at least at E16.5 (Yajima et al., 1997). Given its early onset of expression, it is possible that a complete deletion of mGif/TIEG1 is lethal, thus it was necessary to create the conditional allele. Depending on the phenotype of the knock-out mouse, mice with conditional alleles will be crossed with various CRE mice, in order to study the role of mGif/TIEG1 in specific tissues. During the gene targeting for the null allele we also introduced the LacZ cassette in the genomic locus of mGif/TIEG1, which will also allow us to analyze the expression pattern of the transcription factor.

It has been shown in vitro that mGif/TIEG1 acts as a tumor suppressor (Chalaux et al., 1999; Hefferan et al., 2000; Ribeiro et al., 1999; Tachibana et al., 1997), so it is possible the mutant mice will have a susceptibility to developing tumors, and might therefore be used as a model for human disease.

In addition to be induced by BDNF, mGif/TIEG1 has also been shown to be induced by GDNF. It would therefore be interesting to compare the phenotypes of the mGif-null mice with strains lacking the gene for GDNF or BDNF to dissect the signaling pathways that lead to its induction.

4

MATERIALS AND METHODS

4.1 Primary cultures of cortical progenitors and neurons

The preparation of cortical progenitors from mouse embryos was based on a previously described method (Menard et al., 2002). Cortices were collected from E12-13 mouse embryos, triturated, and then plated on poly-L-lysine (at different densities depending on the final experiment). The culture medium consisted of Neurobasal medium (GIBCO BRL), 0.5 mM glutamine, penicillin-streptomycin, 1% N2 supplement (GIBCO BRL), and FGF (40 ng/ml; R&D system). After 48 h, medium was replaced with the same medium containing 2% B27 supplement (GIBCO BRL) instead of 1% N2 supplement. The cortical neurons generated from these progenitor cells could be maintained for at least 3 weeks under these conditions.

Mature postmitotic neurons were prepared from cerebral cortices of E15.5 mouse embryos derived from intercrosses of wild type, $trkB^{SHC/SHC}$, $trkB^{PLC/PLC}$, $trkB^{WT/WT}$ homozygotes. Cortical neurons were dissociated and plated onto poly-L-lysine hydrobromide (Sigma) in the presence of 10% heat-inactivated horse serum which was replaced to N2-MEM after 3 hr. Cells were cultured for an additional 16 hr prior to ligand stimulation in N2-MEM medium (Minichiello et al., 1998) at 37°C in 5% carbon dioxide. Purified recombinant BDNF (Genentech, Inc., and Regeneron Pharmaceuticals, Inc.) was added to the culture medium at a concentration of 50 ng/ml at different time points.

4.2 Molecular biology

Standard protocols for molecular biology were taken from Molecular Cloning Laboratory Manual (2^{nd} edition). For DNA preparation: MiniPrep, QIAquick PCR purification and Gel extraction kits (Qiagen) were used according to manufacturer instructions. For PCR protocols, AmpTaq polymerase from Applied Biosystem was routinely used. Ligations were performed using T4 DNA ligase from New England Biolabs. Restriction enzymes were purchased from New England Biolabs. If not otherwise stated, E.Coli strain XL1-blue was used.

4.2.1 Plasmids

The pGL3-basic and pRSV-β-Gal vectors were obtained from Promega, while the pBluescript II KS+ vector was obtained from Stratagene.

The following constructs: pMSRF/cebp, pMcebp/ebox, pMebox/AP1, pMebox, pMCRE were constructed by digesting pFC700, as previously described (Ray et al., 1989), with HindIII and XbaI to release a 753-bp fragment. This fragment, after a subcloning step in pBluescript II KS+ vector, was inserted into KpnI-SacI digested pGL3-basic.

The different mutations were introduced using the QuickChange Site-Directed Mutagenesis Kit, and the following mutagenic oligonucleotide primers:

pMebox/AP1: tgtccatattaggacatccatggcagcaggtttccacggcc

pMebox: tgtccatattaggaaatctgcgtcagcaggtttccacggcc

pMCRE: ccgtggttgagcccgtgatgtttacactcattcat

pMSRF/cebp, pMcebp/ebox were kindly provided from C. Nerlov.

