INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Analysis of the Rat Tα1 α-Tubulin Gene Promoter

David Howard Rogers

Centre for Neuronal Survival

Department of Neurology and Neurosurgery

Montreal Neurological Institute

McGill University

Thesis submitted May 2000.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of doctor of Philosophy.

Abstract

The mature nervous system is composed largely of two cell types, glial cells and postmitotic neurons. All potmitotic neurons of the mature nervous system derive from proliferating neural precursor cells. To generate a neuron, a precursor must cease dividing and express a number of genes that are characteristic of the neuronal phenotype. How these changes in cell behaviour and phenotype are brought about in mammals is still poorly understood.

This thesis describes experiments that were designed to explore cell intrinsic mechanisms regulating the generation of neurons from neural precursor cells. Specifically, the regulatory region of the rat *Tα1* α-tubulin gene, which encodes an isoform of α-tubulin expressed in neurons throughout the nervous system immediately following cell cycle exit, was analyzed to identify DNA sequences directing early neuronal gene expression.

A novel 10-nucleotide regulatory sequence, named the neuronal restriction element (NRE), has been identified. In the context of the *Tα1* gene, the NRE inhibits precocious expression in neural precursor cells. Interestingly, the NRE is conserved in the *α-1* α-tubulin gene and is found in a number of neural genes expressed widely and early in development. As such, the NRE may affect the onset time of a battery of neuronal genes and modulate the timing of neuronal differentiation. *In vitro*, the NRE binds Su(H), a highly conserved transcription factor involved in the repression of neuronal differentiation.

A second novel regulatory element has been identified, the forebrain response element (FRE), which acts to enhance gene expression specifically in the neocortex. The FRE overlaps the NRE and also contains a conserved 30-nucleotide sequence constituting a putative homeodomain recognition sequence. We speculate that the FRE consists of two subelements that act synergistically to promote gene expression in newborn and mature neocortical neurons.

Résumé

Il y a deux types de cellules dans le système nerveux; les cellules gliales et les neurones postmitotiques. Tous les neurones du système nerveux sont dérivés à partir de cellules progénitrices neuronales. Afin de générer une neurone, une ceullule progénitrice neuronale doit arreter sa division et commencer d'exprimer des gènes charactéristiques du phenotype neuronal. Cépendant, la façon dont ces changement cellulaires se présentent dans les mammifières n'est pas determinée.

Cette thèse décrit des expérients conçus afin d'examiner les méchanismes cellulaires qui sont responsables de contrôler la génération des neurones à partir de progénitrices neuronales. En particulier, la régionregulatrice du gène Tα1 α-tubuline, qui est éxprimée par les neurones du système nerveux immédiatement après leur sortie du cycle cellulaire, a été analysée.

On a identifié une nouvelle séquence régulatrice de 10bp, qu'on a appelée "l'élément de réstriction neuronal" (NRE). En plus, on a démonstré que dans le contexte du gène Tα1, le NRE est requis pour la répression de l'expression précoce des gènes neuronaux. Cette séquence de 10bp est bien conservée, et se retrouve dans plusieurs gènes neuronaux. Ensemble, ces résultats suggèrent que le NRE peux moduler le debut de l'éxpression des gènes neuronaux et le chronométrage de la différentiation neuronale.

"Supressor of Hairless(Su(H)), un facteur de transcription, se lie dans la region du NRE. Cela suggère que Su(H) est possiblement impliqué dans la répression de l'activité du promoteur Tα1 dans les précurseurs neuronaux et les neurones immatures par son abilité a se lier dans le NRE.

Une deuxième séquence règlementaire à était identifiée. Celle-la contient une module d'activation de la transcription, spécifique au néocortex. Cette séquence, nommée "l'element de résponse de l'avant-cerveau" (FRE), inclus le NRE, en plus d'une séquence conservée de 30bp qui sert comme endroit de reconnaissance canonique pour les facteurs

à homéodomaine. L'hypothèse est que le FRE contient deux éléments qui se comportent ensemble à stimuler l'activation de la transcription dans le néocortex.

Acknowledgements

I would like to thank my supervisor, Dr. Freda Miller, for her support, encouragement, and thoughtful guidance in these matters and others over the course of my Ph.D. It goes without saying that I owe a great debt to all members of the Miller laboratory, past and present. Above all, I would like to thank them for their friendship and for making my time in Montreal so wonderful.

To the other members of my graduate committee, Drs. Alan Peterson and Stefano Stifani, I offer my gratitude for their time, sincerity, and guidance. A "good teacher" is difficult to define, but easy to recognize. My committee consisted of wonderful teachers, and each of them taught me something new.

Through daily interactions with Drs. Stifani and Miller, I have gained an appreciation for the scientific method rigorously employed, and have grown envious of the understanding, perspicacity and confidence they posses which makes them such remarkable scientists and people. I was very lucky to have been in this place at this time, and I can't thank the two of them enough.

My family has supported me in all ventures, but I imagine this one has made them more proud than some of the others. And so, this thesis is dedicated to my mother and father.

And there is one other, my wife Shernaz, whose effect on me could never be recorded. I met her in the lab, and we will always look back on our time in Montreal with fond memories.

Table of Contents

Claims for Originality

Three novel findings are reported in this thesis, all of which stem from an analysis of the previously defined *Tα1 α-tubulin* gene promoter. First, deletion of a 66-nucleotide segment of the *Tα1* promoter, the activity of which is normally restricted to postmitotic neurons, may have led to precocious promoter activity in neural precursor cells. As such, the 66-nucelotide segment may constitute a repressor of precocious neuronal gene expression. It is argued that a 10-nucleotide sequence, the NRE, located within the 66-nucelotide segment may contribute to the repressive activity ascribed to the segment. The importance of the NRE is inferred from its conservation within the *α-1 α-tubulin* gene. It is located in a similar position in the goldfish *α-1 α-tubulin* gene and may function as a transcriptionally repressive sequence in this context. In addition, the location of the NRE in several neuronal genes, either alone or at the core of the previously described neuronal restriction silencing element (NRSE), suggests it may be widely involved in the regulation of neuronal gene expression. Moreover in the context of the neural *L1* gene, the NRE has been shown to be required for the repression of precocious promoter activity. The identification of the NRE highlights a possible scheme for constituent neuronal gene repression prior to neuronal differentiation, one which may contribute to the timing of neuronal differentiation. Regardless of mechanism, these studies have potentially unmasked transcriptional activity in neural precursor cells that may impinge on neuronal genes prematurely and which may normally require early opposition.

The second finding in this thesis supports the argument for NRE function by providing a possible link between the NRE and a transcriptional repressor known to be required for the repression of precocious neuronal gene expression in mammals, namely the *"suppressor of hairless/recombination signal-binding protein-Jk"* gene product (Su(H)/RBP-Jk). Su(H)/RBP-Jk bound the NRE in a sequence specific manner *in vitro*. Though the relevance of the interaction has yet to be demonstrated, its potential importance is intimated by the fact that *Su(H)/RBP-Jk* has been evolutionarily conserved in sequence and to some extent function, as it is required for the inhibition of ectopic

neuronal differentiation in both Drosophila and C. elegans and the repression of precocious neuronal differentiation in mammals. Further, the putative Su(H)/RBP-Jk binding site has been conserved in the *α-1 α-tubulin* gene. The intrinsic repressive activity of Su(H)/RBP-Jk and the situation of the NRE in many neuronal genes may in part explain why the targeted disruption of *Su(H)/RBP-Jk* in transgenic mice led to precocious neuronal gene expression and differentiation in the developing nervous system. *Su(H)/RBP-Jk* may provide critical negative regulation of neuronal genes and sit at a pivotal point in the transcriptional hierarchy governing neuronal differentiation. Furthermore, by virtue of its conservation in the Notch signaling cascade, a connection between Su(H)/RBP-Jk and the NRE potentially places constituent neuronal genes under direct control of the Notch pathway, which is believed to repress neuronal differentiation early, and inhibit neurite outgrowth following differentiation. With respect to differentiation, this would represent a new and direct mechanism of regulation by the Notch pathway, which is presently believed to repress neuronal differentiation solely through the negative regulation of differentiation-promoting transcription factors, which in turn regulate neuronal genes. The mechanisms responsible for the repression of neurite outgrowth have yet to be discerned.

Third, in both embryos and adults, deletion of the same 66-nucleotide segment of the *Tα1* promoter diminished activity specifically in the neocortex while deletion of an encompassing 184-nucleotide segment led to a further decrease in activity specifically in the neocortex. The deletions affected neocortical gene expression in both embryos and adults, implicating a single regulatory module in the regulation of gene expression in immature and mature neocortical neurons. It is argued that the 184-nucleotide segment contains a bipartite cis-regulatory module that contributes the neocortical aspect of activity to the pan-neuronal and neuron-specific activity of the *Tα1* promoter. We have named this 184-nucleotide sequence the forebrain response element (FRE). This novel finding groups neocortical neurons together molecularly, suggests that they share common transcriptional activity, and suggests that neuronal differentiation in the neocortex may be regulated by a unique transcriptional mechanism. It further suggests that neuronal gene expression in mature neocortical neurons is regulated by

transcriptional mechanisms similar to those operating in developing neocortical neurons. When compared to the expression patterns of several developmentally important neural transcription factors, this molecular grouping and the sequences contained within the 184-nucleotide segment suggest a possible mechanism for the activity changes that resulted from the deletions. Regardless of mechanism, these studies have identified a potentially useful tool for manipulating gene expression in this very important population of neurons.

In summary, this thesis identifies a novel element that putatively represses precocious neuronal gene expression in neural precursor cells. It further suggests a novel role for *Su(H)/RBP-Jk*, and possibly the conserved Notch pathway, in the timing of neuronal differentiation via this novel mechanism of neuronal gene repression. This mechanism may also be relevant to the Notch pathway's purported role in the repression of neurite outgrowth in mature neurons. Finally, it supports the hypothesis that neuronal differentiation is not globally regulated by identifying a novel regulatory module involved in neuronal gene expression specifically in the neocortex. Molecular division within the nervous system is thus revealed using the regulatory region of a structural pan-neuronal gene and suggests that neocortical neurons may depend on transcriptional regulatory mechanisms unique to this neuronal subtype for the regulation of neuronal differentiation.

List of Abbreviations

Ac, anterior commissure

Am, amygdala

AS-C, acahete-scute complex

BF-1 Bf-2, brain factor one, brain factor two

bHLH, basic helix-loop-helix

bp, base pair

Cc, corpus callosum

cer, cerebellum

Cg, cingulum

CNS, central nervous system

Cpu, caudate and putamen

Da, daughterless

Den, dorsal endopiriform nucleus

E, embryonic day

Elav, embryonic lethal abnormal vision

Emc, extramacrochaete

Ems, empty spiracles

EMSA, electrophoretic mobility shift assay

E(spl), enhancer of split complex

Er, entorhinal cortex

Eya, eyes absent

Gcm, glial cells missing

GMC, ganglion mother cell

Gp, globus pallidus

H, hairy

HDAC, histone deacetylase

Hes, hairy enhancer of split homolog

Hi, hippocampus

Hyp, hypothalamus

kb, kilobase

lv, lateral ventricle

Mash, mammalian achaete -scute homolog

Math, mammalian atonal homolog

MGE, medial ganglionic eminence

Nctx, neocortex

Ngn, neurogenin

NRE, neuronal restriction element

NRSE, neuronal restriction silencing element

NSCL, neurological stem cell leukemia factor

Otx, orthodenticle

Pi, piriform cortex

P, pons

PNS, peripheral nervous system

Pros, prospero

RA, retinoic acid

REST, RE1 silencing transcription factor

Rsg, retrosplenial granular cortex

S, septum

Sc, superior colliculus

SOP, sensory organ precursor

SMRT, silence mediating repressive transcription factor

Su(H)/RBP-Jk, supressor of hairless, recombination signal binding protein

SVZ, subventricular zone

Vp, ventroposterior thalamic nuclei

WRPW, tryptophan arginine proline tryptophan

3v, third ventricle

β-gal, beta-galactosidase

List of Figures

Figure 12: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Expression in the Midbrain

Figure 13: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Expression in the Cerebellum

Figure 14: Distribution of Limited β-gal Activity in the Neocortex of *ΔFRE-nlacZ* and *ΔNRE-nlacZ* Mice

Figure 15: Ectopic β-gal Expression is Detected in the Kidney in *ΔNRE-nlacZ* Line 9

Figure 16: Electrophoretic Mobility Shift Assays

Figure 17: Developmental Distribution and Tissue Distribution of NRE and RBP-Jk Consensus Sequence Binding Complexes

Figure 18: Cross Competition Suggests the NRE and RBP-Jk Consensus Sequence Binding Complexes are Related

Figure 19: *In Vitro* Translated RBP-Jk Binds the NRE

Figure 20: NRE-Binding Complex in E10.0 and Cultured Cortical Precursor Extracts Co-Migrates with *In Vitro* Translated RBP-Jk/NRE Complex

Figure 21: Anti-RBP-Jk Antibody Shifts E13.5 NRE-Binding Complex

Figure 22: *In Vitro* Translated RBP-Jk/NRE Complex Co-Migrates with E13.5 NRE-Binding Complex and is Similarly Supershifted by Anti-RBP-Jk Antibody

Figure 23: *In Vitro* Translated REST does not Bind the NRE

Contribution to Papers

Gloster A., El-Bizri H, Bamji S. X., Rogers D., Miller F. D. 1999. Early induction of *Tα1 α-tubulin* transcription in neurons of the developing nervous system. J. Comp. Neurol. 405(1): 45-60.

I took new pictures of old sections and stained and photographed new sections in order to improve the photographic quality and resubmit this paper. I contributed figures 1-7 to the final copy, though I did not perform the original analysis of *Tα1-nlacZ* mice described in the paper.

Toma J. G., Rogers D., Senger D. L., Campenot R. B., Miller F. D. (1997) Spatial Regulation of neuronal gene expression in response to nerve growth factor. Dev. Biol. 184(1) : 1-9.

On this project, I was involved in the Northern blot analysis of neuronal gene expression. I harvested cultures along with Dr. Jean Toma, and we performed RNA extractions and Northern blot analyses together.

Publications

Refereed Papers

Gloster A, El-Bizri H, Bamji S X, **Rogers D**, Miller FD (1999) Early induction of *Tα1 α-tubulin* transcription in neurons of the developing nervous system. J. Comp. Neurol. **405**(1): 45-60.

Toma JG, **Rogers D**, Senger DL, Campenot RB, Miller FD (1997) Spatial Regulation of neuronal gene expression in response to nerve growth factor. Dev. Biol. **184**(1) : 1-9.

Book Chapters:

Miller FD, **Rogers D**, Bamji SX, Slack RS, Gloster A (1996) Analysis and manipulation of neuronal gene expression using the *Tα1 α-tubulin* promoter. Seminars in Neurosci. **8**:117-124.

Published Abstracts

Rogers D, Laferriere N, Brown D, Peterson A, Yang X-M, El-Bizri H, Gloster A, Miller FD (1997) Transcriptional regulation of a neuron-specific pan-neuronal gene. Soc. Neurosci. Abst.

Yang X-M, **Rogers D**, Gloster A, El-Bizri H, Peterson A, Nepveu A, Miller FD (1997) Cux homeodomain protein binds to the homeodomain consensus element within the *Tα1 α-tubulin* promoter which is required for cortical expression in transgenic mice. Soc. Neurosci. Abst.

Rogers D, Gloster A, Laferriere N, Brown D, Peterson A, Miller FD (1995) Identification of cis-elements in the *Tα1* promoter responsible for neuron-specific gene expression in transgenic mice. Soc. Neurosci. Abst.

Rogers D, Laferriere N, Gloster A, Brown D, Miller FD (1994) Analysis of cis-elements in the Tα1 α-tubulin promoter. Soc. Neurosci. Abst.

Toma JG, **Rogers D**, Campenot RB, Miller FD (1993) Neuronal responses to NGF are spatially regulated: NGF exposure at terminals versus cell bodies differentially alters neuronal gene expression. Soc. Neurosci. Abst.

Rationale

In the mammalian nervous system, neurons are generated from proliferating neural precursor cells. The molecular mechanisms governing cell cycle exit and neuronal differentiation are poorly understood. The present studies were undertaken to probe the intrinsic genetic mechanisms that underlie these developmental changes. The appropriate timing and placement of neuronal differentiation is presumed to be paramount to the formation of an integrated nervous system and fidelity of function. Such understanding may yield insight into mechanisms responsible for naturally occurring developmental abnormalities, as well as pathologic states in which cell growth and differentiation are perturbed. Further, with the advent of cell replacement therapy in the mature nervous system and the use of stem cells in such procedures, an understanding of the mechansims regulating neuronal differentiation may facilitate the direction of pluripotent precursor cells towards desired cell fates.

This thesis focuses on the genetics of neuronal differentiation. The outlined experiments were undertaken to examine intrinsic genetic mechanisms that regulate early differentiation events that immediately follow or are coincident with cell cycle exit in developing neurons. Analysis of the regulatory regions of neuronal genes has been used to probe mechanisms of neuronal differentiation. This approach seeks to identify important transcription factors through the identification of important target sequences. I have used the previously characterized 1100-nucleotide *Tα1 α-tubulin* gene promoter (Gloster et al 1994) for such analysis.

The rat *Tα1* gene encodes a pan-neuronal and neuron-specific isoform of α-tubulin that is expressed as a function of neuronal growth. The 1100-nucleotide *Tα1* promoter specifies a neuron-specific and pan-neuronal pattern of gene expression in transgenic mice. This 1100-nucleotide fragment has been used to identify cis elements that confer early pan-neuronal and neuron-specific activity, thereby probing transcriptional mechanisms involved in neurogenesis.

Two deletions have been made in the *Tα1* promoter and the resultant mutant promoter sequences have been fused to reporter genes and used to generate transgenic mice. Analyses of mutant promoter activity were conducted both *in vitro* and *in vivo*. Neural precursor cells were cultured from the immature embryonic neocortex of transgenic mice, and transgene expression was monitored as cells underwent neuronal differentiation and cell cycle exit *in vitro*. *In vivo*, transgene expression was assayed at different developmental stages in whole embryos and in dissected adult tissues.

These experiments have led to the identification of important cis-sequences and to a potentially important interaction between one of these sequences and a highly conserved transcription factor. These findings have been evaluated in the context of our current understanding of regulatory mechanisms, and new conclusions about the coordinated control of neuronal differentiation in the developing embryo have been drawn. Specifically, these novel findings suggest that neuronal differentiation may involve the derepression of neuronal genes, that this mechanism may involve the transcription factor Su(H)/RBP-Jk, and that neocortical neurons posses unique transcriptional activity that may contribute to the regulation of their differentiation.

Introduction

(A) Overview

Studies using a variety of organisms have revealed that transcription factors play critical roles at multiple stages of neuronal development. Genetic analyses performed in Drosophila melanogaster have yielded many transcription factors that function early in neural specification. Cell manipulation studies and the analysis of mosaic mutants has further revealed that local intercellular communication regulates neural specification via the modulation of such regulatory transcription factors. Importantly, many of the transcription factor-encoding genes identified and the intercellular signaling components regulating them are conserved in vertebrates and appear to contribute, often in a similar manner, to the regulation of vertebrate neurogenesis. Drosophila studies have thus been fruitful not only in identifying particular genes and gene families, but also in revealing molecular mechanisms relevant to vertebrate neural development.

Piecing together the molecular mechanisms identified in Drosophila, neural specification may be viewed as a series of successive molecular events. Beginning with a sheet of equivalent and uncommitted cells constituting the ventral ectoderm, a number of genes, many of which encode transcription factors, act in succession to progressively create and distinguish neural progenitor cells from epidermal progenitor cells within this domain. The choice of which cells become neural and which become epidermal is determined by local intercellular communication involving the "neurogenic" genes, several of which encode transcription factors. The domain of action of the neurogenic genes is established by upstream transcription factors, which in a coarse manner demarcate potential neural territory within the early ventral ectoderm, and direct the neurogenic genes to divide the potential neural territory into dermoblasts and neuroblasts with the high resolution of a locally acting system. This high resolution occurs in part because the neurogenic genes are regulated by the intercellular communication they initiate, thus refining their own expression within proneural domains. The early neural potentiating transcription factors

that establish proneural domains are members of the "proneural" gene family and are individually expressed in distinct but partially overlapping subdomains of the ventral ectoderm. Collectively, the proneural genes regulate neurogenesis throughout the embryo. Their expression is progressively restricted to and increased within a subset of cells in the proneural domains, the presumptive neural progenitor cells, through the action of the neurogenic genes. Thus the proneural genes initially regulate, and are subsequently regulated by, the neurogenic genes. The early phase of proneural gene expression, and thus neural potential, in turn falls under the control of a collection of spatially restricted transcription factors, or "pre-pattern genes", which translate positional information into a pattern of potential neural territory.

Viewed in this way, neural specification involves a hierarchy of transcription factors that act in succession to translate spatial information into the local specification of cell fate. Early pre-pattern genes converge to some extent on a set of "proneural genes" which in turn confer neural potential to partially-overlapping subdomains of the ectoderm. The proneural genes initiate local signaling events within these subdomains via the neurogenic genes. The neurogenic genes control neural specification locally with intercellular communication by restricting subsequent proneural gene expression to presumptive neural progenitor cells via transcriptional regulation.

While Drosophila studies have provided a great deal of incite concerning invertebrate neural specification and the events leading to it, they have been less revealing about the proximate regulation of neuronal differentiation. Enhancer trap analysis has identified genes commonly expressed in neuroblasts, the so-called "neural progenitor genes", and at least one gene commonly expressed in newborn neurons. The existence of such factors suggests that neuronal differentiation may involve convergence upon molecular mechanisms common for the cell type. However, the mechanisms involved are unclear, and regulation in the mammalian neural lineage following neural specification (including neuronal differentiation) appears to involve many genes of the type regulating neural specification in Drosophila.

Neural specification in the mammalian presumptive CNS occurs by a fundamentally different mechanism involving signals derived from the organizer and underlying mesendoderm during gastrulation. Following neural specification, the neural fate appears to be obligatory given the environment that cells are subsequently restricted to. It is possible however that genes similar to those involved in Drosophila neural specification are involved in sustaining the neural fate in the CNS following neural induction. In the presumptive PNS, neural specification may indeed occur by mechanisms similar to those in Drosophila, where alternative non-neural cell fates are an option even after neural specification has occurred in the neural plate. Regardless of specification mechanisms, in both the CNS and PNS cellular development following neural specification appears to involve genes similar to the Drosophila proneural genes.

Mammalian and invertebrate neuronal differentiation may be regulated in fundamentally different ways. Mammalian neural progenitors on average exhibit a comparatively longer period of time and a greater number of cell divisions between the acquisition of neural competence and terminal neuronal differentiation and generate a greater variety of neuronal subtypes. In Drosophila, the generation of neurons may follow in a relatively simple manner from neural specification, involve a relatively small number of intermediate stage genes, and perhaps draw on spatial determinants as has been suggested previously. In mammals, this does not appear to be the case, as early progenitor cells have the potential to go through many cell divisions, change their phenotype and behaviour as they pass through developmental stages, migrate great distances through different environments before reaching their final destinations, and ultimately give rise to many cells of different types and subtypes. In essence they may display more natural variability and generate more descendants in vivo than their Drosophila counterparts, and more proximate mechanisms for regulating neuronal differentiation may be necessary.

Vertebrate studies have begun to fill the gap between neural specification and neuronal differentiation with genes that are sequentially expressed, and progressively drive progenitor cells through to neuronal differentiation. Studies in Xenopus have contributed a great deal to this understanding, and homologs of many such "bridging" Xenopus genes

have been isolated from mammals. Moreover, many of the genes thus isolated are similar in form and ability to the "proneural" Drosophila genes, suggesting that amplification of prototypic transcription factor genes involved in neural specification may have occurred to provide a larger number of factors for regulation at distinct stages in the protracted process of neurogenesis in vertebrates. The limited analysis of these transcription factors to date has suggested that they differ subtly in function and not simply in expression.

The "neurogenic" genes have also been conserved in mammals, although the morphology of the developing mammalian nervous system provides them with a very different setting within which to function. While the ectodermal placodes resemble the Drosophila nervous system to some extent, the mammalian neural tube is a very different and privileged environment where resident cells have already been specified as neural. Furthermore, within the multi-layered neural tube, regions of cell proliferation and differentiation are segregated, and progenitor cells can divide many (and possibly variable) times in proliferative zones as neural specified cells before leaving the zone and differentiating. Genetic analyses have however suggested related functions for neurogenic gene homologs, as there are similar neural consequences for the manipulation of homologous neurogenic genes in different organisms. Furthermore, molecular interactions and relationships among neurogenic gene products appear to have been conserved. Mechanistically, the products of the neurogenic genes appear to collectively constitute a pathway from the cell membrane to the nucleus. Functionally, this pathway appears to negatively regulate neuronal differentiation, either directly or indirectly. In Drosophila, these genes function early to regulate neural specification and may function later to inhibit neuronal differentiation in support cell precursors. In the mammalian CNS, these genes function later and appear to influence the choice of whether or not neuronal progenitor cells differentiate.

The primary transcription factor in the signaling cascade deployed by the neurogenic genes is encoded by the highly conserved suppressor of hairless *Su(H)* gene. Interestingly, much is known about mammalian Su(H) protein activity, as viral studies have focussed on it due to its involvement in the transformation of B cells following

Epstein Barr virus infection. Targeted disruption of the *Su(H)* gene in mice has revealed a conserved role in mammalian neurogenesis as well.

Gene disruption in transgenic mice has played a critical role in the analysis of mammalian neurogenesis. Interestingly, the disruption of genes encoding regionally restricted transcription factors and genes involved in local intercellular communication within the nervous system has produced a number of regionally restricted neural defects. In many cases, the processes affected by the lack of function of the disrupted gene are unclear. However, these findings suggest that neuronal differentiation may be regulated as a function of position by transcriptional mechanisms. In this scenario, it might be suggested that regionally restricted transcription factors converge on a limited set of more widely expressed proneural-like genes, similar to the manner in which spatially restricted transcription factors converge on proneural genes in Drosophila to establish proneural domains. However, some of these mammalian proneural factors also appear to confer subtype specificity, as Drosophila proneural genes do in developing sensory organs. Whether subtype specification is separable from cell type specification is a matter of debate. A specific Drosophila mutant, and transplant studies in vertebrates have suggested that cell type and position-based cell subtype are separable.

Perhaps the most cellularly diverse and complicated region of the nervous system, where regional control of differentiation is of great interest, is the telencephalon. Many studies have focussed on the molecular division of the telencephalon based on transcription factor expression profiles, combined these profiles with morphological analysis throughout development, consequently broken the telencephalon into primordial morphogenetic units, and generated an hypothesis for telencephalic development based on these divisions. While redundancy may cloud the functional analysis of such transcription factors, certain mutations have yielded profound phenotypic alterations in the developing brain and provided evidence for the spatial regulation of neurogenesis and the specification of discrete brain structures. Furthermore, at least one of these genes has been found to be involved in a human syndrome characterized by morphological abnormalities in the brain. While the differential expression of transcription factors may

be reflective of subdivisions in the CNS, whether these divisions reflect differences in neurogenic mechanisms between populations is not clear. Exactly how to group cells in this region of the nervous system based on shared developmental mechanisms rather than gene expression is unclear. Different cells may use different factors for the same purpose. By this reasoning, a true description of "mechanistic cohorts" might come from the analysis of what cells are able to do (as pertains to neuronal gene expression) with what they express, as opposed to what transcription factors they express.

An approach complimentary to the direct analysis of transcription factors involves the analysis of regulatory regions of transcriptionally regulated neuronal genes. This approach attempts to identify important transcription factors from the identification of important target sequences. In some instances the two approaches have met and important cis sequences situated in neuronal genes appear to be target sites of conserved transcription factors with critical roles in neurogenesis. Analysis of the *Tα1* promoter appears to have provided such a window for regarding mechanisms of regulation.

Neuronal differentiation involves multiple processes, including morphological differentiation, expression of differentiation-stage genes, and cell cycle exit. Whether these are separate events or are bundled molecularly is under investigation. Given the extensive proliferation evident in the mammalian nervous system as well as the apparent exclusiveness of cell proliferation and differentiation, it is perhaps not surprising that molecules involved in mammalian cell cycle regulation also affect neurogenesis. What is interesting is how direct these effects may be and how terminal mitosis and other aspects of neuronal differentiation may be molecularly tied to the cell cycle machinery.

Finally, the neural lineage appears to diverge very early, as differences in potential are observed as early as the neural plate stage. Lineage divergence is also observed subsequently at various times during development and in various sublineages. Together, lineage divergence at various developmental points, multiple stages of progenitor cell development, and the non-uniform nature of neuronal differentiation throughout the

nervous system have led to the conclusion that the "progenitor cell" population at any time is a collection of related but distinct cell types.

(B) Literature Review

(i) Neurogenesis in Drosophila

(a) Overview

Much of what we know today about the molecular mechanisms regulating neurogenesis in mammals stems from earlier studies of neurogenesis in Drosophila melanogaster. Neurons in CNS and PNS of Drosophila are derived from ectoderm in a process that involves the separation of their precursors from those committing to the epidermal lineage (Campos-Ortega and Hartenstein 1985). Genetic and phenotypic analyses have revealed a number of loci required for the appropriate allocation and segregation of epidermal and neural progenitor cells in the CNS and PNS. Beginning with an epithelial sheet of approximately 1800 cells, the primordium of the embryonic CNS (with the exception of the brain) is formed in three waves of neurogenesis over a period of approximately three hours. Approximately 25% of the epithelial cells form neuroblasts which delaminate from the epithelial sheet in a stereotypical manner and move inward towards the mesenchyme. Neuroblasts divide asymmetrically to generate one ganglion mother cell (GMC) and another neuroblast. The GMC divides symmetrically and terminally to give rise to two neurons, while the sibling neuroblast divides again in stem cell-like fashion to generate another neuroblast and a GMC (Campos-Ortega, 1994). The embryonic PNS is formed from a different region of ectoderm, but also involves a physical segregation of neuroblasts (sensory organ precursors "SOPs" from the remaining epithelium (Bodmer et al 1989, Hartenstein 1988). Upon segregation, SOPs undergo several rounds of asymmetric division to generate sensory neurons and their associated structures. In the developing adult PNS, ectodermal cells set aside during embryogenesis (imaginal discs) undergo a similar process whereby neuroblasts (SOPS) are segregated from an epithelial sheet (Modolell 1997). The SOPS thus generated divide

asymmetrically and go on to form neurons and associated sensory structures of the adult PNS, much like the SOPs of the embryonic PNS (Bodmer et al 1989).

Early genetic analyses of Drosophila development identified many loci involved in early stages of neuronal development. These loci appeared to participate widely in the specification and segregation of neuroblasts from dermoblasts, generally in both the CNS and PNS. Subsequent analyses identified genes at, and in some cases multiple genes within, these loci. Most of the genes identified and characterized have been divided into three classical groups, namely proneural genes, neurogenic genes, and neural progenitor genes. These groups of genes are believed to act sequentially to progressively distinguish neuroblasts from dermoblasts, render them competent to generate neurons and associated cells, and to drive cell differentiation (Campos-Ortega and Jan 1991, Salzberg and Bellen 1996). More recent work has identified a number of genes that lie outside these classical divisions but also contribute to these processes and are important for neurogenesis. Included in this group are genes that repress neuronal gene expression within and outside the nervous system, which may contribute to the appropriate timing of neuronal differentiation and the generation of cellular diversity, respectively. Prevalent among all of these groups of genes, classical and non-classical, are those that encode transcription factors. It appears that a transcriptional hierarchy exists in which a cascade of transcription factors specifies neuroblasts and pushes them towards the generation of descendant neurons. Importantly and as discussed in subsequent sections, many of these genes are conserved and function in vertebrate neurogenesis (Salzberg and Bellen 1996).

(b) The Proneural and Neurogenic Genes

The "proneural genes" are collectively required for embryonic CNS and PNS development, as well as the development of adult sensory structures (Jimenez and Campos-Ortega, 1979, 1987, Campos-Ortega and Jimenez 1980, White 1980, Dambly-Chaudiere and Ghysen 1987). All but one of the genes identified in this group lie within the *achaete-scute complex* locus *(AS-C)* (Campuzano et al 1985, Alonso and Cabrera

1988, Balcells et al 1988, Gonzales et al 1989, Caudy et al 1988b, Cronmiller et al 1988). Four distinct transcription units arising from this locus have been identified. These transcripts encode related factors which share the basic helix-loop-helix motif (Villares and Cabrera 1997, Alonso and Cabrera 1988, Gonzales et al 1989), a protein motif common to DNA-binding proteins and originally recognized in the proto-oncogene c-myc (Murre et al 1989a). The basic region of the motif mediates DNA binding while the HLH portion of the motif facilitates protein-protein interactions, which are required for DNA binding by this class of transcription factors (Murre et al 1989b, Cabrera et al 1991). The products of the *AS-C* encode sequence-specific DNA binding proteins, and activate transcription in the context of a number of identified target promoters. These proteins heterodimerize with the ubiquitously expressed *daughterless* gene product (Da), also a bHLH factor, and recognize the DNA sequence CANNTG commonly referred to as the E-box (Murre et al 1989b, Cabrera and Alonso 1991). Expression studies and functional analyses suggest that *AS-C* genes serve unique roles, but exhibit similar activity and can substitute for one and other to varying degrees. The mapping of transcripts (and some proteins) expressed from the *AS-C* has revealed widespread and partially overlapping expression domains (Cabrera et al 1987, Romani et al 1987), consistent with the widespread but unique functions of the members. Loss of function mutations and phenotypic analyses reveal incomplete complementation by other members of the complex (Garcia-Bellido 1979, Garcia-Bellido and Santamaria 1978). Loss of individual *AS-C* gene function results in neural hypotrophy, with the absence of select neuronal populations and associated sensory organs (Dambly-Chaudiere and Ghysen 1987, Ghysen and Dambly-Chaudiere 1988, Ruiz-Gomez and Modolell 1987, Gonzales et al 1989). The use of gain of function alleles, as well as ectopic expression and the manipulation of gene dosage have all suggested that these genes share common activities, with each capable of causing neural hypertrophy (Hinz et al 1994, Jarman and Ahmed 1998, Giebel et al 1997, Jarman et al 1993, Brand et al 1993). In the embryonic PNS, loss of *da* function has a much more severe phenotype than loss of individual *AS-C* genes (Caudy et al 1988), consistent with the ubiquitous expression of *da* and its widespread requirement as a heterodimerization partner for *AS-C* gene products. This

relationship is also supported by the genetic interaction observed between *da* and *AS-C* (Dambly-Chaudiere et al 1988).

Deletion of the entire *AS-C* does not lead to the loss of all peripheral sensory structures (Jimenez and Campos-Ortega, 1979, 1987, Campos-Ortega and Jimenez 1980, White 1980, Dambly-Chaudiere and Ghysen 1987). The chordotonal organs appear refractory to this loss of function. An additional proneural gene, *atonal*, is expressed in developing chordotonal organs (Jarman et al 1993). *Atonal* encodes a bHLH transcription factor that interacts with da, and binds to the E-box (Jarman et al 1993). Loss of *atonal* function results in a loss of chordotonal organ formation specifically, suggesting it is a proneural factor designated for this peripheral sensory organ subtype. In addition, loss of *atonal* function severely perturbs neural development in the eye imaginal disc, another neural domain that is refractory to the effects of *AS-C* loss of function mutations and appears to depend on *atonal* for proneural activity (Jarman et al 1994).

The expression pattern of genes of the *AS-C* correlates with the definition of proneural clusters in the developing CNS and PNS, as well as the adult PNS (Cabrera et al 1987, Romani et al 1987, Cubas et al 1991, Simpson 1990, Culi and Modolell 1998). Clusters of cells expressing these genes are believed to be equipotent with respect to their neurogenic potential, with any member capable of generating a neural progenitor cell. This is based on findings from three types of experiments. First, in laser ablation experiments, the destruction of a presumptive neuroblast results in the conversion of an adjacent presumptive dermoblast to a neuroblast in a compensatory mechanism (Taghert et al 1984, Doe and Goodman 1985). Second, the homotopic and isochronic transplantation of cells between mutant and wild type strains has suggested that signals pass between cells in the cluster to determine their fate (Technau and Campos-Ortega 1986, Technau et al 1988, Campos-Ortega 1988). These studies imply that cells giving rise to neuroblasts are not intrinsically predetermined to do so and that disruption of local intercellular signaling, as with laser ablation, alters the fate of ectodermal cells in a cluster. Third, genetic mosaic analyses in individual fly strains have confirmed the role of

intercellular communication in neural specification and identified many molecular participants (Artavanis-Tsakonas et al 1995).

After initially expressing one or several of the *AS-C* genes in a cluster, proneural gene expression is gradually restricted to and elevated within one (or a few) cells of the cluster. This restriction and elevation is achieved by a local intercellular signaling mechanism employing members of the second classical group, the neurogenic genes (Martin-Bermudo et al 1995, Skeath and Carroll 1991, Simpson and Carteret 1989, Cabrera et al 1990, Cubas et al 1991, Culi and Modolell 1998, Heitzler et al 1996). Early analyses showed that mutant alleles of most the neurogenic genes interact with one and other genetically and result in the formation of either more or fewer neuroblasts at the expense of or to the benefit of dermoblasts, respectively (Poulson 1937, Lehmann et al 1981, Lehmann et al 1983, Campos-Ortega et al 1984, Dietrich and Campos-Ortega 1984, Vassin et al 1985, Shepard et al 1989, Brand and Campos-Ortega 1989, Xu et al 1990). Some of these interactions were allele specific, suggesting they reflected interaction at the protein level. It is now recognized that the interacting genes collectively define a molecular pathway extending from the cell surface to the nucleus, namely the "Notch" pathway (Artavanis-Tsakonas et al 1995).

Signal reception in the Notch pathway begins with the product of the *Notch* gene itself. The *Notch* gene encodes a 300kD transmembrane protein which serves as a receptor at the cell's surface (Wharton et al 1985, Kidd et al 1986, Kidd et al 1989, Johansen et al 1989, Fehon et al 1990, Rebay et al 1991). In the developing CNS, Notch gain of function mutations result in fewer neurons being generated without perturbing epidermal differentiation (Struhl et al 1993, Rebay et al 1993, Lieber et al 1993). Similarly in adult sensory structures, gain of function mutations result in the inability to generate neurons and sensory structures from imaginal discs (Struhl et al 1993). Conversely, *Notch* loss of function mutations are embryonic lethal and cause all ectodermal cells to assume the neuroblast fate in the developing CNS (Poulson 1937). Similarly, in the adult PNS, conditional mutants have been used to show that loss of *Notch* function correlates with excessive neurogenesis in imaginal discs (Hartenstein and Posakony 1990). Importantly,

persistent activation of the Notch pathway is required for cells to avoid a neuroblast fate, as transient *Notch* activation merely delays the process of neuroblast specification and segregation, without committing cells irreversibly to an epidermal fate (Struhl et al 1993). Thus Notch activation does not appear to cause epidermal commitment.

In addition to its early role in the segregation of neuroblasts from dermoblasts, *Notch* also plays a subsequent role in lateral specification between sibling neurons in at least one CNS lineage (Spana E et al, 1996). Following the specification of neuroblast subtype, the MP2 neuroblast undergoes a characteristic division to generate two distinct sibling motor neurons. The formation of distinct neurons is dependent on Notch signaling, and is perturbed by manipulating *Notch* or *numb*. The *numb* gene product is an intracellular protein that interacts molecularly with Notch protein and is believed to inhibit Notch's ability to transduce a signal, thereby mimicking a *Notch* loss of function mutation. *Notch* is expressed in both neurons, while *numb* is selectively expressed in one neuronal type. Loss of *Notch* function yields two neurons that resemble the neuron which normally expresses *numb*, while loss of *numb* function yields two neurons that resemble the neuron that normally does not express *numb*. *Notch-numb* double mutants yield two neurons similar to the wildtype *numb* expressing neuron, indicating that *numb* is not directly required for subtype specification, but rather for the inhibition of Notch activity which consequently affects subtype specification. In vitro experiments have demonstrated that the inductive cue for differentiation originates outside the lineage. This suggests that Notch signaling and lateral specification distinguishes the presumptive motor neurons and regulates their receptivity to extrinsic cues in a differential manner to generate distinct motor neuron subtypes.

Two genes encoding ligands for Notch have been identified in Drosophila, namely *delta* and *serrate* (Kopczynski et al 1988, Vassin et al 1987, Flemming et al 1990, Thomas et al 1991, Fehon et al 1990, Klueg and Muskavitch 1999). Expression data show the two ligands do not completely overlap and loss of function data suggest they are redundant in some contexts but not in others. Further, complementation experiments show that they share some activity, but cannot complement each other in all developmental contexts.

Delta loss of function mutations are similar to *notch* loss of function mutations in the embryo and adult, resulting in a neurogenic phenotype (Lehmann et al 1983, Heitzler and Simpson 1991, Parody and Muskavitch 1993, Parks and Muskavitch 1993, Corbin et al 1991). In contrast, *serrate* loss of function mutations are not associated with excessive neurogenesis, however *serrate* overexpression can partially substitute for loss of *delta* function during embryogenesis (Gu et al 1995). In contrast, in late stage SOP development, *serrate* and *delta* appear redundant, and a loss of function *Notch*-like phenotype is not produced unless both genes are disrupted (Zeng et al, 1998). Finally, during wing development, both *delta* and *serrate* are required and neither can substitute for the other. These discrepancies may be partly due to differences in *delta* and *serrate* expression and the expression of selective ligand modifiers such as "*fringe*" in the developing wing (Klein et al 1998). The situation in C. elegans appears to be similar, where two Notch ligands are differentially required but can substitute for one and other to some extent (Lambie et al 1991, Fitzgerald et al 1993, Yochem et al 1988, Yochem and Greenwald 1989, Austin and Kimble 1989).