All C/EBPβ derivatives were introduced in pcDNAI (Invitrogen) for in vitro labeling as BamHI-EcoRI fragments. The basic region point mutants (BRM-1: V287A, E290A; BRM-2: I294A, R297A; BRM3: D301A, K304A; BRM-5: Y285A) were introduced by overlapping PCR using rat C/EBPβ, and confirmed by DNA sequencing. All C/EBPβ derivatives were provided from C. Nerlov and described in Porse et al., 2001.

The GCN4 zipper exchange mutant (C/EBPβ :Gz) has been previous described (Agre et al., 1989). Full length C/EBPβ :Gz was amplified by PCR from pC/EBPβ :Gz using primers BamHI/5'gcttggatccccatggagtcggccgacttc, and gaggaattcagcgttcgccaactaatttc 3'/EcoRI. The PCR product was cloned into the polylinker of pcDNAI using the BamHI and EcoRI sites.

Mouse Mash1 full-length cDNA was obtained by RT-PCR using RNA isolated from cortical neurons RNA by RT-PCR (GeneAmp kit from Applied Biosystem) and the primers EcoRI/5'ggaattcccggcatggagagctc, and gctctagaggacggtcctcagaa 3'/XbaI. The PCR product was cloned into the polylinker of pcDNA3 using the EcoRI and XbaI sites.

Mouse NeuroD full-length cDNA was obtained by RT-PCR using RNA isolated from cortical neurons and the primers BamH1/5'cgggatccgtggaaacatgacc, and ggaattcactgacgtgcctcta 3'/EcorI. The PCR product was cloned into the polylinker of pcDNA3 using the BamH1and EcoRI sites.

Rat Myogenin full-length cDNA has been previously described (Wright et al., 1989). The full-length cDNA was obtained by cutting a myogenin cDNA containing pEMSV with EcoRI, and was subcloned into the EcoRI sites of pcDNA3. The correct orientation was checked by KpnI digestion.

4.2.2 Generation of mGIF/TIEG1 targeting constructs

The genomic BAC clone, containing the mGIF/TIEG1 transcription factor was obtained by library screening (Incyte Genomics). Gene targeting was performed using both a knock-out and a conditional-approach. An ET-recombination strategy was used to obtain the targeting vectors (Angrand et al., 1999).The plasmids used for ET-recombination were: plZKeoX1 (provided by F. Stewart), pSVKeoX1 (provided by F. Stewart), and pSVKeoneoFLP, which was derived from pSVKeoX1 by the insertion of two FRT sites and the elimination of one loxP site (provided by B. Porse). The bacterial strain for the recombination was JC8679 (Angrand et al., 1999).

The targeting vector for the knock-out consisted of a long arm of 5978 bp, followed by a stop codon at the beginning of exon 3 of the gene, an IRES-LacZ-polyA cassette, blaP-Sve-neo-polyA cassette flanked by loxP sites, and short arm of 2732 bp.

The targeting vector for the conditional allele consisted of a long arm of 6891 bp, a blaP-Sve-neo-polyA cassette, positioned in the opposite direction to the mGif/TIEG1 gene and flanked by FRT sites, and a short arm of 2732 bp..

4.2.3 Targeting of mGif wild-type allele

The targeting vector (100 μg) for the knock-out allele was linearized with an XhoI restriction endonuclease, while the targeting vector (100 μg) for the conditional allele was linearized with an EcoRI restriction enzyme.

After linearization, 30 μg of DNA was electroporated into ES cells (10^7 cells) at 220V and 480 μF in a Biorad Gene pulser. The cells were incubated for 20' on ice and subsequently plated on gelatine coated 10 cm dishes in ES medium. After 40 hr, selection with G418 was begun and continued for 10 days. To identify the recombinant clones

carrying the targeted allele, southern blot screening was carried out using an external probe in the 5' region and an internal probe in the neo cassette (describe below).