A conserved and functionally important sequence in the Notch intracellular domain interacts with the product of the *Su(H)* gene, which encodes a transcription factor that is ubiquitously expressed (Tamura et al 1995). The binding of Notch by Delta or Serrate leads to the transcription of genes found in the *enhancer of split complex (E(spl))* by Su(H) (Lecourtois and Schweisguth 1995, Bailey et al 1995). This stimulation is direct, as Su(H) directly binds regulatory regions in these genes and activates their expression as a consequence of Notch activation. The sequence recognized by Su(H) and found in the *E(spl)* genes is CGTGGGAA (Lecourtois and Schweisguth 1995, Bailey et al 1995). In the eye imaginal disc, gain of function mutant alleles of *Su(H)* can rescue the phenotype caused by loss of function mutant alleles of *Notch*, thereby suppressing excessive neuroblast formation (Fortini et al, 1994). Furthermore, loss of function mutations in *Su(H)* result in the formation of excess neuroblasts, while overexpression prevents neuroblast specification in other imaginal discs (Schweisguth et al 1995, Schweisguth et al 1992, Furukawa et al 1992). Combined with numerous genetic interactions between alleles of *Su(H)* and those of other genes of the Notch pathway (Campos-Ortega et al

1984, Dietrich and Campos-Ortega 1984, Vassin et al 1985, Shepard et al 1989, Brand and Campos-Ortega 1989, Xu et al 1990), these studies support the notion that *Su(H)* operates downstream of *Notch* and positively propagates the Notch signal.

Within the *E(spl)* complex are seven related genes encoding transcription factors which share the bHLH motif, similar to the *AS-C* genes. These bHLH factors bind to DNA as dimers and recognize the sequence CACNAG commonly referred to as the N-box. An eighth gene named *groucho* is also found in this complex. *groucho* is unrelated to the other genes of the *E(spl)* complex and does not contain the bHLH motif. *groucho* genetically and molecularly interacts with members of the *E(spl)* complex and is required for their activity in vivo (Paroush et al 1994). Groucho has been shown to function as a transcriptional cofactor, accounting for its functional interaction with the gene products of E(spl). In vitro transcription assays have demonstrated that Groucho acts as a transcriptional repressor, but is unable to bind DNA on its own (Fisher et al 1996, Jimenez et al 1997). Gain of function mutations in *groucho* and other members of the *E(spl)* complex, as well as the overexpression of normal alleles results in the formation of fewer neuroblasts in the embryo and the adult. Persistent expression of two *E(spl)* genes also suppresses embryonic neural development in the CNS (Nakao et al, 1996), revealing that the downregulation of *E(spl)* expression is a prerequisite for neural development and neuronal differentiation. Conversely, loss of function mutations in *groucho* and the other *E(spl)* genes generate neurogenic embryonic phenotypes, with the mutant *groucho* phenotype being the most severe. The *E(spl)* gene products repress expression of *AS-C* genes, linking Notch activation to the down-regulation of proneural gene expression. In this manner, local intercellular communication acts to restrict proneural gene expression to presumptive neuroblasts within the proneural clusters.

Notch pathway activation commences with the binding of ligand to the receptor Notch (Fehon et al 1990, Klueg and Muskavitch 1999, Rebay et al 1991). That binding event stimulates cleavage of the intracellular domain of Notch, and the cleavage event releases an intracellular fragment of Notch which is subsequently found in the nucleus (Schroeter et al 1998, Lecourtois and Schweisguth 1998, Sestan et al 1999, Kidd et al 1998) . The

release of the intracellular portion of Notch is thought to be important for the localization and/or transcriptional activity of Su(H), and the subsequent activation of *E(spl)* genes (Lecourtois et al 1995, Bailey et al 1995). Finally, to close the loop, the ligand encoding genes themselves, *delta* and *serrate*, are under the positive control of the proneural genes (Heitzler et al 1996). Thus as a result of Notch activation, *AS-C* activity is repressed by activation of *E(spl)* and ligand production is not stimulated by AS-C. In this manner, a cell becomes a signal emitter (Notch not activated, proneural gene expression, ligand expression stimulated, emit signal) or a receiver (Notch pathway activated, proneural gene repression, ligand expression not stimulated), and also becomes a neuroblast (signal emitter, proneural gene activity) or an epidermoblast (signal receiver, proneural genes repressed by Notch activation).

The details of the Notch signaling pathway remain incomplete. There are a number of genes which interact genetically with *Notch* or mimic its mutant phenotypes. Included in this group are *mastermind* (Yedvobnick et al 1988, Hartenstein et al 1992), *deltex* (Xu et al 1990), *big brain* (Rao et al 1992, Hartenstein et al 1992), *strawberry notch* (Coyle-Thompson and Banerjee 1993), *vestigial* (Rabinow and Birchler 1990, Abu-Issa and Cavicchi 1996), *neuralized* (Hartenstein et al 1992), *pecanex* (LaBonne et al 1989), *almondex* (LaBonne and Mahowald 1985), *wingless* (Couso and Martinez Arias 1994), *star* (Mayer and Nusslein-Volhard 1988, Heberlein et al 1993), *scabrous* (Baker et al 1990, Mlodzik et al 1990, Rabinow and Birchler 1990) and *shaggy* (Ruel et al 1993). It is not clear that all of these genes have a role in neurogenesis. Those that do may function in the specification of neuroblast subtypes rather than in neural specification, with the possible exception of *mastermind* and *deltex*. *Mastermind* encodes a nuclear factor of unknown function, while *deltex* encodes a cytoplasmic protein that functions upstream of *Notch* genetically (Gorman and Girton 1992), and interacts molecularly with its cytoplasmic domain (Diederich et al 1994, Matsuno et al 1995). In addition, Delta and Notch may interact on the same cell and elicit a cell intrinsic signal, the ligands Delta and Serrate may normally be secreted (Sun and Artavanis-Tsakonas 1997), and soluble Delta may act as an agonist of *Notch* activity (Qi et al 1999).

(c) *Emc* and *Hairy*

Functioning in parallel with the neurogenic genes are *extrachromachaete* (*emc*) and *hairy* (*h*). These two genes are not part of the classical neurogenic pathway, and do not appear to be regulated by Notch activity. However they encode nuclear proteins that function to inhibit the activity of the proneural genes and are important regulators of neurogenesis. *Emc* encodes a HLH protein which notably lacks a basic stretch like that found in the Da, AS-C and E(spl) bHLH proteins (Garrell and Modolell 1990, Ellis et al 1990). As a consequence, Emc does not bind to DNA, however it is able to heterodimerize with AS-C factors and Da (Martinez 1994, Martinez et al 1993, VanDoren et al 192). These heterodimers are incapable of binding DNA (with only one basic stretch provided per dimer) and as a consequence are thought to be functionally inactive. Overexpression of *emc* mimics a loss of *AS-C* function, and results in the formation of fewer neuroblasts, while loss of *emc* function is neurogenic (Cabrera et al 1994, Cbas et al 1992, Cubas et al 1994). *Hairy* encodes a bHLH factor similar to the E(spl) factors, but is separate from this locus (Rushlow et al 1989). Like E(spl), H interacts molecularly with the transcriptional co-repressor Groucho (Fisher et al, 1996, Jimenez et al 1997), and this interaction is important for hairy function *in vivo* (Wainwright and Ish-Horowicz 1992). Like *emc* and *E(spl)*, overexpression of *hairy* inhibits neuroblast formation while loss of *hairy* function yields a neurogenic phenotype (Rushlow et al 1989, Ohsako et al 1994). Hairy is believed to negatively regulate the expression of the proneural genes directly (VanDoren et al 1994).

Unlike the *E(spl)* genes, *h* and *emc* functions appear to be independent of the Notch pathway, and active prior to neurogenic gene function. Their function and expression patterns suggest that they play an early role in setting up the preliminary boundaries where proneural clusters may initially form. They also appear to function later within those boundaries to partially determine where a neuroblast will form (Cubas and Modolell 1992). Specifically, *h* and *emc* are expressed in pattern reciprocal to that of the *AS-C* genes in the ectoderm and the lowest levels of *emc* expression prefigure a region

from which a neuroblast will form. As a result, it appears that mechanisms besides those involving the neurogenic genes contribute to the specification and segregation of neuroblasts from dermoblasts. How *h* and *emc* are regulated is unknown.

In the developing eye disc, both *hairy* and *emc* have been manipulated and results show that they play a cooperative role in the timing of neurogenesis (Brown et al 1992, Brown et al 1994). In the developing eye disc, differentiation occurs simultaneously for cells at the same A/P position across the entire width of the disc. This differentiation begins at the posterior rim of the disc and cells progressively anterior differentiate in succession. The result is a wave of cellular differentiation which spans the width of the eye field and sweeps across the disc from posterior to anterior, leaving differentiating and ordered cells in its wake. The wave is morphologically evident as a consequence of shape changes and positional changes taking place among constituent cells, and it is named the morphogenetic furrow (MF). Ahead of the MF are presumptive neuroblasts, while behind the MF are determined neuroblasts and neurons. Mosaic analysis has revealed that the combined loss of *h* and *emc* functions results in precocious neuronal differentiation ahead of the normal MF accompanied by precocious expression of *atonal* in a cell autonomous fashion (Brown et al 1994). This study provides an elegant example of the molecular basis of coordinated neuronal differentiation and will be further addressed in the discussion.

Together, *emc*, *h*, the proneural genes and the neurogenic genes are believed to act in succession to demarcate neurogenic territory from which candidate neuroblast precursors are chosen, and then to restrict the number of neuroblasts derived from the ectoderm. Importantly, these genes have been conserved in mammals. Notably, the number of such genes is much larger in mammals, and multiple homologs display distinct but partially overlapping expression patterns in the developing mammalian nervous system. The functional analysis of individual genes has suggested that many of them play a role in the regulation of mammalian neurogenesis. Furthermore, analysis of Notch pathway constituents collectively has revealed that the signaling pathway has been conserved and conducts signals in response to ligand/receptor interactions much as the Drosophila

signaling pathway does. Moreover, the Notch pathway in mammals appears to repress neurogenesis, as it does in Drosophila.

(d) Neural Progenitor Genes

The last group of Drosophila genes to be considered, the neural progenitor genes, are expressed commonly in most or all developing neuroblasts or neurons and are thought to be important in carrying early neuroblasts up to and through neuronal differentiation. Among these genes are *embryonic lethal abnormal vision (elav)*, *prospero*, *scratch* and *deadpan*.

The *prospero* gene (*pros*) encodes a homeodomain containing transcription factor. During embryogenesis, *prospero* is specifically expressed in nearly all neuroblasts and SOPs. Prospero is localized in the basal cortex of CNS neuroblasts during mitosis and is selectively distributed to ganglion mother cells (GMCs) during mitosis. Both *pros* mRNA and protein are observed in the GMC, while the *pros* gene is not transcribed in the cell. Neither *pros* mRNA nor protein are observed in differentiated neurons. *Prospero* gene function is required for the establishment of GMC-specific gene expression, and for the proper differentiation of neurons. Loss of function mutations result in neuronal malformation, with defects in axonal outgrowth (Doe et al, 1991; Vaessin et al, 1991). The normal function of Pros and its mechanism of action in GMCs is unclear, though putative targets include the homeobox genes *fushi tarazu*, *even-skipped* and *engrailed*, which display altered expression in specific neuroblast progeny in *pros* mutants.

In the embryonic PNS, SOPs divide asymmetrically to generate the IIa and IIb cells. The IIa cell divides and generates the socket and hair cells, while the IIb cell generates neuron and sheath cells. *Prospero* is expressed in SOPs and then localized to the IIb daughter cell. It is then transiently found in neurons, but persists in sheath cells (Knoblich et al 1995; Spana and Doe 1995). Loss of *prospero* function in the SOP lineage leads to axonal outgrowth defects in the resultant neurons, similar to the effect in CNS neurons

(Doe et al 1991, Vaessin et al 1991). In the adult SOP lineage, Pros is not localized to the IIb cell from the SOP, but rather is first detected in the IIb cell. Prospero is found in the nucleus of the IIb cell, in the cytoplasm during mitosis of IIb, and then in the nucleus of the neuron and sheath cell. Prospero is soon lost from the neuron, but persists in the sheath cell as observed in the embryonic PNS. Loss of function results in a transformation of IIb to IIa, with a resultant "double bristle no neuron sense organ" phenotype. Forced misexpression of *pros* in adult SOPs causes the converse IIa to IIb transformation, and flies lack sensory bristles but have duplicate neurons and sheath cells. *Prospero* is also required for the proper differentiation of the R7 photoreceptor in the developing eye, where loss of *pros* function causes a defect in axonal outgrowth and connectivity (Kauffmann et al 1996). Together these results indicate that *pros* is required for neuronal differentiation to some degree and that prospero is sufficient to induce neuronal specification and differentiation, directly or indirectly, in certain contexts. *Prospero* has been evolutionarily conserved, and homologs have been identified in C. elegans (Burglin 1994) as well as mice (Oliver et al 1993). In mice, *prospero* is also expressed in neuroblasts of the CNS.

Interestingly, the Notch pathway influences these cell fate decisions in a manner similar to its effect on the MP2 lineage in the CNS (Guo et al 1996). A loss of, or decrease in, *Notch* function at the IIa/IIb stage of the adult SOP lineage results in a IIa to IIb transformation, and the generation of ectopic neurons and sheath cells at the expense of hairs and socket cells. Notch activation at this same point leads to the opposite transformation, whereby extra hair cells and socket cells are generated at the expense of neurons and sheath cells. Notch activity is differentially regulated to generate the distinct IIa and IIb daughter cells. This is achieved by the *numb* gene product, which interacts molecularly with Notch and is believed to inhibit Notch's ability to transduce a signal. Numb protein is asymmetrically localized to the IIb daughter cell (Rhyu et al 1994), and is believed to inhibit Notch signal transduction within it. In support of this model, *numb* loss of function mutations cause a IIb to IIa transformation in a *Notch*-dependent manner (Guo et al 1995; Uemera et al 1989). The *tramtrack* gene, which encodes a putative transcription factor possessing zinc-fingers, functions genetically downstream of *Notch* in

the IIa cell. A loss of *tramtrack* function results in transformation of the IIa cell to a IIb cell, like loss of *Notch* function. *Numb* is believed to block the activation of *tramtrack* by *Notch*, and thus inhibit the specification of the IIa cell fate in the IIb cell. In the absence of *tramtrack*, loss of *numb* function does not lead to a IIb to IIa transformation, despite the presence of *Notch*. It is believed that pros lies downstream of *tramtrack*, either directly or indirectly, and that *tramtrack* activation inhibits *pros* activation in the IIa cell (Campos-Ortega 1996).

The *elav* gene encodes a nuclear protein which contains an RNA-binding motif and is widely expressed in nearly all neurons (Robinow and White 1991). Neither elav mRNA nor protein are detectable in neuroblasts or glia, and the onset of *elav* expression correlates with the first appearance of neurons (Robinow and White 1988; Robinow et al 1989). *Elav* mutations are embryonic lethal, but a post embryonic function for *elav* has also been discerned from conditional mutants (Campos et al 1985; Homyk et al 1985). Neural defects have been noted in the optic lobes and eyes of *elav* mutants, and mosaic analysis suggests *elav* functions in a cell autonomous manner in eye development (Campos et al 1985; Homyk et al 1985; Yao and White 1991). The cellular function of Drosophila *elav* and its mechanism of action may involve the regulation of neuron-specific splicing events (Koushika et al 1996). Studies of mammalian homologs of *elav* (Okano and Darnell 1997; Wakamatsu and Weston 1997) have suggested that *elav* may participate in the regulation of growth through the post-transcriptional regulation of growth-associated mRNA's (King et al 1994; Lazarova et al 1999; Ross et al 1997).

The *deadpan* gene encodes a bHLH transcription factor related to the *hairy* gene product, and like hairy, deadpan interacts molecularly with the transcriptional co-repressor groucho (Bier et al 1992). The *scratch* gene encodes a zinc-finger protein, and both *deadpan* and *scratch* are expressed in nearly all neural precursors. *Deadpan* and *scratch* interact genetically, and double mutants display a marked loss of embryonic neurons not observed in individual mutants (Roark et al 1995). Loss of *scratch* gene function alone leads to a loss of photoreceptors in the eye, while ectopic expression of *scratch* alone generates supernumerary neurons (Roark et al 1995). Both deadpan and scratch are

believed to function as transcriptional repressors, and one hypothesis suggests that they repress non-neuronal effectors of differentiation.

(e) Non-Classical Genes

Among the genes lying outside of the classical divisions but still required for proper neurogenesis are *glial cells missing* and *yan*. These are factors believed to repress neuronal gene expression outside and within the neural lineage respectively, so that neuronal genes are expressed in a cell-type-specific and timely manner.

The generation of an appropriate number and pattern of neurons in Drosophila depends on the function of the non-neuronal gene *glial cells missing* (*gcm*) (Jones et al 1995, Hosoya et al 1995). It encodes a nuclear protein with a unique DNA-binding domain that binds to DNA in a sequence-specific manner. *Gcm* is specifically expressed in glial progenitor cells and is required for the generation of all but a few glial cells in the Drosophila embryo. Loss of function *gcm* mutations result in the conversion of prospective glial cells to neurons. Moreover, neurons thus generated assume a phenotype appropriate for their position, suggesting that positional specification among neurons and glia is commonly coded and independent of cell type. Gain of function mutant *gcm* alleles when expressed in neuroblasts of the developing embryo conversely generate supernumerary glial cells at the expense of neurons. The ectopic glial cells thus generated also appear to assume a phenotype appropriate for their position.

The mechanism of gcm action remains unknown, but one possibility is that the induction of glial cell differentiation by gcm involves the direct or indirect repression of neuronal gene expression. Gcm may repress positive regulators of neuronal differentiation, may engage neuronal genes directly and repress them, may promote the expression of negative regulators of neuronal gene expression, or may promote the expression of positive regulators of glial gene expression which in turn interface with neuronal genes at some level. Many scenarios are possible, but the fact that gcm can convert mesoderm to a glial-

like state suggests that *gcm* does not simply avert neuronal differentiation, but rather induces proactive glial differentiation activity that is capable of overriding neuronal and non-neuronal differentiation programs (Bernardoni et al 1998). As fate acquisition seems to be exclusive (though there are a number of genes commonly expressed) an emergent question is where the program of glial cell differentiation and neuronal cell differentiation interface (Anderson 1995). Such an intersection of exclusive differentiation programs may be similar to the situation earlier in development where the choice between ectoderm and neuroectoderm appears exclusive, and transcription factors regulating these early states of commitment may be interconnected in a cross-regulatory network (Sasai 1998).

A question that follows from the examination of *gcm* function is whether transcription factors which promote neuronal differentiation operate in a converse manner to oppose glial cell programs of differentiation while promoting neuronal differentiation. However, many of the neural transcription factors studied to date in Drosophila function in neural specification, regulating the choice between neural and epidermal fates rather than glial and neuronal fates. Little is known about the molecular mechanisms governing neuronal specification. In vertebrates however, the transcription factor NeuroD, which belongs to the basic helix-loop-helix family of transcription factors, is involved in the proximate regulation of neuronal differentiation (Lee et al 1995). *NeuroD* is expressed in all Xenopus neurons prior to or coincident with their differentiation. It is capable of converting ectoderm to a neuronal fate, though is incapable of converting mesoderm or endoderm. The ectopic expression of *NeuroD* in developing Xenopus ectoderm leads to the creation of supernumerary neurons and precocious neuronal differentiation, but whether this is at the expense of glial cells or their precursors remains a question. In short, it is not known whether transcription factors promoting neuronal differentiation do so in part by opposing glial cell differentiation. In support of such an intersection, distinct extrinsic cues are believed to trigger either neuronal or glial cell differentiation from common precursors in the mammalian PNS. However within the mammalian CNS, neuronal genesis and gliogenesis are not completely coincident, suggesting an immediate choice between glial cell fate and neuronal fate may not always be necessary. Still somewhat surprisingly, the mammalian homolog of *gcm* is not restricted to mammalian

neural precursor cells, nor even highly expressed in the nervous system during development. Apparently *gcm* function has diverged evolutionarily.

Another Drosophila transcriptional repressor widely involved in the regulation of neuronal differentiation is encoded by the *yan* gene. *Yan* encodes a transcription factor of the ETS (E twenty-six) family. These transcription factors are evolutionarily conserved and typically lie at the end of a signal transduction pathway which usually involves a homolog of the mammalian proto-oncogene *Ras* (Wasylyk et al 1998). The ETS transcription factors are thus poised to respond to intracellular responses to extracellular signals. *Yan* is expressed in precursors in the dorsal neuroectoderm of the embryo which will give rise to the anterior CNS. Loss of *Yan* function leads to hyper-proliferation in this region of the neuroectoderm. *Yan* is also expressed in the developing eye disc, and its expression dramatically decreases with cell differentiation. Homozygous null mutations are embryonic lethal, however conditional mutation and mosaic analysis has revealed that loss of yan function also leads to precursor hyper-proliferation in the developing eye disc.

Genetically, *yan* functions as a negative regulator of photoreceptor development (Lai et al 1992; O'Neil et al 1994). Molecularly, yan represses transcription and is negatively regulated by the Ras1/MAPK pathway (O'Neil et al 1994). Yan contains eight putative MAPK phosphorylation sites and can be directly phosphorylated in vitro (Brunner et al 1994). A proposed gain of function mutant *yan* allele causes a near complete loss of photoreceptors correlated with extensive and excessive cell death, and completely disrupts regular ommatidial morphology when expressed in the morphogenetic furrow of the developing eye (Rebay et al 1995). The activated yan isoform inhibits both neuronal and non-neuronal differentiation in the developing eye, and also inhibits neuronal differentiation in the embryonic CNS following ectopic expression. In addition, this isoform of yan inhibits the differentiation of mesoderm, which may be relevant as *yan* is expressed in mesodermal precursors but not in differentiated mesodermal derivatives. The activated yan does not however repress differentiation universally, and is unable to inhibit epidermal differentiation. A naturally occurring gain of function allele harbors a mutation in a putative phosphorylation site. To create an activated *yan* allele, mutations

were introduced at putative MAPK phosphorylation sites to render it refractory to regulation by Ras/MAPK signal transduction. It has been suggested that yan may normally repress cellular differentiation until instructed not to do so by a phosphorylation event, and that this regulation could control the timing of neuronal differentiation. Biochemical evidence suggests that such phosphorylation could regulate the subcellular distribution and/or the stability of yan protein. The targets of yan and hence its mechanism of action are unknown.

(ii) Neurogenesis in C. elegans

Homologs of *Notch*, *delta*, *groucho*, *E(spl)*, Su(H) and *AS-C* have been identified in C. elegans. Although C. elegans studies have revealed much about the nature of Notch signaling, they have revealed little about its regulation of neurogenesis. The conventional Notch pathway appears to play a limited role in neurogenesis in C. elegans. The proneural and *E(spl)* orthologs do however play important roles in neurogenesis.

The *lin-32* gene encodes a homolog of the *AS-C* genes, and is expressed in many neuroblasts (Zhao and Emmons 1995). Mutations in the *lin-32* gene correlated with a loss of neurons and their associated sensory structures, similar to the loss of *AS-C* function and *atonal* function phenotypes observed in Drosophila. In the absence of *lin-32* expression, the presumptive neuroblast cells undergo a transformation of cell fate and generate epidermal derivatives. In C. elegans, as in most other animals, the neuronal lineage is closely related to the epidermal lineage, and neurogenesis involves the progressive distinction of the two. The *lin-32* gene appears to confer the ability to generate neurons and their associated sensory structures, much the same as *AS-C* genes and *atonal* do in Drosophila.

The *lin-22* gene encodes a bHLH transcription factor related to the *hairy* and *E(spl)* genes (Wrischnik et al 1997). Lin-22 differs from the Drosophila gene products in that it does not contain the C-terminal WRPW sequence which is crucial for interaction with groucho

and important for biological function in the Drosophila proteins. *Lin-22* appears to function by repressing the activity of *lin-32* in a subset of precursors that do not normally give rise to neurons or sense organs. In the absence of *lin-22*, ectopic neurons are generated at the expense of epidermal derivatives, and the ectopic neurogenesis is correlated with ectopic expression of *lin-32* in the same cells. This process does not involve either of the two *Notch* genes identified, nor their ligands. Furthermore, the fact that lin-22 lacks the motif necessary for groucho interaction suggests that lin-22 may act in a different manner to specify the fate of these cells. Biochemical analysis of the nature of hairy and E(spl) function has suggested that there are two mechanisms by which these repressive factors operate (Dawson et al 1995). One of these mechanisms does not rely on groucho, and may therefore be analogous to the action of lin-22 in C. elegans. Whether or not *lin-22* function depends on *unc-34* function, the C elegans homolog of groucho, has not been reported. Moreover, the only neurogenic process reported to be altered by *unc-37* mutation is the specification of a small number of motor neurons (Pflugard et al 1997). Other mutations in *unc-37* are lethal however, and may mask a broader involvement in neurogenesis.

The *lag-1* gene encodes a factor highly homologous to Drosophila Su(H) and mammalian Su(H).RBP-Jk (Christensen et al 1996). Furthermore, the *lag-1* gene product binds the consensus sequence identified for Su(H) (LeCourtois and Schweisguth 1995, Bailey et al 1995, Jarriault et al 1995, Dou et al 1994, Henkel et al 1994), and *lag-1* interacts genetically with the notch homologs *lin-12* and *glp-1*. The function of *lag-1* in neuronal development in uncertain.

Like *gcm*, the C. elegans *lin-26* gene encodes a zinc-finger protein which is expressed in non-neuronal cells but is required for the formation of an appropriate number and arrangement of neurons (Labouesse et al 1996). The Lin-26 protein is found in all non-neuronal ectodermal cells of the embryo and adult. *Lin-26* expression begins at various times relative to terminal differentiation in various lineages, but is induced prior to or coincident with the final division of all such progenitor cells. In instances where an ectodermal progenitor cell gives rise to a neuron and a non-neuronal cell, both cells

inherit Lin-26 protein from the mother cell, but Lin-26 protein is then lost from the daughter cell that adopts a neuronal fate.

Loss of function alleles of *lin-26* demonstrate that it is required for the differentiation and/or maintenance of all non-neuronal ectodermal cells, including glial cells. In the absence of wild type *lin-26*, glial cells of the embryo either die or are malformed, generating dysfunctional sensory organs which have been assayed both functionally and ultrastructurally. Death and malformation do not appear restricted to subsets of glial cells. In one particular region, the malformed glial cells morphologically resemble adjacent neurons, suggesting trans-differentiation may have occurred. Gain of function alleles and overexpression studies have not been reported as yet.

Thus the repression of neuronal differentiation in non-neuronal cells is important for the generation of appropriate numbers and arrangements of neurons and non-neuronal cells. Interestingly, the mammalian gene *neuronal restriction element silencing transcription factor (REST/NRSF)* encodes a zinc-finger protein that functions outside the neural lineage to repress neuronal gene expression (Chong et al 1995, Schoenherr and Anderson 1995). The expression of *REST/NRSF* in non-neural cells and its absence in neurons has been hypothesized to contribute to the neuron-specific expression of many genes and in vitro results support this hypothesis. *REST/NRSF* may distinguish non-neural lineages from the neuronal lineage, but unlike *lin-26* and *gcm*, *REST/NRSF* does not appear to be involved in the distinction of glial and neuronal lineages.

(iii) Neurogenesis in Xenopus and Mouse

(a) Overview

In comparison to invertebrates, the vertebrate neural lineage has a larger number of intermediate stages between neural specification and neuronal differentiation. Vertebrate studies have begun to identify genes that are sequentially expressed and may foster progressive development of the neuronal lineage. The identification of these genes has

revealed that progenitor cells pass through a number of molecularly distinct stages en route to neuronal differentiation.

A number of genes encoding transcription factors and intercellular signaling molecules have been found to play important roles in vertebrate neurogenesis. Many display regionally restricted patterns of expression, while others are expressed widely throughout the developing nervous system. Both types are usually temporally controlled, suggesting that they function at particular stages of development in the neural lineage.

Many regionally restricted transcription factors and signaling molecules are expressed in progenitor cells and newborn neurons and their loss often leads to regional deficits in the nervous system. These factors may direct appropriate cell division, survival, specification, and differentiation in specific regions of the developing nervous system.

Many of the vertebrate transcription factor encoding genes are orthologs of Drosophila genes or are similar to such orthologs. Other genes encode factors with functional domains that are common among transcription factors, but appear newly formed or recruited for the purpose of managing gene expression and responsiveness at particular stages or in particular subdomains of the nervous system. These have been found to alter specification, survival, proliferation, and differentiation in specific populations.

The analysis of neurogenesis in zebrafish, Xenopus, and mouse has revealed that neurogenic mechanisms have been conserved from Drosophila. Xenopus studies have been particularly fruitful in adding genes and mechanisms that bridge neural induction to neuronal differentiation in vertebrates. Importantly, Xenopus provides a powerful tool for the functional assessment of genes potentially involved in neurogenesis. As such, genes identified in mammals have often been functionally analyzed in Xenopus, either directly in interspecies assays or following the isolation of Xenopus orthologs. Gene targeting in mice has served as the ultimate test for gene function in mammalian neurogenesis. However these experiments are often complicated by the increased redundancy and pleiotropic nature of gene function in the mouse.

Neurogenesis in Xenopus, like that in Drosophila, occurs in multiple waves. The first wave, referred to as primary neurogenesis, generates a primitive nervous system allowing the tadpole to function and respond to external stimuli. This first wave of neurogenesis provides an assay system where gene expression and phenotypic changes may be scored following genetic manipulation. Such studies utilize whole embryos or naive ectodermal explants (animal caps) which may be induced to generate neural tissue.

The neurogenic and proneural genes have been conserved in Xenopus and mouse where homologs of *Notch*, *Su(H)*, *E(spl)* and *AS-C* have been identified. Homologs of *emc* (*Id* genes), *h* (*Hes* genes) and *da* (*E2A*) have also been identified. Additional genes are also involved in vertebrate neurogenesis. Some of the novel genes encode proteins similar to the classic proneural bHLH gene products in both form and neurogenic ability. Thus the number of proneural-like genes appears larger in vertebrates, with members functioning at different developmental stages and possibly in distinct ways along the extended neural lineage.

(b) The Neural bHLH Genes

The products of the *neurogenin* genes, as well as the products of the *mammalian achaete-scute homolog* (*Mash*) genes are bHLH transcription factors related to *AS-C* gene products (Lee 1997). There are three neurogenin family members, *ngn1*, *ngn2* and *ngn3*. All three are expressed in neural progenitor cells (Sommer et al 1996, Ma et al 1997), and are believed to be capable of triggering ectopic neurogenesis (Ma et al 1996, Blader er al 1997). There are two MASH family members, *Mash1* and *Mash2* (Johnson et al 1990), but only *Mash1* is expressed in the nervous system and involved in neurogenesis (Guillemot et al 1994, Guillemot et al 1993). *Mash1* is also expressed in neural progenitor cells (Lo et al 1991, Guillemot and Joyner 1993). *Mash1* and *neurogenin* gene products are believed to heterodimerize with products of the *E2A* gene, the ubiquitously

expressed homolog of *da*. Mash1/E2A heterodimers bind the E-box DNA sequence and can activate transcription in an E-box dependent manner *in vitro* (Johnson et al 1992).

In the mouse central nervous system, *ngn1*, *Mash1* and *ngn2* are widely expressed in partially overlapping domains (Ma et al 1997, Sommer et al 1996, Lo et al 1991, Guillemot and Joyner 1993). Their expression in the PNS is more restricted and exclusive (Ma et al 1998, Fode et al 1998). The disruption of *ngn1*, *ngn2*, and *Mash1* individually in transgenic mice led to a lack of neuronal development in those tissues in which they were exclusively expressed. Precursors of the sympathetic, parasympathetic and enteric nervous system express *Mash1* exclusively, as does a subset of cells in the olfactory epithelium. In contrast, the cranial ganglia express either *ngn1*, *ngn2*, or both but do not express *Mash1*. In addition, a portion of the olfactory epithelium does not express *Mash1* but does express *ngn2* (Cau et al 1997). The sympathetic and most enteric ganglia do not develop in *Mash1* mutant animals, nor does the portion of the olfactory epithelium that normally expresses *Mash1* (Guillemot et al 1993, Cau et al 1997). In contrast, the cranial ganglia appear unaffected by *Mash1* loss of function, as does the portion of the olfactory epithelium that normally expresses *ngn2*. The disruption of *ngn1* and *ngn2* individually affects distinct sets of cranial ganglia which express them selectively. Ganglia that express both *ngn1* and *ngn2* are affected, though to a lesser extent, by the disruption of either gene (Fode et al 1998, Ma et al 1998).

The loss of *Mash1* and the neurogenins affects development at the progenitor cell stage. In the olfactory epithelium, *Mash1* mutant cells appear to be specified as neural progenitor cells but apoptose prior to differentiation. In addition, support cells in the olfactory epithelium appear to survive in the absence of *Mash1* (Cau et al 1997). In the sympathetic nervous system, neural crest cells appear to be specified as sympathetic precursor cells in *Mash1* mutant mice, but do not differentiate to form sympathetic neurons (Guillemot et al 1993). These cells likely die in the absence of differentiation. In the ectodermal placodes that give rise to the cranial ganglia, neural progenitor cells do not appear to form in the absence of functional *ngn2*, and corresponding ganglia are absent (Fode et al 1998). In *ngn1* mutant mice, the normal pattern of bHLH gene

expression is not observed in presumptive progenitor cells emanating from the ectodermal placodes, these cells do not appear to differentiate in the anlagen of their respective ganglia, and the corresponding ganglia are absent (Ma et al 1998).

Mash1 mutant mice also display a complex mutant CNS phenotype (Casarosa et al 1999; Hirsch et al 1998; Torii et al 1999; Horton et al 1999). In *Mash1* mutant mice, changes in gene expression occur in regions that normally express *Mash1*. Included among these genes are those that encode transcription factors and signaling molecules thought to play important roles in neural development within their domains of expression. Accompanying changes in gene expression are cell deficiencies. A decrease in the progenitor cell domain is evident in the SVZ of the medial ganglionic eminence (MGE), and discrete cell populations appear absent in the basal ganglia, cerebral cortex, and brain stem. Within the brainstem, expression of the *Phox2a* gene is dramatically reduced. *Phox2a* encodes a homeodomain-containing transcription factor and that is normally expressed in developing noradrenergic neurons of the locus ceoruleus, and is required for their development *in vivo* (Morin et al 1997). *In vitro* experiments suggest that *Mash1* lies upstream of *Phox2a* (Lo et al, 1998), and such a relationship may account for the loss of the locus ceoruleus and *Phox2a* gene expression in the brainstem of *Mash1* mutant animals.

In addition to the cell deficiencies and changes in gene expression, the trajectory of specific axons that normally encounter *Mash1* expressing cells appears altered in *Mash1* mutant mice (Tuttle et al 1999). Stereotypical thalamocortical pathways that encounter *Mash1* positive cells appear altered in *Mash1* mutant mice. Axons travelling from the thalamus to *Mash1* expressing clusters in the forebrain do not enter the designated territory in *Mash1* mutant animals, but rather appear to tangle or alter their trajectory at the border of these territories. This non-autonomous effect may reflect incomplete or inappropriate cell differentiation within the clusters in the absence of *Mash1*.

Mash1 is also expressed in a subpopulation of progenitor cells in the developing retina, its temporal pattern coinciding with the generation of rod, horizontal and bipolar cell

subtypes. Retinal explants cultured from *Mash1* null mice exhibit delayed rod, horizontal and bipolar cell differentiation as well as a significant loss of bipolar cells and a significant surplus of Mueller glial cells (Tomita et al 1996a). Conversely, forced expression of *Mash1* in retinal progenitor cells resulted in an increase in bipolar cells (Tomita et al 1996a). Interestingly, *Hes1*, an *E(spl)* ortholog, and *Mash1* exhibit opposing effects in the developing retina. While retinal development in *Mash1* mutant mice is delayed, rod and horizontal cell differentiation is accelerated in *Hes1* mutant mice (Tomita et al 1996b). Premature cell differentiation in *Hes1* mutants has morphological consequences and the retina appears grossly abnormal, with extensive bipolar cell death ensuing soon after cell differentiation. Also in contrast to *Mash1*, forced expression of *Hes1* inhibits retinal progenitor cell differentiation rather than promoting it (Tomita et al 1996b). *Hes1* encodes a bHLH transcription factor similar to the bHLH products of *E(spl)* and binds the N-box sequence CACNAG (Sasai et al 1992). *In vitro* studies have suggested that Hes1 can antagonize *Mash1* activity in two ways. First, by repressing its transcription, and second, by interacting molecularly with E2A proteins and limiting the formation of E2A/Mash1 heterodimers, which are believed to be the active Mash1 species (Sasai et al 1992).

The relatively late expression of *Mash1* and *ngn2* in the retina suggests that they do not regulate ganglion cell development. The vertebrate *Math5* gene encodes a bHLH transcription factor similar to *Mash1* that is expressed exclusively in the developing retina and prior to *Mash1*, *ngn2*, and *NeuroD* expression (Brown et al 1998). Its expression pattern suggests that it may be involved in the specification or differentiation of early retinal cell types such as ganglion cells. Forced expression of *Math5* in vertebrate retinal progenitor cells leads to an increased frequency of bipolar cell differentiation, and injection of *Math5* into Xenopus embryos prior to retinal formation causes a subsequent expansion of retinal territory (Brown et al 1995). Thus *Math5* possesses neurogenic activity and is expressed early in the developing retina, prior to the differentiation of the first retinal neurons. However it does not appear to be sufficient to promote ganglion cell differentiation specifically.

Antagonism between *Mash1* and *Hes1* has also been demonstrated in cultures of immature hippocampal neurons (Castella et al 1999). These genes are reciprocally expressed as hippocampal neurons differentiate, with a decrease in *Hes1* expression accompanying an increase in *Mash1* expression during differentiation. Forced expression of *Mash1* in newborn hippocampal neurons leads to an increase in neuronal differentiation, as indicated by neurite outgrowth. Forced expression of *Hes1* leads to an inhibition of unprovoked neuronal differentiation, and is sufficient to inhibit the neuronal differentiation triggered by forced *Mash1* expression. Additional evidence supporting a reciprocal relationship between *Hes1* and *Mash1* comes from *Hes1* null mice, where premature neuronal differentiation in the CNS is correlated with *Mash1* upregulation (Ishibashi et al 1995).

The *neurogenins* and *Mash1* share the ability to induce the expression of *delta* and normally prefigure its expression *in vivo*, much like the activity of *AS-C* in Drosophila (Ma et al 1996, Chitnis e al 1995, Henrique et al 1995, Bettenhausen et al 1995, Ma et al 1998). In addition, these early bHLH factors are capable of inducing two subsequently expressed neural bHLH genes, *NeuroD* and *neurological stem cell leukemia factor* (*NSCL*) (Ma et al 1996, Ma et al 1998). Three *NeuroD* genes and two *NSCL* genes have been identified to date (McComick et al 1996, Gobel et al 1992, Begley et al 1992). The *NSCL* genes are so named because their sequences resemble that of the heamatopoietic regulatory gene *SCL*, which is required for development of the heamatopoietic lineage (Porcher et al 1996). In contrast to *Mash1* and the *neurogenins*, *NSCL* and *NeuroD* family members are not generally expressed in early neural progenitor cells. These genes are expressed following *neurogenin* and *Mash1* expression, and in Xenopus, *NeuroD* is clearly restricted to a subset of *ngn1*-expressing cells (Ma et al 1998, Fode et al 1998, Cau et al 1997, Ma et al 1996, Sommer et al 1996, Chitnis and Kintner 1996). Furthermore, the expression of *NeuroD* tightly correlates both spatially and temporally with N-tubulin expression in Xenopus. In the murine P19 embryonal carcinoma cell line (P19 EC), bHLH transcription factor genes are expressed sequentially as cells differentiate along the neuroectodermal lineage, with *Mash1* and the *ngns* being expressed first, followed by *NeuroD* and *NSCL* (Itoh et al 1997). *In vivo*, both *NeuroD*

and *NSCL* are specifically expressed in the mantle zone of the developing mouse neural tube, complementary to the expression of the *ngns* and *Mash1* in the ventricular zone (Lee et al 1995; Sommer et al 1996; Ma et al 1996; Ma et al 1997; Begley et al 1992; Gobel et al 1992). The expression of *NeuroD* and *NSCL* also appears to follow *Mash1* and *ngn* expression in the developing retina, olfactory epithelium, and cranial sensory ganglia (Cau et al 1997; Morrow et al 1999; Ma et al 1997; Sommer et al 1996; Lee et al 1995). A comparison of the extent of expression of *Mash1*, the *ngns* and *NeuroD1* reveals potential convergence onto *NeuroD1* by the more restricted and disparate *Mash1* and *ngn* genes. If such convergence occurs, it does so despite the number of *NeuroD* genes identified, and may suggest that the *NeuroD* genes perform redundant functions. Manipulation of *Mash1* and *ngn* gene expression also alters the expression of *NeuroD* and *NSCL* in a predictable manner, suggesting a causal relationship (Guillemot et al 1993; Fode et al 1998; Ma et al 1998; Ma et al 1996). Thus it has been hypothesized that *NeuroD* and *NSCL* lie at the end of a transcriptional regulatory pathway that spans the neuronal lineage and is largely responsible for the progressive differentiation of neurons (Lee et al 1997). *In vitro*, the biochemical behaviour of NeuroD1 and NeuroD2 is largely indistinguishable from that of MASH-1. NeuroD1 and NeuroD2 bind the E-box and activate transcription in an E-box dependent manner (McCormick et al 1996). Interestingly, the activities of NeuroD1 and NeuroD2 appear to differ slightly, as NeuroD2 is capable of activating transcription from a fragment of the *GAP-43* gene, while NeuroD1 is not (McCormick et al 1996).

The analysis of *NeuroD* function *in vivo* has been complicated by the fact that *NeuroD* is required for development of pancreatic β-cells. Mice null for the *NeuroD* locus die shortly after birth from severe diabetic complications (Naya et al 1997). Early neural development appears overtly unaffected by the loss of *NeuroD*, possibly owing to redundancy within the *NeuroD* gene family. However studies utilizing retinal explants, and a conditional *NeuroD* null mutant, have revealed multiple functions for *NeuroD* in neural development.