4.2.4 Isolation of DNA from ES clones and southern blot analysis

For screening of the ES cells, the confluent plates were washed with PBS. 50 µl of lysis buffer (10mM Tris, pH 7.5; 10 mM EDTA pH 8, 10 mM NaCl 0,5% sarcosyl, 100 µg/ml proteinase K) was added to each well and incubated ON at 55^0C. The DNA was precipitated using 100 µl EtOH/NaCl, washed three times with 70% ethanol, dried and digested with EcoRI ON at 37^0C. 18 µl of the ES DNA preparation was separated using 0.7% agarose gels, run at 30V for 15-18 hr.

Before blotting, the gels were stained with ethidium bromide, and then depurinated for 15' in 0.25 M HCl, denaturated with 0.4 M NaOH, 2 times for 30', and finally neutralized with SSC 20X (NaCl 3M, $C_6H_5Na_3O_7$ 0.3M) for 1 hr.

Gels were blotted ON onto a GeneScreen Plus membrane (Perkin Elmer) in presence of SSC 20X (capillary blotting). The membrane was baked at 80^0C for 1 hr and then washed for 1 hr in 0.1X SSC and 0,5% SDS at 65^0C to reduce background. Prehybridization was then performed for 2 hr at 42^0C (50% deionized formanide, 5X SSC, 5% denhart's solution, 50 mM Na_3PO_4 pH 7,2, 1% SDS 350 µg/ml DNA salmon sperm), followed by hybridization with probe ON at 42^0C (50% deionized formanide, 5X SSC, 5% denhart's solution, 50 mM Na_3PO_4 pH 7,2, 1% SDS 100 µg/ml DNA salmon sperm, 5% destran sulfate). The probes were prepared using Random Primed DNA Labeling Kit (Roche).

Probe 1 was 600 bp in length and was obtained by PCR using the following primers:

5'gacttaatttggctgtagga 3'

5'cccactcctcaagtccatcct 3'

Probe 2 was 700 bp and was obtained by digesting the plZKeoX1 vector with NcoI.

Following the hybridization, the blots the were washed in 2X SSC and 1% SDS, twice at room temperature and once at 50^0C, and then washed in 0.1X SSC and 1%SDS, twice for 30' at 55^0C. Hybridized probes were visualized using phosphoimager system.

4.2.5 RNA isolation

Following BDNF stimulation cortical neurons were washed twice with cold PBS, and then lysed in 1 ml of TRIzol (GIBCO Life Technologies). Homogenized samples were incubated for 5' at room temperature, then 200 µl of chloroform was added, the tubes were shook by hand for 15" and then incubated for 3' at room temperature. The samples were centrifugated to separate the two phases. The upper phase was transferred to a fresh tube and the RNA was precipitated by adding 500 µl of isopropanol, and by centrifugation for 15' at 15000rpm. The RNA pellet was washed once with 70% ethanol and briefly dried. RNA yield was quantified by spectrophotometric analysis using the convention that 1 absorbance unit at 260 nm equals 40 µg RNA per ml. The absorbance was routinely checked to determine RNA purity. The A_{260}/A_{280} ratio was close to 2.0.

To obtain a concentration of 2 µg/µl, different samples were pooled and precipitated by adding 1/10 volume 3 M NaOAc, pH 5.2, and 2.5 volumes ethanol, and incubated at –20°C for 1 hour. The samples were centrifugated at 12000rpm for 20' at 4 °C, and the pellet was washed with 70% ethanol. The pellet was air dried and resuspended in DEPC-treated H_2O. At this point the RNA was sent for Affymetrix analysis performed using Murine Genome U74Av2 chips.

4.2.6 Northern blot

15 µg of RNA was dissolved in 4.5 µl of water plus: 3.5 µl of 37% formaldeide, 10 µl of deionized formamide, 2 µl of MOPS 10X (MOPS 0.2 M, CH_3COONa 50 mM, EDTA 5mM) and incubated 15' at 55°C. 2 µl of RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenolblue) and 2 µl of ethidium bromide (1 mg/ml) were added to samples before loading on a formalin- 1.5% agarose gel, in 1X MOPS running buffer. The gel was run at 100V for 3 hours. Before blotting the gel was soaked in water for 30' and then in SSC 20X for 15'. The gel was blotted overnight onto GeneScreen Plus (PerkinElmer) using 20X SSC. After blotting the membrane was UV-crosslinked using a Stratalinker (Stratagene).

The probes for c-fos, Egr1 and mGif were obtained from ESTs by RZPD. The cDNA fragments were purified by agarose gel and 20 ng for each of them were used for labeling

using a Random Primed DNA Labeling kit (Roche). The blots were prehybridized at 42°C for 3 hr, and then hybridized ON at 42 °C using the same buffer (SSC 5X, 50% deionized formamide, Denhart's solution, 50 mM Na_3PO_4 1% glycine) containing different concentration of DNA salmon sperm: 350 mg/ml in the prehybridiziation and 100 mg/ml in the hybridiziation buffer. Washes were performed first at room temperature, and then two times for 10' at 42 °C in SSC 1X and SDS 0,1%. The membrane was then exposed to Biomax film at –80°C, ON.

4.3 Reporter gene assay

Cortical neurons were plated at $3x10^6$ cells per 60-mm well. The transfections were carried out on 3 or 4 DIV using Lipofectamine 2000 Reagent (Invitrogen) according to the protocol supplied by the manufacturer. One 60-mm well of cells was transfected with 2.3 μg of reporter plasmid plus 3 μg of pRSV-β-gal. Twenty-four hours after transfection, cells were left unstimulated or stimulated with 50 ng/ml BDNF for 2 hours. The plates were then washed twice with cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, 1.4 mM KH_2PO_4) and the cells were lysed with 250 μl Triton lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mM DTT). The cell debris was removed by centrifugation, and the lysate was used in either a luciferase or β-gal assay. Relative luciferase units were defined as the ratio of β-gal normalized luciferase activity in lysates of stimulated cells relative to the normalized luciferase activity from unstimulated cells.

4.3.1 Measuring luciferase activity

The luciferase activity was measured using a Lumat LB 9507 luminometer. The samples were measured using 50 μl lysate in 350 μl reaction solution (25 mM Gly-Gly pH 7.8, 2 mM ATP, 10 mM $MgSO_4$). 100 μl injection solution (25 mM Gly-Gly pH7.8, 0.2 mM Luciferin) was injected in each reaction.

4.3.2 Measuring β-gal activity

The β-gal activity was measured as follows: 0.4 ml ONPG (20mg/ml in ethanol) and 1.4 μl MeSH was added to 10 ml of the β-gal buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$). 50 μl lysate was placed in a well on a 96 well plate and 150 μl β-gal buffer was added and incubated at 37°C. The reaction was stopped with 50 μl 1 M Na_2CO_3 and the absorbance was measured at 414 nm in an ELISA-reader.

4.4 Biochemistry

4.4.1 Nuclear extract preparation

Following treatment cortical neurons were washed twice with cold PBS, and then lysed on ice for 10' in buffer A (10 mM KOH-Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1mM DTT, PMSF 0.5 mM). Nuclei were collected by centrifugation at 13000 rpm for 1 min and then lysed in the buffer C (20 mM KOH-Hepes pH 7.9, 1.5 mM $MgCl_2$, 417 mM NaCl, 25% glycerol, 0.2 mM EDTA, 1 mM Dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF) 0.5 mM) for 20' on ice. Insoluble material was removed from nuclear extracts by centrifugation at 13000 rpm for 2'at 4°C.

4.4.2 Immunoprecipitation

Immunoprecipitations were performed by incubating 50 μg of nuclear extracts in buffer C, with specific antibodies for 2 hr at 4°C. The antibodies used were the followed: 2 μl goat anti-NeuroD (sc-1086X), 10 μl rabbit anti-Mash1 (provided by JE Johnson), 2 μl 14AA anti-C/EBPα (sc-61X; Santa Cruz Biotechnology), 4 μl C-103 anti-C/EBPβ, 2 μl C-19 anti-C/EBPδ (sc-150X; Santa Cruz Biotechnology), 4 μl Δ198 anti-C/EBPδ (sc-746; Santa Cruz Biotechnology).