NeuroD is expressed in retinal progenitor cells and neurons in the developing retina, but is excluded from glial cells. Retinal explants derived from *NeuroD* null mice exhibit a 3 to 4 fold increase in the number of glial cells produced (Morrow et al 1999). There is also a change in the population distribution of neuronal subtypes in these explants. A larger proportion of bipolar and a smaller proportion of amacrine cells develop in these explants as compared to explants from wild type mice. In addition, *NeuroD* appears to be required for the survival of a subset of photoreceptors in vitro. Conversely, forced expression of *NeuroD* in retinal explants inhibits glial cell formation, increases the number of neurons formed, and shifts the subtype distribution toward amacrine cells and away from bipolar cells (Morroe et al 1999). Studies in chick also support a role for *NeuroD* in photoreceptor development. *NeuroD* is expressed in progenitors and developing neurons in chick and forced expression of *NeuroD in vivo* leads to the production of photoreceptors specifically, with no change in amacrine, bipolar or ganglion cell formation (Yan amd Wang 1998). In the chick, *NeuroD* has the ability to generate photoreceptors *de novo* from an explant of retinal pigment epithelium, without inducing the formation of other retinal cell subtypes.

Recently the mutant pancreatic phenotype of *NeuroD* null mice was rescued, permitting an analysis of postnatal neural development in the absence of *NeuroD* (Miyata T et al 1999). These conditional *NeuroD* mutant mice carry a second transgene in which *NeuroD* is positioned behind the insulin promoter. Mutant mice survive postnatally and display postnatal neuronal differentiation defects. Granule neurons of the dentate gyrus in the hippocampus fail to form, and granule neurons of the cerebellum are largely absent in these *NeuroD* mutant mice. In the cerebellum, an increase in apoptosis is observed in the external granule cell layer where newborn granule neurons reside. The domain of apoptotic cells in the external germinal layer (EGL) appears expanded as well. Apoptosis is also observed in the internal granule layer, indicating that some mutant neurons die only after migration. *NeuroD* expression in the hippocampus is unusual in that it is observed in progenitor cells that give rise to granule cells. Mutant animals never form a dentate gyrus, and exhibit increased apoptosis among presumed granule neuron progenitor cells.

The *NSCL2* gene has been targeted and mice null for the *NSCL2* locus are infertile, display sexual dysfunction, and have altered levels of circulating gonadotrophins (Good et al 1997). *NSCL2* is normally expressed in the developing hypothalamus and in the primordium of the pituitary. Despite the physiological consequences of *NSCL2* disruption, these structures appear overtly normal in *NSCL2* mutant mice. It is presumed that *NSCL2* affects the differentiation or function of these cell populations without noticeably compromising their survival or organization.

The Xenopus *Xash3* gene encodes a bHLH factor which is expressed early in neural progenitor cells but in a more restricted domain than the *ngns* (Zimmerman et al 1993). It is concentrated in the anterior portion of the neural plate. Forced expression of *Xash3* results in an expansion of the neural plate, as epidermal progenitor cells adjacent to the normal neural plate domain are converted to a neural fate. Somewhat paradoxically, *Xash3* was found to repress primary neuronal differentiation. Thus *Xash3* promotes neural precursor formation within a limited domain and adjacent to pre-existing neural tissue, but inhibits neuronal differentiation within normal and ectopic neural territory. The exact role of *Xash3* is unclear, and a murine homolog has not been identified.

A number of genes related to the Drosophila bHLH proneural gene *atonal* have also been identified in Xenopus and mice. These genes are collectively referred to as the *mammalian atonal homolog* genes (*Math* genes). These genes are expressed in distinct and partially overlapping domains. Their expression is also dynamic and suggestive of a function in the early stages of neurogenesis. *Math1* is first expressed at embryonic day 9.5 in cells of the developing cranial ganglia and rostral alar plate of the central nervous system (Akazawa et al 1995). By embryonic day 10.5, expression throughout the dorsal neural tube is evident, but by embryonic day 18 expression is restricted to the external granule layer of the developing cerebellum. *Math1* is expressed in progenitor cells and temporarily in their derivatives in the cerebellum, but is not detected in the adult. Disruption of the *Math1* gene in transgenic mice results in a cerebellum devoid of an external germinal layer and lacking granule cells (Ben-Arie et al 1997). *Math1* is also

normally expressed in the developing inner ear sensory epithelia, and mutant mice also lack cochlear and vestibular hair cells (Bermingham et al 1999). In contrast to *Math1*, *Math2* is not expressed in progenitor cells. At embryonic day 11.5, *Math2* is expressed in the cortical plate and the mantle zone of the developing brain and spinal cord respectively (Shimizu et al 1995). By embryonic day 13.5, expression has increased in the cortical plate, and decreased elsewhere in the nervous system. The adult cerebrum also shows a high level of *Math2* mRNA. Both Math1 and Math2 form dimers and bind in a sequence-specific manner to the E-box sequence (Akazawa et al 1995; Shimizu et al 1995). In addition, Math1 is a transcriptional activator, the activity of which may be antagonized by co-expression of *Hes* genes (Akazawa et al, 1995), similar to Mash1 and the ngn's. *Math5* expression, as previously mentioned, is restricted to the developing retina where expression is seen in early retinal progenitor cells and is required for their development. Further, *Math5* also possesses neurogenic ability in a Xenopus assay, where it expands developing retinal territory when mis-expressed.

The Xenopus homolog of *Math1*, *Xath1*, is restricted to the dorsal hindbrain during neural development, much like the latter phase of *Math1* expression in mouse (Kim et al 1997). Injection of *Xath1* in Xenopus embryos results in ectopic neurogenesis within and outside of the normal neurogenic domain of ectoderm. Interestingly neither *NeuroD* nor neurogenin are induced by forced expression of *Xath1*, and *Xath1* induces N-tubulin expression without inducing *NCAM* expression. These results suggest that *Xath1* functions in terminal differentiation rather than neural specification, and does so independent (or downstream) of *NeuroD*. The relevance of these results are unclear.

Similarities between the *ngn's*, *Mash1* and *NeuroD* raise the question of whether they differ solely in expression or also in function. Ectopic expression studies in Xenopus have suggested that they differ in their sensitivity to lateral inhibition, and in their ability to activate each other (Chitnis and Kintner 1996). *Neurogenin* induces the expression of *delta*, and presumably initiates lateral inhibition. It also induces *NeuroD* and *N-tubulin*, suggesting that neurons are formed. However *NeuroD* does not appear to be capable of inducing *ngn*. In the presence of an activated Notch signal, *neurogenin* cannot stimulate

neuronal formation. In contrast, *NeuroD* can induce ectopic neuronal differentiation in the absence or presence of Notch activation. It has been suggested that determination factors and differentiation factors may have evolved differential sensitivity to inhibitory cues, with determination factors being sensitive, and differentiation factors being insensitive. This may also be the case in muscle development, where distinct bHLH factors similarly lie at the determination event and at the terminal differentiation event (Weintraub et al 1991; Lee et al 1995). Such a mechanism may allow cells to commit while delaying differentiation, and allow them to be modified and to proliferate in the interim.

(c) The Neurogenic Genes

Turning to the negative regulation of neuronal differentiation, the Notch pathway has maintained its molecular integrity in both Xenopus and mouse (Chitnis and Kintner 1995; Artavanis-Tsakonas et al 1999). In Xenopus, the inhibition of *delta* function results in excessive neuronal differentiation, while ectopic *delta* expression leads to the inhibition of endogenous neuronal differentiation (Chitnis et al 1995). Similarly, expression of a *Notch* allele encoding an activated isoform of Notch inhibits the formation of neurons, while a dominant negative isoform causes excessive neuronal differentiation in Xenopus (Coffman et al 1993). At the next step in the pathway, overexpression of *Su(H)* results in the suppression of neuronal differentiation, while a dominant negative form of the transcription factor leads to excessive neuronal differentiation in Xenopus (Wettstein et al 1997). Overexpression of a Xenopus *E(spl)* homolog inhibits neuronal differentiation, while inhibition of endogenous gene activity enhances it. Xenopus homologs of *groucho* have been cloned (Choudhury et al, 1997), and recent experiments suggest that they too function as transcriptional co-repressors (Roose et al, 1998), though their role in neuronal development is not clear. A Xenopus homolog of *h* has also been isolated which contains a C-terminal WRPW repeat that is required in functional assays (Dawson et al, 1995), suggesting it may interact with a groucho homolog and that this interaction may be important for function.

Several *Notch* genes have been cloned in mice. They exhibit widespread and partially overlapping expression domains (Williams et al 1995). With respect to neural development, the *Notch* genes appear to be expressed primarily in progenitor cells of the ventricular zone within the developing neural tube. However, these genes are later expressed in regions where mature neurons reside, in specific regions of the spinal cord and brain. Three genes encoding Notch ligands have been identified in mice. *Jagged1*, a *serrate* ortholog, and *delta1* are expressed widely throughout the nervous system and coincide in many progenitor cell domains with the expression of the *Notch* genes (Lindsell et al 1996). *Jagged2*, a second *serrate* ortholog, exhibits a more restricted pattern of expression among progenitor cells, but is found in differentiated neurons of the CNS and PNS, as well as retinal progenitor cells (Valsecchi et al 1997; Shawber et al 1996).

A comparison of expression patterns shows that specific *Notch* genes are not co-expressed with specific ligand-encoding genes, that nearly all regions of the nervous system express at least one *Notch* and one ligand encoding gene, and that regions may express more than one ligand-encoding and *Notch* gene. However at the cellular level it is not known whether multiple *Notch* and ligand-encoding genes are co-expressed. Regardless, the disparate patterns of expression raise the issue of whether the encoded receptors and ligands differ intrinsically or are capable of complementing one and other functionally. Finally, this group of genes is of great interest as three human disorders are associated with mutations in three distinct members, illustrating the broad scope of Notch pathway activity in humans. Alagille syndrome, a developmental disorder affecting the face and eyes, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a late onset systemic vascular disorder affecting brain structure and behaviour and predisposing to victims to stroke, and T-cell acute lymphoblastic leukemia (TAL) are associated with mutations in Jagged-1, Notch-3, and Notch-1 respectively (Joutel et al 1998; Ruchoux et al 1997; Oda et al 1997).

Deletion of the *Notch1* gene is homozygous lethal, and embryos die by embryonic day 10, shortly following the onset of neuronal differentiation (Swiatek et al 1994; Conlon et al 1995). These mice exhibit a defect in the segmentation of somites from presomitic mesoderm, but neural defects are not evident. This may be due to their early embryonic death. Conversely, forced expression of a *Notch* allele encoding an activated Notch isoform in neural progenitor cells led to an expansion of the progenitor cell population, a corresponding expansion and deformation of the neural tube, and death by embryonic day 12.5 in transgenic mice (Lardelli et al 1996). In addition, the expression of a similar *Notch* allele in naive P19 EC cells inhibited neuronal differentiation that was normally triggered by a pulse of retinoic acid (Nye et al 1994).

In the developing mammalian retina, *Notch1* is expressed at successive stages of development, coincident with the differentiation of different cell types (Bao et al 1997). The mammalian Notch1 ligand encoded by *delta1* is also found in the retina during development (Morrow et al 1997). An activated Notch1 isoform causes abnormal growth in retinal progenitor cells *in vivo* and perturbs normal retinal cell-type differentiation (Bao et al 1997; Austin et al 1995), similar to the effects of forced *Hes1* expression (Ishibashi et al 1994). Retinal ganglion cells are the first cell type to form in the developing retina, and the number of retinal ganglion cells decreases in the presence of activated Notch. Conversely, the introduction of *Notch* antisense oligonucleotides into retinal progenitor cells in vivo results in an increase in the number of retinal ganglion cells formed (Austin et al 1995).

The Notch pathway also appears to be involved in inner ear development. The sensory epithelium of the inner ear is composed of spatially arrayed hair cells, regularly interspersed with non-hair support cells such that neighbouring sensory hair cells are separated from one and other by an intervening support cell. The early epithelium is thought to consist of equivalent cells, and local interactions are thought to specify cell fates within the epithelium. *Notch1* and its ligand encoding gene *Jagged2* are expressed in alternating cell types in the developing epithelium, and disruption of *Jagged2* results in a significant increase in the number of sensory hair cells, apparently at the expense of

support cells (Lanford et al 1999). This is reminiscent of the situation in Drosophila where the Notch pathway is used to segregate dermoblasts and neuroblasts in an epithelial sheet, and where Notch pathway manipulation in cells of the ventral ectoderm renders them incapable of lateral specification, resulting in either excessive neuroblasts, or excessive epidermoblasts, at the expense of the alternative cell type.

In addition to its role in neurogenesis, *Notch* has also been implicated in cell proliferation. Such activity may be relevant to neurogenesis, where proliferation and differentiation appear to be mutually exclusive. This activity has been observed in C. elegans, where the mutations in the *glp-1* gene result in ligand independent and cell autonomous tumour formation in the germ cell line (Berry et al 1997) . In mice, the *Notch4* locus is a frequent target of the mouse mammary tumour virus in mammary tumours. *Int-3*, the viral oncogenic form of *Notch4* encodes a protein lacking the extracellular domain that is predicted to be constitutively active (Uyttendaele et al 1996). In rats, an activated isoform of *Notch1* alters the proliferation of retinal progenitor cells in vivo (Bao et al 1997). In humans, *Tan-1* is a naturally occurring translocation in the *Notch1* locus associated with B-cell lymphoma. The *Tan-1* gene predicts a truncated Notch protein similar to the activated isoforms described above. Furthermore, retroviral expression of this *Notch* allele is sufficient for the transformation of primed mammalian cells in culture (Capobianco et al 1997).

Mutations in *Jagged1* are associated with human Alagille syndrome, the symptoms of which include eye and facial dysmorphogenesis (Oda et al 1997). The disruption of *Jagged1* in mice is lethal and embryos die at an early stage from hemorrhage, apparently due to a vasculature deficiency (Xue et al 1999). The early embryonic death precludes an analysis of the requirement for *Jagged1* in neural development. Interestingly, heterozygous mutant mice exhibit eye dysmorphology.

The murine *RBP-Jk/Su(H)* gene product protein also appears to be involved in the repression of neuronal differentiation in mice. Murine *RBP-Jk/Su(H)* has been cloned and is ubiquitously expressed in the developing nervous system. Targeted disruption of *RBP-*

Jk/Su(H) is homozygous lethal, similar to *Notch* disruption, with mice dying by embryonic day 11.5 (de la Pompa et al 1997). In contrast to the *Notch* mutant mice, *RBP-Jk/Su(H)* mutant mice display a clear neural phenotype, possibly owing to their relatively prolonged viability. These mutants display premature neuronal differentiation throughout the nervous system. *In vitro*, RBP-Jk/Su(H) exhibits intrinsic transcriptional repression activity and binds the sequence CGTGGGAA (Hsieh and Hayward 1995; Henkel et al 1994), the natural target sequence of Drosophila Su(H) located in *E(spl)* genes (LeCourtois and Schweisguth 1995; Bailey and Posakony 1995). Interestingly, this sequence is also located in *Hes1*, where it is required for induction by Notch activation, revealing conservation of the Notch signaling cascade (Jarriault et al 1995).

A number of *E(spl)* genes have been cloned in mice, namely *Hes1*, *Hes2*, *Hes3*, and *Hes5*. These genes show distinct and dynamic patterns of expression, with some restricted to particular cell types. *Hes3* is first expressed in presumptive progenitor cells in the rhombencephalon and then later restricted to Purkinje cells (Lobe 1997; Sasai et al 1992). *Hes2* is expressed as early as embryonic day 9.5 and is detected in a number of embryonic and adult tissues (Ishibashi et al 1993). *Hes1* is expressed early throughout the developing nervous system and is localized to the ventricular zone from the caudal neural tube to the forebrain (Sasai et al 1992). Disruption of the *Hes1* gene results in premature neuronal differentiation in the forebrain and a defect in anterior neural tube closure, possibly as a result of progenitor cell depletion (Ishibashi et al 1995). Most *Hes1* mutant mice die perinatally. Premature and abnormal neuronal differentiation was also observed in the retina of *Hes1* mutant mice (Tomita et al 1996). Conversely, forced expression of *Hes1* in developing retinal progenitor cells (Tomita et al 1996) as well as CNS progenitor cells surrounding the lateral ventricles (Ishibashi et al 1994) results in the inhibition of neuronal differentiation.

Hes5 is expressed specifically in the nervous system, and is confined to the ventricular zone throughout the neural tube (Akazawa C et al 1992). In the developing retina, *Hes5* is expressed along with *Hes1* in retinal progenitor cells. Interestingly, retinal progenitor cells cultured from *Hes1* or *Hes5* null mice are inhibited from generating neurons in

response to Notch activation, while cells derived from double null mice do not respond to Notch activation (Ohtsuka et al 1999). This indicates that the two genes are redundant for at least some aspects of Notch signaling, but that one of these genes is minimally required for this response in retinal progenitor cells. The retinal phenotype of *Hes1* mutant mice may indicate that *Hes5* cannot normally compensate for *Hes1 in vivo*, that Notch/HES signaling is normally limiting, or that *Notch* independent *Hes1* activity is required for retinal development *in vivo*. Both Hes5 and Hes1 bind the N-box sequence *in vitro*, and behave as transcriptional repressors *in vitro* (Akazawa et al 1992; Sasai et al 1992).

Multiple vertebrate homologs of Drosophila *groucho* have been identified (Stifani et al 1992; Leon and Lobe 1993; Choudhury et al 1997). These genes are widely expressed and interact with a number of transcription factors in different tissues, suggesting widespread and diverse functions (reviewed Fisher and Caudy 1998). The encoded proteins have affinity for the nucleus (Stifani et al 1992; Husain et al 1996), can oligomerize (Grbavec et al 1998; Chen et al 1998) and posses intrinsic transcription repression activity (Grbavec et al 1998, Fisher et al 1996). They are believed to function as transcriptional co-repressors that are recruited to DNA by their affinity for DNA-binding transcription factors (Fisher et al 1996). These factors are expressed in neural precursor cells along with *Hes* genes (Grbavec et al 1998; Fisher et al 1996; Grbavec and Stifani 1996; Allen and Lobe 1999; Leon and Lobe 1993; Koop et al 1996; Dehni et al 1995) and interact with HES-1 *in vitro* and *in vivo* (Fisher et al 1996; Grbavec et al 1998 respectively). As mentioned previously, *Hes-1* is required for neuronal development. In addition, vertebrate *groucho* homologs mediate signal transduction in the *wnt* pathway (Roose et al 1998) as *groucho* appears to do in Drosophila (Cavallo et al 1998). In vertebrates, a *groucho* gene has been implicated in neuronal survival (Arndt et al 1999), but the function of individual vertebrate *groucho* genes may be redundant, as implied by the lack of a neural phenotype resulting from targeted disruption of a single *groucho* gene in mice (Mallo et al 1995).

These results suggest that the intercellular communication pathway established by the neurogenic gene products is conserved, is able to inhibit neurogenesis, and may normally

do so. Interestingly, in Drosophila this function takes place largely at the stage of epidermal/neural decision making, just prior to neural specification. In Xenopus this may also be the case, however in mammals it appears that Notch functions at a later stage of development, following neural specification but prior to neuronal differentiation. The neurogenic pathway does however target related factors of the bHLH class in different organisms and at different stages of lineage development in order to perform these functions. Some interesting genes that are expressed at intermediate stages of neuronal development and appear refractory to the inhibitory actions of the Notch pathway have recently been isolated. These factors may in fact enable cells to make the transition from neural specification to neuronal differentiation, and that transition may require resistance to the influence of lateral inhibition and the neurogenic genes. Some of these factors posses a bHLH domain, while others possess zinc-finger motifs for binding DNA. It is tempting to suggest that these factors render neural progenitor cells "deaf" to the inhibitory effects of lateral inhibition, thereby committing cells to the expression of neuronal differentiation genes such as *NeuroD*, and irreversible neuronal differentiation.

(d) Intermediate Stage Genes

X-MyT1 is a Xenopus gene which is expressed following neurogenin but prior to *NeuroD* in a pattern which prefigures neuronal differentiation (Bellefroid et al 1996). *X-MyT1* encodes a zinc-finger protein that is found in the nucleus of neural progenitor cells. *X-MyT1* mRNA injection in Xenopus embryos results in an increase in neurogenesis within the normal neurogenic domains. Co-injection of *ngn* mRNA or low levels of *Xash3* mRNA results in excessive ectopic neurogenesis both within and outside of the normal neurogenic domains. The effect of co-injection differs from injections of *ngn* or *Xash3* alone as the neurogenesis observed cannot be inhibited by Notch pathway activation. That is, an activated Notch isoform inhibits ngn and Xash3 triggered neurogenesis, but not when *X-MyT1* is expressed. Further, inhibition of normal *X-MyT1* function by expression of a dominant negative isoform of the protein revealed that *X-MyT1* function is required for neurogenesis. The authors of this study have suggested that *X-MyT1* may

be an intermediate factor that is expressed in neural progenitor cells as they make the transition from specified progenitor to differentiating neuron, and that it may act to cause an intrinsic inhibition of Notch pathway activation at some level.

Another gene that is expressed at a similar timepoint in Xenopus neural development is *Xcoe2* (Dubois et al 1998). This gene belongs to a family of related genes that all share a common HLH domain. Murine and human homologs have been identified, and in situ analysis has revealed that *Coe2* is expressed at an intermediate stage of development in mice as well as Xenopus. This factor is excluded from the ventricular zone and expressed specifically in the intermediate zone in the developing central nervous system. Forced *Xcoe2* expression in Xenopus embryos results in excessive neurogenesis within the normal neural domains, much like forced *X-MyT1* expression. However, whether or not *Xcoe2* renders the neurogenic effects of bHLH factors immune to the inhibitory influences of the Notch pathway has not been reported, nor has a dominant negative form of the protein been generated to assay the normal requirement for *Xcoe2* in neurogenesis.

A murine gene highly related to *NeuroD*, called *NeuroM*, has been isolated and is expressed in the intermediate zone of the developing CNS (Roztocil et al 1997). *NeuroM* and *NeuroD* are expressed in mutually exclusive domains. *NeuroM* is expressed by cells in the intermediate zone of the developing brain and spinal cord and appears to mark an intermediate developmental and molecular stage between *ngn/Mash* and *NeuroD* expression. The function of *NeuroM* has not yet been reported.

(e) Additional Genes Involved in Vertebrate Neurogenesis

Studies of neural differentiation in the murine P19 teratocarcinoma-derived cell line have also identified a novel transcription factor with interesting properties (Boudjelal et al 1997). The *Stra13* gene is expressed early following neural induction of the parent stem cell line, and encodes a bHLH transcription factor similar to the Hes proteins. Notably, Stra13 is unable to bind the normal target sequence of the Hes proteins, though it is

capable of interacting with the basal transcriptional machinery to effect transcriptional repression. Forced expression of *Stra13* is unable to induce neural differentiation in P19 cells, but is capable of directing differentiation along the neural lineage once induction has been triggered by an agent that normally promotes myogenesis. Thus *Stra13* is incapable of inducing these stem cells to differentiate, but appears to be capable of specifying their fate once they leave the stem cell state. The activity of *Stra13* in a Xenopus neurogenesis assay has not been reported. *In vivo*, *Stra13* expression is largely restricted to the ventricular zone within the developing nervous system, though expression is not restricted to the nervous system.

The neurogenic role of a murine zinc-finger-containing factor has been intensively studied recently (Schoenherr and Anderson 1995; Chong et al 1995). The *REST/NRSF* gene was isolated in an expression screen looking for factors binding to a previously defined target DNA sequence. This 24-nucleotide DNA element is found in a number of neural genes and will be discussed in the subsequent section. Briefly, this element, called the neuronal restriction silencing element (NRSE), has been shown to repress neural gene expression in non-neural cells in vitro. *REST* is expressed in non-neural cells and excluded from most developing neural tissue, suggesting REST may function as the cognate transcription factor for the NRSE. This is supported by the fact that *REST* expression is sufficient to repress neural gene expression in neural cell lines. However, disruption of the *REST* gene in transgenic mice resulted in limited and non-uniform neural gene expression outside of the nervous system, and did not cause a transformation of fate among those cells in which ectopic expression was observed (Chen et al 1998). Thus the role of *REST* remains elusive.

Gata2 and *Gata3* are two members of the Gata family of transcription factors, and are the only two members reported to be expressed in the developing nervous system (Nardelli et al 1999). *Gata2* expression begins at E9 and is localized to neural progenitor cells in the ventral neural tube. *Gata3* expression follows shortly, first being detected at E9.5. Like *Gata2*, *Gata3* expression is also restricted to neural precursors in the ventral neural tube. Both genes are expressed along the anterior/posterior axis and remain restricted to the

ventral neural tube as development proceeds. In sections of the developing spinal cord, *Gata2* expression is observed in the ventricular and subventricular zones, while *Gata3* expression is confined to the subventricular zone. Notably, neither gene is expressed in the mantle layer, where differentiating neurons reside.

Gata2 null mice die by embryonic day 10.5, preventing an in depth analysis of neural consequences (Nardelli et al 1999). Despite their early death, a number of morphological and molecular defects are observed in the nervous system of mutant embryos. The expression of *Isl-1*, a transcription factor essential for the formation of motor neurons and a subset of interneurons (Pfaff et al 1996), is dramatically downregulated in the ventral spinal cord and hindbrain of mutant embryos. Morphologically, the differentiation of cranial nerves III, IV, V and VII appears abnormal. All of the nerves appear smaller and some display abnormal trajectories. In addition, the trigeminal, facial, and vestibuloacoustic ganglia of mutants also appear smaller than their wild type counterparts.

The "forkhead" gene family is comprised of genes encoding transcription factors that contain the forkhead or "winged helix" domain and are important for the development of a variety of tissues (Hromas and Costa 1995; Hacket et al 1996). *HNF3β* is essential for the development of the node and notochord in mice, as well as for the development of the floor plate and motor neurons (Ang et al 1994; Weinstein 1994). *HNF3β* is normally expressed in all of these cell types (Monaghan et al 1993; Sasaki et al 1993) and *HNF3β* mutants display perturbations in dorsal/ventral organization while anterior/posterior organization of the neural tube appears relatively normal. This suggests that the widespread defects may be the result of cell autonomous effects. Conversely, the misexpression of *HNF3β* in the developing midbrain/hindbrain region under the control of the *engrailed-2* promoter results in loss of the inferior colliculus, a reduction in the cerebellum, and displacement of dorsal cell types with the concomitant formation of ventral (floor plate-like) cell types in their place, within the *HNF3β* expression domain (Sasaki et al 1994). In addition, axonal trajectories from efferent dorsal neurons are perturbed.

HNF3β appears to interact genetically with the transcription factor-encoding gene *goosecoid* in mice. *HNF3β* heterozygous and *goosecoid* homozygous double mutant mice display phenotypic defects not observed in either mutant individually (Filosa et al 1997). Double mutant mice exhibit a loss of *HNF3β* and *sonic hedgehog* expression in the ventral neural tube and notochord, severe forebrain growth defects, and the absence of optic vesicles. These results suggest that the two transcription factors interact directly or indirectly to regulate development of these neural and mesodermal structures.

Several forkhead genes similar to *HNF3β* have been isolated from Xenopus. The *pintallavis* gene encodes an HNF3β-like transcription factor that is expressed in a similar manner, in the developing floor plate. Overexpression of *pintallavis* perturbs the differentiation of anterior and dorsal neural cells types (Ruiz i Altaba et al 1992), and in combination with *sonic hedgehog*, *HNF3β* can induce ectopic floor plate development in Xenopus (Ruiz i Altaba et al 1995).

Additional murine forkhead genes involved in neural development include *Mf1*, *Mf3* and *TWH*. *Mf1* is expressed in neural crest cells and a mouse mutation that results in hydrocephaly and malformations in skeletal elements derived from the neural crest maps to the *Mf1* locus (Sasaki et al 1993). *Mf3* is expressed in the developing diencephalon, midbrain and spinal cord. *Mf3* null mice display poorly defined diencephalic and midbrain defects that vary with background (Labosky et al 1997). *TWH* is expressed in subset of spinal motor neurons and interneurons in a restricted axial domain (Dou et al 1997). Mice null for the *TWH* locus display retarded growth and motor weakness. In addition, molecular analysis indicates that neuronal patterning and population distribution is altered in the ventral spinal cord.

Retinoic acid (RA) is an important regulator of vertebrate neural development (Maden and Holder 1992). Widepsread neural tube and neural crest defects result from its absence (Dickman et al 1997). Retinoic acid is also a teratogen which when administered

exogenously perturbs many aspects of neural development, including the segmentation of the hindbrain and development of the neural crest (Lee et al 1995; Gale et al 1996).

Retinoid signals are transduced by retinoic acid receptors, which are members of the nuclear steroid hormone receptor superfamily (Beato 1989). There are a number of distinct retinoic acid receptor genes and a number of them generate transcripts that are alternatively spliced (Lohnes et al 1995). Functionally, loss of retinoic acid receptor function in Xenopus embryos using a dominant negative strategy leads to an inhibition of primary neuronal differentiation (Sharpe et al 1997) and disorganized hindbrain development (van der Wees et al 1998). Conversely, expression of a constitutively activated retinoic acid receptor leads to ectopic primary neuronal differentiation (Sharpe et al 1997). In addition, forced expression of an activated retinoic acid receptor at the neural plate stage causes a "posteriorization" of anterior neural plate tissue, while a dominant negative isoform "anteriorizes" posterior neural plate tissue (Blumberg et al 1997). Retinoic acid itself appears to have posteriorizing ability in Xenopus, as it promotes posterior cell fates in animal caps in conjunction with the neural inducing factor noggin (Papalopulu et al 1996). This posteriorizing activity may relate to the effects retinoic acid has on the developing murine hindbrain via *HOX* gene modulation.

Somewhat surprisingly, mice with mutations in individual retinoic acid receptor genes appear normal. For example, mice lacking all four alternatively spliced isoforms of the *RARβ* gene are overtly normal and maintain a teratogenic response to retinoic acid exposure (Luo et al 1995). Occasionally a fusion of the IXth and Xth ganglia is observed. In addition, mice null for the *RXRα*, *RXRβ*, and *RXRγ* loci individually are overtly normal. Remarkably, compound mutants at two loci also appear to develop normally (Krozel et al 1996). The retinoic acid signal transduction system may therefore consist of a number of redundant factors and/or posses compensatory abilities. This is not absolute however, as retinoic acid deficiency is mimicked by certain compound retinoic acid receptor mutants (Kastner et al 1997; Ghyselinck et al 1997), and certain effects of retinoic acid require specific retinoic acid receptors (Sucov et al 1995; Iulianella et al 1997; Folberg et al 1999). For example, mice null for both *RARβ* and *RARγ* exhibit

retinal defects (Ghyselinck et al 1997). In addition, a dominant negative strategy in murine P19 EC cells has revealed that retinoic acid receptor activity is required for the generation of neurons from parent stem cells following a pulse of retinoic acid.

The *Mef* genes have been intensively studied in the context of myogenesis, where they are involved in cell specification and differentiation and their mechanisms of action are beginning to emerge. Importantly, these factors interact molecularly with the previously identified myogenic bHLH factors, including *MyoD*, to stimulate myogenesis (Black and Olson 1998). By analogy, the recent identification of *Mef* transcripts in the developing nervous system as well as the reported *Mef2* activity in transgenic mice (Naya et al 1999) has raised questions about their involvement in neurogenesis. Biochemical data suggests that the Mef proteins can interact with proneural and differentiation-promoting bHLH factors much the same as they do muscle factors (Black et al 1996; Mao and Nadal-Ginard 1996). However the relevance of this interaction is not yet known as disruption of *Mef* genes in transgenic mice does not lead to overt neural phenotypes.

Interestingly, the Notch pathway appears to affect myogenesis at least partially through the modulation of *Mef2* activity, which may have implications for *Notch* activity in the nervous system. Skeletal muscle gene expression is dependent on associations between Mef2 and myogenic bHLH factors of the MyoD family. Notch activation inhibits MyoD activity by inhibiting an essential cofactor that recognizes the bHLH DNA-binding domain of MyoD. Mef2C interacts with the bHLH domain of MyoD, and an activated isoform of Notch blocks Mef2C binding to DNA, as well as its ability to cooperate with MyoD and myogenin to induce myogenesis. Finally, Mef2C interacts with the conserved and functionally required ankyrin repeats in the intracellular domain of Notch, suggesting that Mef2C may constitute the Notch target which renders MyoD incapable of inducing myogenesis.

One gene that may hold promise in terms of revealing common fundamental mechanisms in neuronal development is *postmitotic neural gene -1 (PNG-1)*. *PNG-1* encodes a putative protein with 6 zinc fingers and is expressed in a pan-neural and neural-specific

manner (Weiner et al 1997). Its expression is restricted to regions containing recently postmitotic differentiating neurons. As such, it represents the only reported putative pan-neuronal transcription factor expressed coincidentally with neuronal differentiation. The function and character of the PNG-1 protein, as well as its cell type distribution within the nervous system, remains to be revealed.

(iv) Neurogenesis in Zebrafish

Examples of neurogenic, proneural and neuronal precursor genes have all been identified in zebrafish, based on homology searches. In addition, genetic screens in zebrafish promise to identify more homologs and novel genes involved in vertebrate neural development. While interesting phenotypes resulting from chemical mutagenesis have been described, at present few culprit genes have been identified.

A highly conserved zebrafish *Notch* gene has been cloned (Bierkamp et al 1993), containing all essential functional components, but distinct in the number of repeated sequences much as C.elegans, Drosophila, Xenopus, mouse and human *Notch* genes differ from one and other. The zebrafish *Notch* gene is widely expressed, and is found throughout the early neural plate but concentrated in longitudinal stripes which prefigure primary neurogenic regions of the plate. Expression falls to an undetectable level prior to differentiation in the neurogenic stripes and is then restricted to the anterior brain region following formation of the neural tube.

Four *delta* homologs have been identified in zebrafish (delta A-D), and they show widespread but distinct patterns of expression (Appel et al 1998; Haddon et al 1998; Dornseifer et al 1997). *DeltaC* does not appear to be directly involved in neurogenesis, as it is not expressed in the neural plate. *DeltaA*, *B* and *D* however are all expressed in the neural plate and appear to be involved in neurogenesis. They are concentrated in the neurogenic stripes and fall within the *Notch* expression domain. These genes all share a very similar structure and are highly related to Xenopus delta. Furthermore, they

commonly posses antineurogenic activity in zebrafish embryos which is indistinguishable from the activity of Xenopus *delta* in zebrafish. Finally, these genes appear to lie within a conserved feedback loop described in Drosophila, as expression of Xenopus *delta* in zebrafish embryos causes a decrease in all three *delta* genes expressed in the neural plate. Conversely, dominant negative Delta isoform increases the density of *deltaA*, *B* and *D* expressing cells within the neurogenic stripes of the neural plate.

Close examination of *deltaA*, *B* and *D* expression revealed differences and suggested potentially distinct roles for the three genes. *DeltaA* and *deltaD* are expressed in a number of adjacent cells simultaneously (a cluster) prior to the appearance of neuronal markers and *delta B* expression, and many of these cells are BrdU positive. *DeltaA* and *D* expression is higher in isolated cells within these clusters. In contrast, *deltaB* is only detectable in isolated cells and expression begins coincident with the appearance of a neuronal marker. Approximately 95% of these *deltaB* expressing cells are BrdU negative. As a result, it has been suggested that the *delta* genes function in sequence, with *deltaA* and *deltaD* functioning early within a proneural cluster to single out presumptive neurons, and *deltaB* functioning later in the presumptive neurons to inhibit adjacent cells from acquiring the same fate. Interestingly, fewer *delta* genes have been identified in Xenopus and chicken, and studies in chicken have suggested that *delta* gene expression is restricted to postmitotic neurons (Chitnis et al 1995; Henrique et al 1995). In that case, *delta* would not be involved in neuronal specification from a pool of neural progenitor cells, and *delta* expression in presumptive neurons for the purpose of lateral inhibition would have to be achieved by some means other that lateral specification itself. Whether other *delta* genes remain to be isolated, or whether fundamental differences in neuronal differentiation exist between fish and chicken remains a question.

Homologs of *ngn*, *NeuroD* and *Mash* have been identified in zebrafish. *Zash1a* and *Zash1b* are two *Mash*-related genes (Allende et al 1994). They are expressed in neural progenitor cells, but their functional ability has not been reported. *Zash1b* expression is upregulated following neurogenin injection in zebrafish coincident with ectopic neuronal differentiation (Blader et al 1997), suggesting it may be involved in differentiation. The

zebrafish *neurogenin* gene is a homolog of mouse *ngn1*. It is restricted to the neural plate, and is expressed in neural progenitor cells. *Neurogenin* precedes zebrafish *NeuroD* expression (Blader et al 1997; Korzh et al 1998) and neuronal differentiation (Kim et al 1997), and injection causes ectopic expression of *NeuroD*, *deltaB*, *Zash1b*, and several neuronal differentiation markers within and outside the neural plate (Blader et al 1997; Kim et al 1997). This effect is indistinguishable from that promoted by mouse *neurogenin* in zebrafish embryos. Finally, *neurogenin* also appears to be subject to regulation by neurogenic genes, as its expression is decreased and increased in response to expression of Xenopus *delta* and dominant negative Delta isoforms, respectively (Blader et al 1997). Thus the order of neural bHLH gene expression and the activity of individual factors appears conserved between mice and zebrafish, suggesting transcriptional mechanisms regulating development in the neuronal lineage may be conserved.

There are four related *NeuroD* transcripts that have been identified in zebrafish (Liao et al 1999). They display distinct expression profiles and individually appear to cover subdomains of the developing nervous system. Whether these transcripts are derived from a single gene has not bee reported. The function of the encoded factors has also not been reported. Independent cloning and subsequent analysis of one *NeuroD* cDNA revealed that its expression follows and is induced by zebrafish and mouse neurogenin (Blader et al 1997; Korzh et al 1998).

A zebrafish *elav* homolog has also been identified, and appears to be an early pan-neuronal marker as it is in Drosophila. *Elav* expression increases over the course of neurogenesis, commencing earlier than other neuronal markers but becoming colocalized with them. Furthermore, forced expression of *neurogenin* induces ectopic *elav* expression throughout and beyond the neural plate ectoderm (Kim et al 1997). The function of zebrafish *elav* is unknown.

Interestingly a *Xcoe2* homolog has been identified in zebrafish, but whether it has similar activity has not been determined (Bally-Cuif et al 1998). *Zcoe2* is strongly expressed in a

subset of primary progenitor cells within the *neurogenin* and *deltaB* expression domains, and is expressed prior to *NeuroD*. Expression decreases during neurogenesis, and becomes restricted to the olfactory bulb. No neurogenic activity has been demonstrated, though this factor appears to be situated at a similar developmental stage as its Xenopus homolog, indicating it may function in a similar manner. In support of a neurogenic function, *Zcoe2* expression is increased within the neurogenic domain of a mutant zebrafish strain that exhibits excessive primary neuronal differentiation.

A particularly interesting zebrafish mutant exhibits excessive primary neuronal differentiation apparently at the expense of pigment cells, glial cells, subsequent neurons, and neural crest cells. The culprit mutant gene has not been identified, but this mutant, named "white tail" and "mindbomb", is very reminiscent of the neurogenic mutants of Drosophila (Jiang et al 1996; Schier et al 1996). Neurogenesis is affected throughout the nervous system and there is a dramatic increase in neuronal differentiation within the normal neurogenic domain. Precursors of pigment cells, glial cells, later born neurons, and neural crest-derived tissue may adopt a neuronal fate during primary neurogenesis, leaving a shortage of progenitor cells for subsequent differentiation. This hypothesis has been set forth to account for defects in mouse neurogenesis resulting from targeted disruption of murine neurogenic genes. Finally, somitogenesis appears delayed in this mutant, which was also observed in the mouse *Notch* mutant. The gene affected and responsible for these defects awaits identification.

(v) The Regional Regulation of Neurogenesis and Subtype Specification by Transcriptional Mechanisms

As mentioned previously, the advent of homologous recombination for use in mouse genetic studies has revealed that many transcription factors expressed in spatially discrete domains are required for neuronal differentiation specifically within those regions. This suggests that neurogenesis may be regionally regulated at the transcriptional level. Although their cellular mechanisms of action are equivocal in most cases, these

transcription factors may be relevant to a discussion of transcriptional mechanisms of neuronal specification and differentiation and provide a useful preface for the subsequent analysis of regulatory region activity in neural genes.

The *Phox2a* and *Phox2b* genes encode paired box homeodomain containing transcription factors that are very similar and coexpressed in most regions. In the PNS, *Phox2a* is essential for the development of sympathetic, parasympathetic, and cranial sensory ganglia (Morin et al 1997; Pattyn et al 1997). In the central nervous system, *Phox2a* is required for the development of the locus coeruleus (Morin et al 1997). The full extent of *Phox2a* activity may be partially masked by the co-expression of *Phox2b* in most domains. The two Phox genes are expressed in neural progenitor cells and differentiating neurons (Valarchae et al 1993; Tiveron et al 1996; Fode et al 1998) giving rise to these affected structures.

In vitro and *in vivo* analysis suggests that *Phox2a* lies directly or indirectly downstream of *Mash1* (Lo et al 1998; Hirsch et al 1998) in the specification and differentiation of peripheral autonomic neurons and noradrenergic neurons of the CNS. *In vitro* analyses also suggest that *Phox2a* is required for autonomic neuron differentiation, but not sufficient for it (Lo et al 1999). Conversely, *Phox2a* does not lie downstream of *ngn2* in the development of cranial sensory neurons in vivo (Fode et al 1997), yet disruption of either *ngn2* or *Phox2a* is detrimental to the formation of the distal cranial ganglia. These results suggest the existence of a *Mash1* independent mechanism of *Phox2a* induction, as well as parallel pathways of neuronal specification and differentiation within the cranial ganglia. Whether *Phox2a* is directly or indirectly affects these processes is undetermined.