Precipitates were collected with Protein A-Sepharose or Protein G-Sepharose (Amersham Pharmacia) for 1 hr at 4°C. The beads were washed three times in buffer C and the protein was eluted on 20 μl of 2X SDS sample buffer incubated at 95°C for 5'. 10% SDS-PAGE gels analyzed Immunoprecipitates. Protein blots were probed ON at 4°C with specific antibodies, followed by incubation with a horseradish peroxidase-coupled

secondary antibody, and analyzed using the ECL chemiluminescence system (Amersham).

To test the interaction C/EBP-Mash1, C/EBP-NeuroD and C/EBP-Myogenin; pCMV□wt, pCMV□BRM1, pCMV□BRM2, pCMV□BRM3, pCMV□BRM5, were transcribed and translated using TNT T7 kit in the presence of unlabelled methionine, for 90'at 30°C. Instead, NeuroD, Mash1 and Myogenin were transcribed and translated in vitro in the same way but in presence of ^{35}S-methionine. After purification by Microcon YM-10, the labeled proteins were incubated with the unlabeled proteins for 1 hr at room temperature, and then immunoprecipitated in NP40 lysis buffer (20mM TRIS pH7.4, 140mM NaCl, 10% glycerol, 1% NP-40 and protease inhibitors) using 1 □l rabbit 14AA anti-C/EBP□, as described above. The proteins were separated using 10% SDS-PAGE. The gels were dried and subsequently exposed to film (BioMax MR-1 film, Kodak) for 12 hr at –70°C.

4.4.3 UV-crosslinking

50 □g of nuclear extract was incubated with polydI-dC for 15' at room temperature. 2x10^{6}cpm of oligo containing the E-box motif of the c-fos promoter and 5Br-dU (Q) in one strand (5' gaggacatcQgcgtccc), was then added and left 20' at room temperature, followed the UV cross-linking in stratalinker (STRATAGENE) five times 5' and in the middle 5'on ice.

At this point the antibodies bound to the beads were added and incubated 2 hours at 4°C, after the normal immunoprecipitation was performed. Mash1 and NeuroD were bound covalently to protein-G beads using dimhetylpimelidate (Sambrook et al., 1989).

4.4.4 SDS-PAGE, Staining, Western Blot

Protein concentrations were determined using the Bio-Rad Dc protein assay (Biorad).

Proteins were usually analyzed on 1 mm thick minigels (Bio-Rad apparatus).

The tris-glycine separating gel contained 10% acrylamide (Bio-Rad, stock solution 30% Acrylamide/bis-acrylamide 37.5:1 ratio), 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.001% N,N,N',N'-tetramethylenediamine (TEMED). The stacking gel contained 4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1%

ammonium persulphate and 0.001% TEMED. The gels were run in Laemmli running buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS) at a current of 20-40 mA per gel. Proteins were transferred onto Protran nitrocellulose (Schleicher & Schuell) using a Hoefer SemiPhor apparatus for 1 hr at a constant mA (1mA/cm^2 gel area) in transfer buffer (20 mM Tris base, 150 mM glycine, 0.1% SDS, 20% methanol). Membranes were stained in 0.2% Ponceau S solution (Serva) and then washed. Unspecific binding was blocked by incubating membranes in 5% non-fat dry milk in PBS containing 0.1% Tween-20 (BioRad) from 1 hr at room temperature.

Antibodies were diluted 1:1000 in PBS, 5% milk, 0.1% Tween-20, added to the blots, and incubated ON at 4^{o}C. After each antibody incubation the membrane was washed 3 times in PBS, 0.1% Tween-20 for a total of 30'. Horseradish peroxidase conjugated secondary antibodies were used and ECL revealed the signal.

4.4.5 GST pull-down assay

In vitro pull down analysis was carried out using a fusion of: C-terminal of C/EBP to GST, 156-358 domain of C/EBP to GST, 1-96 domain of C/EBP to GST and unfused GST (from pGEX4T-1; Pharmacia) (Nerlov and Ziff, 1995).