The *AP2* gene is expressed in the early neural epithelium, neural folds, and migrating neural crest. Disruption of the *AP2* gene in mice leads to severe dysmorphogenesis of the skull, face, eyes, ears, and cranial ganglia. The neural tube fails to close anteriorly in these mutants, and the two hemispheres develop with their germinal layers facing outward (cranioschisis). With respect to eye development, the optic cup is displaced, and lens placode induction does not take place presumably as a result of misalignment. The

rudimentary retinal layers that do form are dysmorphic. The neural folds are delayed in forming and elevating, and then fail to converge anteriorly. *AP2* is expressed at high levels in the neural folds and emigrating neural crest at this stage in wild type embryos. Further, there is a dramatic increase in cell death in the midbrain and hindbrain at this early stage (E9.0) as indicated by TUNEL labeling. Extensive cell death is also observed in the primordia of the cranial ganglia at this time and soon after. By E10.5, the cranial ganglia are clearly underdeveloped or absent (Schorle et al 1996; Zhang et al 1996), though the DRGs appear less affected. With such dramatically altered morphogenesis, it is difficult to address the cell autonomous effects of the *AP2* mutation, and the phenotype likely results from autonomous and non-autonomous effects. Clearly this is an important factor for craniofacial development.

The POU domain genes encode transcription factors that regulate cell type specific gene expression in mammals (Bodner et al 1988; Clerc et al 1988; Ingraham et al 1988; Ko et al 1988; Scheidereit et al 1988). These genes have been evolutionarily conserved, and a POU domain containing transcription factor has been shown to regulate neural specification in C. elegans (Finney et al 1988; Finney et al 1990). There are at least six distinct classes of POU genes in mammals, and the class III and IV POU genes appear to be expressed widely in the developing nervous system.

The class IV POU domain transcription factors *Brn-3.0*, *Brn-3.1* and *Brn-3.2* are expressed in discrete cell populations of the developing CNS and PNS. The genes have been individually targeted for disruption in transgenic mice, and mutant mice exhibit distinct phenotypes whereby regions that selectively express individual genes are most severely affected. Sensory neurons and specialized sensory cells appear to be particularly susceptible to these mutations.

Brn-3.0 is expressed early in the developing nervous system, in developing sensory neurons and discrete CNS nuclei. Transgenic mice null for the *Brn-3.0* locus exhibit sensory neuron deficits (McEvilly et al 1996). These deficits may not result from defects in neuronal specification or differentiation directly, but may be due to the effect of *Brn-*

3.0 on neurotrophic factor receptor expression (McEvilly et al 1996) and subsequent neuronal survival. Alternatively, in vitro studies have suggested that *Brn-3.0* may have a direct affect on neurite outgrowth (Lakin et al 1995), raising the issue of whether sensory neurons in *Brn-3.0* null mice reach appropriate targets initially.

Brn-3.2 and *Brn-3.1* are expressed in largely overlapping domains but do exhibit selective expression in the retina and inner ear, respectively. *Brn-3.2* is expressed in differentiating retinal ganglion cells in the developing mouse retina, and mice null for the *Brn-3.2* locus exhibit a loss of most retinal ganglion cells (Erkman et al 1996; Gan et al 1996; Gan et al 1999). The retinal ganglion cell loss is correlated with an increase in apoptosis in the retina from E15 to E18, however *Brn-3.2* does not appear necessary for the specification and migration of retinal ganglion cells (Gan et al 1999). In addition, a thinner optic nerve is evident prior to the increase in cell death, indicating a potential defect in process formation. In support of this, cultured retinal ganglion cells display abnormal growth, an inability to fasciculate, and appear perturbed at the ultrastructural level. Thus *Brn-3.2* appears to be required for retinal ganglion cell differentiation and survival. Though expressed in different sensory tissue, the function of *Brn-3.1* appears similar in some respects to *Brn-3.2. Brn-3.1* is expressed in developing hair cells within the inner ear, and mice null for the *Brn-3.1* locus exhibit a loss of hair cells specifically (Erkman et al 1997). The inner ear deficit is a consequence of excessive apoptosis following hair cell specification and concurrent with partial differentiation (Xiang et al 1998). *Brn-3.1* thus does not appear necessary for the early specification of hair cells, but rather for their subsequent differentiation. The function of *Brn-3.1* may be conserved in humans, as a mutation in the human *Brn-3.1* gene is associated with progressive sensorineural hearing loss (Vahava et al 1998).

The class III POU domain genes *Brn-2.0* and *Brn-4.0* are also expressed in the developing nervous system. Mice null for the *Brn-2.0* locus exhibit lack discrete hypothalamic nuclei and the posterior pituitary, regions where *Brn-2.0* is expressed in wild type mice (Schonemann et al 1995; Nakai et al 1995). In vitro, the inhibition of *Brn-2.0* expression in P19 cells prevents them from developing along the neural lineage and

differentiating in response to retinoic acid (Fujii et al 1993). *Brn-4.0* is expressed in the intermediate zone of the developing striatum, between *nestin* expressing cells of the VZ and *β-III tubulin* expressing cells of the mantle zone. *Brn-4.0* is specifically upregulated by brain-derived growth factor (BDNF) or insulin-like growth factor-1 (IGF-1) in both striatal stem cell-derived and primary striatal precursors. Both IGF-1 and BDNF induce neuronal differentiation in these cultures. In addition, *Brn-4.0* is upregulated by BDNF and IGF-1 specifically. The introduction of antisense *Brn-4.0* oligonucleotides blocks neuronal differentiation in these cultures in response to BDNF and IGF-1 (Shimazaki et al 1999), suggesting *Brn-4.0* is required for their differentiation.

A POU domain gene encoding a dominant negative transcription factor has been identified in Drosophila, where it is coexpressed with another POU domain containing protein that is involved in neural gene expression in a subset of CNS neuroblasts. *I-POU* encodes a POU domain containing transcription factor that lacks two basic amino acids in its DNA-binding domain and is incapable of binding to DNA. I-POU is however capable of binding to Cf1a, the POU domain containing factor that is co-expressed with it. Cf1a can bind to DNA on its own, but fails to bind DNA in combination with I-POU (Treacy et al 1991). Thus I-POU appears to function much like Id does in the inhibition of bHLH domain binding to DNA. Whether an *I-POU* ortholog exists in mammals, and whether a similar mechanism of regulation might exist for mammalian POU factors is undetermined.

The Hox genes encode a conserved group of transcription factors that harbour the homeodomain DNA-binding domain (Krumlauf 1994). The mammalian Hox genes are involved in pattern formation in the developing embryo. Their orthologs in Drosophila, the HOM genes, are similarly involved in developmental patterning. Mutations in several HOM genes give rise to the well-known homeotic transformations, where one body region develops inappropriately but much the same as another region. For instance, the "antennapedia" mutation causes a regional transformation whereby ectopic legs are formed in the head region where antennae should normally develop.

Mammalian HOX gene function has been intensively studied in the developing hindbrain, where they are involved in pattern formation (Lumsden and Krumlauf 1996). The mammalian hindbrain is physically and molecularly divided into several segments, or rhombomeres, early in development. These segments differ in their patterns of gene expression and are constrained by regional boundaries that limit cell mixing. Individual rhombomeres constitute cohorts of cells that function as a morphogenetic unit within the developing hindbrain. Importantly, members of the HOX gene family are restricted to particular rhombomeres in the developing hindbrain and contribute to their specification. Altering the pattern of Hox gene expression can lead to malformations in the hindbrain and tissue derived from emanating neural crest.

Some loss of function and gain of function studies have produced what may be interpreted as limited homeotic transformations in neural tissue, whereby the identity of a region appears to have been altered through effects on the primordial cells of the region. The disruption of a single Hox gene in transgenic mice can cause the formation of apparently normal cell subtypes at inappropriate locations, and perturb the formation of appropriate cell subtypes. Such malformations may be due to normal and/or reflexive Hox gene expression in these domains, which may direct a pattern of development reminiscent of another region. It is unclear whether or not the Hox genes are involved in the regulation of cell type specification and differentiation per se, or in a separable process of regional/cell subtype specification. The possible redundancy of Hox genes in certain aspects of neuronal development may make their function difficult to ascertain. Misexpression of the Hox genes in the nervous system leads to similar transformations in regional specification. It is similarly unclear whether a change in subtype specification occurs, separable from cell type specification and differentiation, or whether a distinct neurogenic program is being followed where subtype specification and cell type are inseparable. The activity of one particular Hox gene in C. elegans, an antennapedia homolog, has been examined in great detail and may provide clues as to mammalian Hox gene function.

The C. elegans Hox gene *mab-5* appears to be involved in the control of neuroblast specification (Salser and Kenyon 1996; Cowing and Kenyon 1992; Salser et al 1993). Its proximate involvement in this process may not simply be a consequence of the shortness of the lineage. *Mab-5* expression is regarded as a posterior patterning gene in C. elegans, but it is regulated in a complex manner on a cellular basis within the region, providing a clue as to its diversity of function. *Mab-5* does not appear to prescribe the fate of its respective segment with uniform expression, rather it is dynamically regulated and functions on a cellular basis. In this manner, it appears proximately involved in the specification of distinct lineages within the segment. Among the cell types specified in the segment are neuroblasts. *Mab-5* activity is required for the specification of the neuroblasts in that segment. Moreover, m*ab-5* expression must be actively opposed at a specific time in the siblings of neuroblasts in order for them to avoid being specified as neural. This involves a hairy-like factor (*lin-22*) and an atonal-like factor (*lin-32*), whereby *lin-22* loss of function correlates with the inappropriate expression of *mab-5* as well as *lin-32*. Whether these two events are causally related is not known. Regardless, it appears that in C. elegans, Hox genes may not be far removed from cell specification. This is also true of the POU domain genes, such as *unc-86*. Whether this quality is preserved in mammalian Hox and POU genes, or whether these gene products should be viewed as molecular opportunists with DNA binding domains that do not necessarily follow the example set by C. elegans remains to be seen.

Targeted disruption of the murine *Hoxa-1* gene leads to defects in hindbrain segmentation, changes in gene expression, and defects in the formation of several cranial nerves (Chisaka et al 1992; Carpenter et al 1993; Mark et al 1993). Morphological and molecular analyses indicate that mutant mice lack rhombomere 5 and have a greatly reduced fourth rhombomere, both of which normally express *Hoxa-1*. Similarly, mutation of a RARE in the *Hoxa-1* gene extinguishes anterior *Hoxa-1* expression and generates a cranial nerve phenotype similar to but less severe and less penetrant than that of *Hoxa-1* null mice (Dupae et al 1997). Conversely, ectopic expression of *Hoxa-1* anteriorly induces a transformation in the anterior hindbrain. This change is associated with changes in the expression of other HOX genes. Rhombomeres 2 and 3 show consequent

morphological defects, and specific tissues derived from neural crest in the anterior hindbrain are also malformed (Zhang et al 1994). The zebrafish ortholog of *Hoxa-1* has been identified and characterized (Alexandre et al 1996). Anterior misexpression of *Hoxa-1* in zebrafish leads to neural defects that are reminiscent of those generated by retinoic acid exposure, including the duplication of specific posterior neuronal cell types. In addition, alterations in neural crest cell fate are observed.

Disruption of the *Hoxa-3* locus is homozygous lethal and causes defects in neural crest derived tissue. These defects do not appear to be due to the loss of progenitor cells or a migrational defect, but rather seem to stem from an inability to differentiate or a functional defect following differentiation (Manley et al 1995). Disruption of the paralogous Hoxb3 gene results in defective formation of the IXth cranial nerve (Manley et al 1997). However the stage at which the genetic lesion manifests the phenotypic defect has not been determined.

Hoxb-1 is expressed in rhombomere 4 of the developing hindbrain. Disruption of the murine *Hoxb-1* gene leads to the malformation of identifiable neuronal populations derived from rhombomere 4, and the subsequent loss of the facial motor nerve (Studer et al 1996).

Outside of the developing hindbrain, the *Hoxc-8* gene is expressed in a subset of developing motor neurons in the mouse spinal cord. Loss of *Hoxc-8* function results in the loss of these motor neurons (Tiret et al 1998). The motor neurons are specified and differentiate, but then die during the period of naturally occurring cell death. Aberrant innervation is observed in the natural targets of this population, though these neurons themselves appear to innervate their targets normally. Thus the aberrant innervation effect appears non cell-autonomous. An inability to generate the appropriate type of motor neuron may underlie this effect. The motor neurons formed in the mutant may differ fundamentally or subtly from those in the wild type, but may masquerade as normal neurons until challenged by critical molecular interactions sensitive to the distinction.

The forced expression of the paralogous gene *Hoxb-8* has a peculiar neural phenotype. The rostral dorsal root ganglia are first detectable at about E9.5 in mouse. By E11.5, a transient ganglion at level C1 (Froriep's ganglion) has nearly completed its natural degeneration. The ectopic expression of *Hoxb-8* in developing neural crest leads to the persistence of identifiable Froriep's ganglia with emanating processes (Fanarraga et al 1997). Similar to *Hoxc-8* loss of function mutants, the neurons so formed may be significantly different from the wild type. As with *Hoxc-8*, the function of *Hoxb-8* remains undetermined.

Finally, the ectopic expression of several other Hox genes has been shown to cause abnormalities in craniofacial development. In transgenic mice, the forced expression of *Hox2.2* (Kaur et al 1992), Hox2.3 (McLain et al 1992) and *Hox1.1* (Balling et al 1989) using the chicken *β-actin* promoter leads to subtly distinct craniofacial abnormalities. Whether these effects are direct and reflect the ability of Hox gene products to influence neuronal specification and differentiation is unclear.

The transcriptional regulation of Hox genes has been the subject of a great deal of study. Several conserved transcription factors appear to contribute to the spatial regulation of the Hox genes, including retinoic acid receptors, *Krox-20*, *kreisler*, and the polycomb group of genes. These transcription factors may affect neuronal specification and differentiation directly.

Kreisler and *Krox-20* encode transcription factors that lie upstream of specific Hox genes, and specify rhombomere identity. These genes are expressed in neural progenitor cells within specific rhombomeres and loss of function mutations in either gene results in neural deficits within their specific expression domains. Whether these transcription factors directly or indirectly regulate neuronal specification and differentiation in these regions is unknown.

The murine *Krox-20* gene encodes a zinc finger-containing transcription factor that is expressed very early in rhombomeres 3 and 5. Several Hox genes are direct transcriptional targets of *Krox-20* (Nonchev et al 1996a; Nonchev et al 1996b) and loss of *Krox-20* is homozygous lethal with a dramatic hindbrain phenotype in transgenic mice. The loss of *Krox-20* function results in a loss of cranial nerves and ganglia as well as changes in gene expression which are all consistent with loss or dramatic reduction of rhombomeres 3 and 5 (Schneider-Maunoury et al 1993; Schneider-Maunoury et al 1997).

The *kreisler* gene encodes a transcription factor of the basic leucine zipper Maf family (Cordes et al 1994). Loss of *kreisler* gene function results in a number of molecular and morphological changes that suggest it is required for neuronal specification and differentiation in rhombomeres 5, 6, and 7. *Kreisler* mutant mice display altered Hox gene expression in the hindbrain, and several Hox genes expressed in this region are direct transcriptional targets of *kreisler* (Manzanares et al 1997; Manzanares et al 1999). The *kreisler* mutant mice also display morphological abnormalities of the neural tube, an absence of *Krox-20* gene expression, disrupted segmentation, and excessive cell death posterior to the third rhombomere (McKay et al 1994; Frohman et al 1993). At later time points, these mice display defects in structures derived from neural crest posterior to the third rhombomere (Frohman et al 1993), and lack or have malformed cranial nerves and ganglia normally derived from progenitor cells in the posterior rhombomeres (McKay et al 1997).

An ortholog of *kreisler* has been identified in zebrafish (Moens et al 1996), where it is similarly involved in development of the hindbrain. The *valentino* gene is expressed in the primordium of the hindbrain, and its mutation results in changes in Hox gene expression, disruption of posterior rhombomeres, and defects in structures formed from neural crest emanating from posterior rhombomeres (Moens et al 1996; Moens et al 1998; Prince et al 1998). The *valentino* mutation exerts its effects in a cell autonomous manner.

The polycomb group of genes lie upstream of the Drosophila homeotic genes, and are required for their appropriate expression and segment specification in the developing Drosophila embryo (DeCamillis et al 1994) A murine ortholog of the polycomb group of genes has been identified (Nomura et al 1994). Deletion of the mouse *rae28* gene in transgenic mice is postnatally lethal, and results in neural crest deficits (Takihara et al 1997). Furthermore, these defects are correlated with a rostral shift in the anterior limit of individual HOX gene expression in the developing hindbrain, suggesting the regulatory function of the polycomb group of genes may be conserved.

Two non-clustered homeobox genes potentially involved in neurogenesis are the related genes *Gsh1* and *Gsh2*. *Gsh1* is expressed in the developing pituitary and hypothalamus. Loss of *Gsh1* function results in a pituitary that is morphologically and molecularly abnormal (Li et al 1996). The pituitary is hypocellular, and changes in hormonal gene expression are observed. The hypothalamus of these animals appears morphologically normal, but changes in growth hormone releasing hormone gene expression are observed, suggesting the adenohypohysis axis is perturbed. *Gsh2* is expressed in the developing midbrain, hindbrain and forebrain and expressed at a particularly high level in the ganglionic eminences (Szusik et al 1997). In *Gsh2* loss of function mutant mice, a pronounced decrease in the volume of the ganglionic eminences of the forebrain is observed and is correlated with a conspicuous absence of *Dlx2* gene expression in these areas (Szucsik et al 1997). Discrete nuclei in the hindbrain also appear to be absent in these mutant mice. The developmental stage affected by the loss of *Gsh1* and *Gsh2* function has not been reported.

The *Msx-1* and *Msx-2* homeobox genes are coexpressed in developing limbs, neural tube, neural crest, and branchial arches (Catron et al 1996). *Msx-3*, a third member of the Msx transcription factor family, is also expressed in the neural tube but is not expressed in non-neural tissue (Shimeld et al 1996). Msx-1 and Msx-2 share DNA sequence specificity, and both behave as transcriptional repressors in vitro (Catron et al 1996).

Antisense oligonucleotides directed against *Msx-1* and *Msx-2* individually induce craniofacial and neural tube defects in mouse embryos (Foerst-Potts et al 1997). Each antisense oligonucleotide causes a thinning of the diencephalic epithelium, eye abnormalities that are evident at the optic cup stage, and craniofacial abnormalities. Conversely, *Msx-1* overexpression in Xenopus embryos results in a striking duplication of axial structures, including the neural tube and notochord (Chen et al 1995).

Molecular investigations of eye development have revealed a number of transcription factors that are expressed successively in the developing eye and are critical for its development. The eye is formed from collection of tissues, including an evagination of the neural tube (presumptive optic cup) that contributes neural tissue for formation of the retina, and non-neural overlying surface ectoderm that generates the lens (lens placode). At the cellular level, whether transcription factors critical for eye development are directly or indirectly involved in the specification and/or differentiation of retinal neurons is unclear in most cases.

The *Rx* gene encodes a conserved homeodomain transcription factor that is required for the formation of the optic cup and is normally expressed in this tissue during development (Furukawa et al 1997). Mice null for the *Rx* locus do not form optic cups, and consequently do not develop eyes. In addition, misexpression of *Rx* in Xenopus embryos leads to retinal hypertrophy and the formation of ectopic eye tissue.

The POU domain transcription factor encoded by *Pax6* is also found in the developing eye fields, following the expression of *Rx*. The "small eye" (Sey) mutants of both rat and mouse, named for their phenotypes, harbour mutations in the *Pax6* gene predicted to result in truncated proteins. Detailed developmental analysis of mouse "small eye" mutants has revealed that *Pax6* is involved in both the formation of the lens placode, and the development of the optic cup (Grindley et al 1995). *Pax6* is required for expression of *Math5* and *Hes1* in the optic cup. These two genes are required for development of the neuroretina, and connect *Pax6* to retinal neuron formation (Brown et al 1998). The "eyeless" mutant phenotype of Drosophila is also associated with a mutation in the *Pax6*

gene. Finally, "aniridia" in humans, characterized by small eyes, is also associated with a mutation in *Pax6*, highlighting a remarkable conservation of *Pax6* gene function between species despite structurally divergent sensory structures.

Two additional genes related to *Pax6*, namely *eya1* and *eya2*, are coexpressed with *Pax6* early in the developing lens placode and throughout the developing retina. These two genes depend upon *Pax6* for their expression in these tissues, and they may play roles downstream of *Pax6* in eye development (Xu et al 1997).

Subsequent to *Pax6* expression, the homeobox containing gene *Crx* is expressed in developing photoreceptors of the retina (Chen et al 1997). Misexpression of *Crx* in retinal progenitor cells promotes the formation of photoreceptors (Furukawa et al 1997). Conversely, photoreceptor differentiation is disrupted by the expression of a dominant negative form of *Crx* in presumptive photoreceptors. Photoreceptor-specific targets of *Crx* have been identified (Chen et al 1997), but whether it is involved in neuronal cell type differentiation is not known. A conserved function for the *Crx* gene in humans has been suggested by the finding that *Crx* gene mutations are associated with heritable cone rod dystrophy in humans (Freund et al 1997).

Pax6 is also expressed in other neural progenitor cell populations, and "small eye" mutant rodents have defects in structures arising from these populations as well. Motor neurons of the hindbrain are affected in "small eye" mutant rats (Osumi et al 1997). In addition, axonal morphology among some *Pax6* expressing cells of the spinal cord is disrupted in an autonomous manner, where the ventral growth of motor axons is absent. Outside of the spinal cord, two somatic motor nerves, the abducens and the hypoglossal, are completely absent in these mutant mice. These morphological changes correlate with developmental changes in gene expression. Expression of the transcription factor *Islet-2* is missing in the hindbrain, as is expression of the secreted protein *wnt-7b*.