Mash1, NeuroD and Myogenin were ^{35}S labeled and in vitro translated using pcDNA3 T7 expression vectors, the Promega TNT T7 coupled transcription/translation system and L-^{35}S-methionine (Amersham Biosciences). Bound ^{35}S-labeled proteins were resolved by SDS-PAGE.

4.4.5.1 Purification of GST fusion proteins

The GST-constructs were transformed into a competent BL21 E.Coli strain (Invitrogen) carrying the plasmid pRI952 (Smithkline Beecham) and grown in 25 mg/ml choloramphenicol and 100-mg/ml ampicillin.

A starter culture was incubated ON, then diluted 1:10 and grown 1.5 hr at 30^{o}C. IPTG was added to a final concentration of 0,1 mM and the cells were agitated for 3 hr at 30^{o}C. Cells were pelleted at 4500 rpm, washed in ice-cold STE (10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA), resuspended in 7.5 ml STE with 1 mg/ml lysozime, and incubated on ice for 15'. DTT to a final concentration of 5 mM, 1.25 ml 10% sarcosyl in STE, and one complete mini protease inhibitor cocktail tablet (Boehringer), dissolved in 1 ml

water, were added to the suspension. The suspension was vortexed and sonicated on ice. After sonication, the cell debris was pelleted by centrifugation for 30' at 16000rpm, and the supernatant recovered. TritonX-100, at 2% final concentration, was added to the suspension by vortexing.

Glutathione Sepharose 4B beads (Amersham) were washed in NETN (0.1 M NaCl, 1 mM EDTA, 10 mM Tris pH 8, 0.5% NP40) and resuspended to a concentration of 50%. 200 μl of the bead suspension was added to the lysate and incubated 1 hr at 4°C on a wheel. After incubation the beads were washed 3 times in ice-cold NETN and resuspended in 1ml NETN.

To estimate the relative protein contents of the beads, samples were run on a 12.5% SDS-PAGE gel and coomassie stained (Sigma) according to the protocol supplied by the manufacturer.

4.4.5.2 GST pull downs

Pull down reactions was carried out by preincubating 100μl of the bead suspension (corresponding to about 500 ng protein) with 50μl 10% BSA and 350μl pull-down buffer (50 mM Tris pH 8, 250 mM NaCl 0.5% NP40). The reaction was incubated with gentle shaking for 30' at room temperature before adding 5μl of ^{35}S labeled in vitro translated protein. The incubation was continued for 1 hr. After incubation the beads were washed 3 times with ice-cold NETN, pelleted and then 20μl of 2X SDS buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 125 mM Tris-Cl pH 6.8, 0.2% bromophenol blue) was added. The samples were boiled and the beads were pelleted. The supernatants were run on 12.5% SDS-PAGE gels. The gels were dried and radio labeled protein was detected on a Fuji BAS2040 phosphoimager.

4.5 ChIPs ANALYSIS

Adult mice were perfused with 10 ml of PBS followed by 5 ml of PBS with 1% formaldehyde at 30°C. The forebrains were dissected from the animals and incubated another 5' in a 30°C oven. The reactions were stopped by placing the brains on ice in 10 ml of buffer A (15 mM Hepes, pH 7.6, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, pH 8, 0.5 mM EGTA, pH 8, 0.34 M sucrose, 0.15 mM 2-mercaptoethanol, and 125 mM

glycine) and mincing with small scissors. Crude nuclei were prepared by Dounce homogenization and centrifugated at 4.000 rpm for 10'followed by a subsequent centrifugation at 4000 rpm for 10' in 1.5 ml of 1:1 buffer A: buffer B (15 mM Hepes, pH 7.6, 60 mM KCl, 15 mM NaCl, 0.1mM EDTA, pH 8, 0.1 mM EGTA, pH 8, 2.1 M sucrose, and 0.15 mM 2-mercaptoethanol). Nuclei were resuspended in 5 ml of sonication buffer (50 mM Tris, pH 8, 2 mM EDTA, pH 8, 0.5% N-lauroylsarcosine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 2 mg/ml aprotinin), then incubated 5' at room temperature, 5' on ice, and sonicated with a Bondelin Sonoplus GM200 sonifier mounted with a microtip for two 20-s pulses (duty cycle constant; output 40%). Cross-linked chromatin was separated in a CsCl step gradient (1 ml of 1.75 g/ml, 1 ml of 1.5 g/ml, and 1 ml of 1.35 g/ml CsCl in 0.5% N-lauroylsarcosine and 1mM EDTA, pH 8) at 31000 rpm at 20 ^{o}C for 20 hr. Peak DNA-containing fractions, identified by agarose gel electrophoresis and ethidium bromide staining, were pooled, treated with 25 ng/ml RNAse A for 2' at 37 ^{o}C, and then dialyzed against Tris-EDTA (10 mM Tris, pH 8, and 1 mM EDTA). The purified, cross-linked chromatin was aliquot and stored at -80 ^{o}C. The cross-linked chromatin was dialyzed ON in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS). After a preclearing step with a salmon sperm DNA/Protein A agarose Slurry (50% slurry Upstate), 100 µg of purified chromatin was incubated ON at 4 ^{o}C, with rotation either alone or with 5 µl of rabbit antiserum against Mash1, or 2 µl of rabbit antiserum against C/EBPα, C/EBPβ, NeuroD (Santa Cruz), or 2 µl histone H3 (Abcam), or 2 µl acetylated histone H3 (Upstate Biotechnology). The immuno complex was collected with 60 µl of salmon sperm DNA/Protein A Agarose Slurry for 1 hr at 4 ^{o}C with rotation. Bound material was recovered by centrifugation at 2500 rpm for 2', and immunoprecipitated products were washed three times with 1 ml of RIPA buffer. 100 µl of Tris-EDTA was then added and incubating at 68 oC for 3 hr reversed the crosslinking. To analyze the DNA, the material was treated with proteinase K at 45^{o}C, extracted with phenol:chloroform, and ethanol-precipitated in the presence of 10 µg of the glycogen as carrier. The final immunoprecipitated DNA products were resuspended in 20 µl of Tris-EDTA for PCR analysis.

Each PCR amplification was performed using 2 μl of the immunoprecipitated DNA in a final volume of 50 μl using 50 pmol of each primer, 200 mM of each dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer (Applied Biosystem), 2.5 units of Taq DNA polymerase, and 2.5 % DMSO. The PCR conditions were: initial denaturation at 94°C for 5', 30 cycles of 94°C for 30", 58°C for 20", 72°C for 10"; and final elongation at 72°C for 5'. Amplification products were analyzed on 2.5% agarose gels. The PCR primers were: 5'gctgcaccctcagagttgg3', 5'ccgtggaaacctgctgac3'.

4.6 Immunocytochemistry

Cortical progenitors derived from cerebral cortices of E12.5 mouse embryos were stimulated after 6 DIV with 50 ng/ml of BDNF for 15' at 37 °C. The cells were then fixed in 4% PFA for 20'. Washes were performed with 10 mM PBS/glycine to neutralize the PFA. The cells were permeabilized with 0.5% NP-40/PBS for 5'. Blocking solution was added for 1 hr (10% NHS, 0.3% carragenin, 0.5% tritonX100 in 50 mM TBS) at room temperature, followed by incubation with a rabbit polyclonal anti-pC/EBPβ(Thr 188), diluted 1:500 (cell signaling) in 1% NHS, 0.3% carragenin, 0.5% TritonX100 50 mM TBS, ON at 4 °C. Immunostainings were visualized using a secondary antibody conjugated to Texas Red dye (Jackson lab) diluted 1:200 in TBS. The slides were mounted with Vectashield mounting medium containing DAPI (Vector H2000).

4.7 Generation and genotyping of mice

For the generation of mGif/TIEG1 mutant mice, two clones derived from each construct, null allele and conditional allele, were injected into C57BL/6 blastocysts and the resulting chimeras were bred for germline transmission. Agouti animals were genotyped, in order to distinguish the heterozygous from wild-type animals, using the southern blot analysis as described for the ES cells screening.

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