Within the spinal cord, *Pax6* is believed to respond to a graded ventral signal (sonic hedgehog) to specify progenitor cells within a restricted D/V domain as interneuron

subtype precursors (Ericson et al 1997). The position of these precursors correlates with the subsequent location of a subset of differentiated interneurons that co-express the genes *Pax2*, *engrailed-1* and *engrailed-2* (Burrill et al 1997). *Pax6* is required for the generation of this neuronal subtype, suggesting it may act in progenitor cell specification, and that such specification may pre-pattern neuronal arrangements that are achieved through radial migration following differentiation. Mechanistically, recent studies have suggested that it may specify interneuron precursors indirectly, by restricting the expression of a more ventrally located transcription factor (*Nkx-2.2*) rather than specifying a more dorsal progenitor cell type directly (Briscoe et al 1999).

Roles for *Pax6* in additional regions of the nervous system have been reported. In the developing forebrain between E9.5 and E10.5, *Pax6* expression appears to mark the boundary between prosencephalon and mesencephalon (Mastick et al 1997, Warren et al 1997, Stoykova et al 1996). In "small eye" mutants, the morphological boundary between prosencephalon and mesencephalon is absent, and this change is correlated with shifts in the expression of genes that are normally under tight regional restriction in this area. Caudal prosencephalon markers are lost, while *Dbx*, a mesencephalic marker, is shifted rostrally into the presumptive prosencephalon domain (Mastick et al 1997). In addition, a decrease in cell density is observed in the diencephalon up to E14.5, and the third ventricle appears enlarged (Warren et al 1997). Posterior commisure axons of the prosencephalon are notably absent, and the trajectory of longitudinal axons travelling through the prosencephalon appears altered in domains that normally expresses *Pax6* (Mastick et al 1997; Kawano et al 1999). The morphological changes in mutant mice correlate with a notably low rate of BrdU incorporation in diencephalon progenitor cells between E10.5 and E14.5 (Warren et al 1997), suggesting possible roles for *Pax6* in proliferation, specification, and differentiation.

The *Pax3* gene, like *Pax6*, encodes a POU family member associated with developmental defects linked to a human syndrome exhibiting developmental defects. The "splotch" mutant mouse, named for its pigmentation defect, carries a mutation in *Pax3* which renders the encoded protein nonfunctional (Epstein et al 1991; Chalepakis et al 1994). In

addition *Pax3* is associated with "Waardenburg syndrome" in humans, a disease affecting the neural crest and perturbing pigmentation in humans as well (Tassabehji et al 1992). *Pax3* mutations associated with "Waardenburg syndrome" similarly encode nonfunctional proteins (Calepakis et al 1994). "Splotch" mutants display a loss of DRG and sympathetic neurons (Franz et al 1993). In the thoracic and lumbar segments, sympathetic neurons appear to be completely absent. DRG neurons appear to be affected along the anterior/posterior axis, though the cranial ganglia appear overtly normal. In addition, premigratory neural crest cells appear to be "stuck" at particular A/P locations at the dorsolateral margin of the tube, where they are confined to the epithelial layer.

In the CNS, *Pax3* mutants display increased apoptosis and gross malformations of the midbrain/hindbrain region (Phelan et al 1997). This phenotype is also found in the embryos of diabetic mice, and correlates with a decrease in midbrain/hindbrain *Pax3* expression. Finally, *Pax7*, another POU family member expressed in the nervous system, is required for survival and mice null at the *Pax7* locus have facial malformations indicative of neural crest cell defects (Mansouri et al 1996). *Pax7* is expressed in both the neural crest and the spinal cord, but no CNS phenotype is evident in these mice. However *Pax7/Pax3* double mutant mice display a mutant phenotype in the spinal cord (Mansouri et al 1998). Both of these genes are normally expressed in the dorsal spinal cord and ventrally in commisural neurons. Double mutant mice display changes in gene expression, characterized by the dorsal expansion of ventral markers. Morphologically, ventral commisures are reduced in the cord, possibly indicating a loss of *Pax3/Pax7* commisural neurons.

The midbrain/hindbrain junction of the developing CNS is an important region with demonstrated organizing activity. Molecular genetic studies focussing on genes expressed in this region and their activities has revealed several important and conserved transcription factors critical for morphogenesis. Two additional POU domain transcription factors, *Pax2* and *Pax5*, are expressed in progenitor cells of this region early in development. The disruption of each gene individually in transgenic mice leads to mild

defects in the region, while *Pax2/Pax5* double mutant mice exhibit changes in the structure of the midbrain/hindbrain region (Schwarz et al 1997).

The *engrailed-1* (*En1*) and *engrailed-2* (*En2*) homeobox genes are also expressed in the developing midbrain/hindbrain region. They are orthologs of the Drosophila *engrailed* gene which encodes a transcription factor involved in segment specification during embryogenesis. Mice null for either the *En1* or *En2* locus lack midbrain and anterior hindbrain structures (Wurst et al 1994; Millen et al 1994). In addition, disruption of the *wnt-1* gene, which encodes a protein secreted by expressing cells in this domain, results in a similar loss of midbrain and anterior hindbrain structures (McMahon and Bradley 1990). During development, *En2* is not expressed in the primordia of the midbrain in *wnt-1* mutant mice. Interestingly, the forced expression of *En1* in progenitor cells of the presumptive midbrain can rescue the mutant phenotype generated by *wnt-1* gene disruption, suggesting one of the critical functions of this secreted organizing factor is to regulate *En1* expression in this region (Danielian and McMahon 1996).

The generation of motor neuron subtype diversity within the spinal cord is believed to be under the control of a number of related transcription factors. A number of genes belonging to the Nkx family of transcription factors are expressed in distinct dorsal/ventral and anterior/posterior domains of the developing neural tube (Qiu et al 1998; Pabst et al 1998). These factors reveal molecular distinctions between progenitor cells in different regions of the nervous system, and are believed to contribute to subtype diversification at this early stage. Shortly after terminal mitosis, other transcription factor families are expressed. *Islet-1* and *Islet-2* are homeobox genes expressed in newborn motor neurons (Ericson et al 1992), with *Islet-2* being expressed in a smaller number of cells (Tsuchida et al 1994). A number of transcription factors belonging to the LIM domain family are also expressed at this time (Tsuchida et al 1994). The expression patterns of these transcription factor encoding genes suggests that they may constitute a combinatorial code for positional identity (Tsuchida et al 1994). The cellular organization revealed by gene expression studies prefigures the formation of motor neuron columns and the formation of motor axon pathways.

In terms of function, targeted disruption of *Islet-1* in transgenic mice leads to a loss of motor neurons, and indirectly to a loss of interneurons (Pfaff et al 1996). The remainder of the cells in the neural tube appear to differentiate normally, but display a consequent ventral shift in their localization due to the loss of motor neurons and interneurons. Mice with disruptions in either the *Lhx3* or *Lhx4* LIM gene form motor neurons, but axonal trajectory is altered in a cell autonomous manner (Sharma et al 1997). In addition, misexpression of *Lhx3* in neural progenitor cells alters their consequent axonal trajectory in a predictable manner, giving them *Lhx3*-like neuronal axon trajectories. Interestingly, the Drosophila ortholog of *Islet-1* is expressed in motor neurons but is not required for their survival. Loss of *Islet-1* function is however associated with axonal trajectory defects and neurotransmitter synthesis defects in motor neurons of Drosophila (Thor et al 1997). In addition, Drosophila LIM and Islet proteins appear to form a combinatorial code for axonal trajectories, and the misexpression of Drosophila *Lim3* alters axonal projections in a predictable manner, yielding *Lim3*-like neuronal axon projections (Thor et al 1999).

Additional LIM proteins have been ascribed functions in the regional regulation of neural development. In *Lim1* mutant mice anterior head structures fail to develop, but structures posterior to the hindbrain appear to develop in remarkably normal fashion. Disruption of the *Lhx2* gene is homozygous lethal. Mutant mice do not form eyes and also display a hypocellular neocortex that correlates with an observable decrease in progenitor cell proliferation in the prosencephalon (Porter et al 1997). Mice homozygous null for the *Lhx5* locus display a hippocampal defect (Zhao et al 1999). Progenitor cells of the presumptive hippocampus appear to be specified, by gene expression criteria, but subsequently falter during hippocampal morphogenesis. Loss of *Nkx2.1* function, one of the Nkx genes restricted to the forebrain, results in a lack of pituitary formation (Takuma et al 1998). This is likely an indirect effect caused by a subtle differentiation defect in the diencephalon. *Nkx2.1* is expressed in the developing diencephalon, but not in the pituitary nor its primordia.

Homeodomain and LIM domain transcription factors also appear to be involved in the specification and/or differentiation of neuronal subsets in C. elegans. The C. elegans *Lim6* gene is expressed in a small number of motor neurons, sensory neurons, and interneurons. *Lim6* mutant animals exhibit changes in axonal morphology and trajectory, as well as changes in neurotransmitter phenotype (Horbert et al 1999). These changes appear to be due to a cell autonomous effect.

Similarly, the *lin-11* homeobox gene appears to be involved in neuronal differentiation (Horbert et al 1998). Focussing on a particular interneuron that expresses *lin-11*, the homeodomain transcription factor has been shown to be necessary for morphological differentiation. Furthermore, aberrant neuronal differentiation in its absence is correlated with the loss of function of a scoreable neuronal circuit in which the interneuron lies. The morphology of other *lin-11* expressing neurons outside the circuit also appears abnormal. The *ttx3* homeodomain transcription factor is expressed in a distinct neuronal constituent of the same scoreable neuronal circuit. Loss of *ttx3* function is functionally equivalent to the ablation of the expressing neuron in the circuit. This loss of function also correlates with atypical axonal morphology and trajectory in mutant animals (Horbert et al 1997).

Unc-4 encodes a homeodomain transcription factor that is required for the differentiation of a well studied motor neuron. Loss of *unc-4* function results in morphological changes in the expressing motor neuron, whereby the dendritic tree is altered changing the synaptic input received by the motor neuron. There is a functional consequence to this morphological change, and *unc-4* mutant animals exhibit a motor deficit.

(vi) Cis Elements Involved in Neural Gene Expression

One means of identifying transcriptional regulatory mechanisms in neurogenesis has been to analyze the regulatory regions of neural genes. The neural genes studied serve a variety of functions and are expressed in various cell types and subtypes at various developmental stages. This approach has identified gene segments regulating cell type specific gene expression, restriction to subsets of cells in the nervous system, and temporal control of gene expression within the neural lineage. Constituent neuronal genes that encode structural proteins constitute a major group that has been analyzed. These genes are typically expressed early in neuronal differentiation and in a wide variety of neuronal subtypes. Their regulation may involve mechanisms common to many different neuronal subtypes and therefore potentially fundamental to neuronal differentiation. Neuronal gene analysis has occasionally identified common functional sequences, implying the existence of such widespread mechanisms, as has been suggested by complimentary studies focussing on transcription factors.

Neural gene analysis has been carried out largely in Drosophila and mice, though mammalian gene analysis in most cases has not proceeded beyond the identification of functional segments to the identification of functional elements. These analyses have revealed modular organization within regulatory regions, whereby distinct regulatory modules contribute distinct characteristics to the overall expression pattern of a gene. Some modules contribute spatial specificity, which may often be regarded as subtype specificity and vice versa. Some modules contribute cell type specificity, and others contribute to dynamic regulation, inducing or repressing gene expression at specific developmental stages or mediating responsiveness to extrinsic (eg. growth factors) or intrinsic (eg. injury) cues.

Focussing on two genes that are expressed in nearly all neuroblasts of the Drosophila nervous system, E. Bier and colleagues have identified common regulatory sequences and organizational schemes involved in the transcriptional control of progenitor cell development (Emery et al 1995). The deadpan gene encodes a bHLH transcription factor

(Bier et al 1992), while the scratch gene encodes a transcription factor with zinc-fingers (Roark et al 1995). Both genes are expressed in nearly all neuroblasts of the developing nervous system, and both are involved in neural development (Bier et al 1992; Roark et al 1995). Each gene contains two regulatory modules, one that targets gene expression to the CNS and another that targets gene expression to the PNS (Emery et al 1995). Found within these modules are repeats of the E-box sequence (CANNTG), which is the consensus sequence for bHLH transcription factors such as those encoded by AS-C, making deadpan and scratch putative direct targets of the proneural genes.

Studies of the AS-C genes have also revealed transcriptional mechanisms involved in neurogenesis (Gaomez-Skarmeta et al 1995). Strewn throughout the complex are enhancer elements that appear to commonly contribute to the spatial and temporal expression of several genes of the complex. An artificial promoter constructed from some of these elements can target reporter gene expression specifically to neuroblasts in Drosophila. Mutation of specific reiterated elements within the artificial promoter leads to precocious reporter gene expression in many cells of a proneural cluster, prefiguring selection of individual neuroblasts. Moreover, reporter gene expression is not restricted to segregating neuroblasts with time, indicating that premature expression was gained and an ability to respond to lateral specification was coincidentally lost with the deletion of a single element. Among the elements found within the *AS-C* regulatory region are E-boxes, and the sequence which when lost specifically causes premature reporter expression resembles the Su(H) binding element.

Genomic DNA for the Drosophila *β-1 tubulin* gene has been analyzed, and the gene's promoter has been identified (Kohler et al 1996). A 3kb fragment of the gene delivers high level reporter gene expression in neurons of the CNS similar to *β-1 tubulin* expression. The 3kb fragment is composed of three modules, which cooperatively yield a high level CNS expression. One of the modules has been further analyzed, and in conjunction with a basal promoter is sufficient to promote expression in some CNS neurons in Drosophila. This module contains a 6bp sequence, CAAAAT, which is essential for CNS expression, and which resembles the mammalian C/EBP consensus

sequence. Furthermore, in gel shift assays a CNS-specific nuclear protein complex forms with the 6bp CNS element, however the identity of the complex is unknown.

The Drosophila *elav* gene promoter has been isolated in order to study transcriptional mechanisms involved in the regulation of a pan-neuronal gene (Yao et al 1994). Elav is an RNA binding protein expressed early in nearly all neurons of the nervous system (Robinow and White 1991). Analysis of genomic DNA has identified a 3.5kb promoter fragment that confers an elav-like expression pattern to a reporter gene in Drosophila. A 333bp fragment within this 3.5kb is required for this pattern of expression. The functional sequences within this fragment have not been identified as yet.

Analysis of neuronal gene expression in transgenic mice has also been fruitful. The rat *Tα1* gene was isolated in a subtractive hybridization screen designed to identify genes preferentially expressed in the developing nervous system versus the adult brain (Miller et al 1987). These studies further revealed that the *Tα1* gene is highly and specifically expressed in a pan-neuronal and neuron-specific manner (Miller et al 1987). *Tα1* mRNA constitutes approximately 98% of the α-tubulin mRNA and approximately 2% of the total mRNA in the developing rat brain (Miller et al 1987). *Tα1* is also dynamically regulated as a function of growth in the developing nervous system (Miller et al 1989; Mathew and Miller 1990; Mathew and Miller 1993). It is expressed early during neural development coincident with neuronal differentiation and neurite outgrowth. It is subsequently downregulated as neurons reach their targets and cease extending neurites. Following axonal injury in the peripheral nervous system, *Tα1* gene expression is upregulated, coincident with neurite regrowth, and is maintained until target reinnervation.

Nuclear run-on assays have revealed that the *Tα1* gene is regulated at the transcriptional level (Miller et al 1991). Moreover, Dr. Miller's laboratory has previously demonstrated that 1.1kb of contiguous sequence located immediately 5' of the *Tα1* gene's 5' untranslated region is sufficient to target reporter gene expression specifically to neurons, and in a spatio-temporal manner that mimics that of the endogenous gene both during

development (Gloster et al 1994), and adulthood (Bamji and Miller 1996). During development, *Tα1* promoter activity commences coincident with terminal mitosis throughout the nervous system. This coincidence between terminal mitosis and onset of *Tα1* promoter activity is also observed in vitro (Gloster et al 1999). Thus, the *Tα1* promoter possesses sequences sufficient for very early and pan-neuronal gene expression. As such, the 1.1kb *Tα1* promoter has served as a tool for the genetic analysis of neuronal differentiation reported in this thesis.

The regulation of the goldfish *α-1* α-tubulin gene has also been analyzed *in vitro* and *in vivo* (Hieber et al 1998). The goldfish *α-1* α-tubulin gene is neuron-specific and regulated as a function of growth, similar to the rat *Tα1* gene. For instance, *α-1* is expressed at high levels in the developing retina, but decreases as retinal ganglion cells cease to grow (Hieber et al 1998). Following optic nerve crush, the goldfish optic nerve regrows, and α-1 is expressed at high levels retinal ganglion cells once again (Hieber et al 1998). A 1.7kb fragment of the 5' region of the goldfish *α-1* α-tubulin gene is sufficient to target reporter gene expression specifically to the nervous system and preferentially to neurons in zebrafish embryos and adult goldfish retinal explants (Hieber et al 1998). Furthermore, this promoter is induced following axonal injury *in vivo* and *in vitro* (Hieber et al 1998). Thus the 1.7kb fragment contains cis elements sufficient for neuronal specificity and dynamic regulation, yielding a pattern of expression and behaviour similar to the endogenous *α-1* α-tubulin gene. A comparison of the goldfish and rat *α-1* α-tubulin gene regulatory regions reveals a number of conserved sequences. Some of these, including a putative Su(H) target sequence and a putative homeodomain binding site, are found within a segment of the goldfish *α-1* promoter that contributes to neuronal specificity (Hieber et al 1998).

The *peripherin* gene encodes an intermediate filament protein that is expressed in a variety of neuronal populations in the CNS and PNS (Portier et al 1983). *Peripherin* gene expression is limited to the nervous system, and similar to the *Tα1* gene, its expression is growth associated. A 5.8kb fragment of the *peripherin* gene is sufficient to confer to a reporter gene a pattern of expression similar to that of the endogenous

peripherin gene (Belecky-Adams et al 1993; Leconte et al 1996). A portion of intragenic *peripherin* gene sequence must be included in the 5.8kb segment in order to ensure cell-type specific expression, as well as dynamic regulation following injury. The 5.8kb gene fragment mediates dynamic regulation following nerve injury in a manner similar to that observed for peripherin. The specific sequences mediating this activity have not been reported. Interestingly, the induction of peripherin gene expression in PC12 cells in response to NGF appears to involve de-repression and this effect is mediated by the cis element GGCAGGGCGCC. Complexes binding this sequence are found in undifferentiated and differentiated PC12 cells, before and after NGF exposure respectively. The complexes appear to be distinct however, and may be involved in the dynamic regulation of peripherin at least in vitro (Thompson et al 1992).

The *GAP43* gene is similar to the *Tα1* gene in that it is widely expressed, neuron-specific and growth associated (Karns et al 1987). Analysis of various portions of the *GAP43* gene regulatory region in transgenic mice has revealed a modular organization (Vanselow et al 1994). A rather large segment of the gene is able to target reporter gene expression to the nervous system in a pattern similar to the endogenous *GAP-43* gene. Restriction to the nervous system requires intronic sequence, suggesting that an extra-neural repressor is located in this region. Deletion analysis has identified a repressive segment downstream of the *GAP-43* TATA box which is highly position-dependent (Weber et al 1997). This segment represses heterologous gene expression in non-neuronal cells, and binds to a protein complex specific to non-neuronal cells. Further analysis has identified a novel sequence element in the segment, named the SNOG element as it is found in a similar position in the *S*NAP-25 gene and the neuronal *n*itric *o*xide *g*ene. The 11kb *GAP-43* promoter also displays dynamic activity mimicking endogenous *GAP-43* gene expression. Following sciatic nerve crush, the promoter was reactivated in mature sensory and motor neurons, where expression of the endogenous *GAP-43* gene was also induced. The *Tα1* gene and 1.1kb *Tα1* promoter behave similarly following sciatic nerve crush, as does the peripherin gene and the 5.8kb peripherin promoter. These similarities raise the question of whether common regulatory elements underlie this parallel in injury stimulated transcriptional activation in neurons.

The *nestin* gene encodes an intermediate filament protein that is specifically expressed in neural and muscle precursors (Zimmerman L et al 1994). *Nestin* expression is extinguished with cell differentiation. Analysis of nestin regulatory sequences in transgenic mice has identified separable muscle and neural enhancers, located in the first and second introns respectively (Zimmerman et al 1994). Cloning and analysis of the human *nestin* gene has revealed conserved sequence within the neural enhancer (Lothian and Lendahl 1997), and immunohistochemical analysis has shown that *nestin* is also expressed in CNS progenitor cells in human embryos (Tohyama T et al 1992). A 374bp segment of the human *nestin* gene corresponding to the conserved neural enhancer is sufficient to target reporter gene expression to progenitor cells throughout the developing CNS in transgenic mice (Lothian et al 1999). Within this 374bp sequence is a 120bp sequence that is required for activity. Also within this 374bp fragment is a midbrain specific enhancer. Collectively these studies reveal that the widespread expression of nestin involves complex regulation based on cell type and subtype and/or spatial regulation. The specific elements involved in promoting transient *nestin* expression in the neural lineage causing it to be expressed in neural progenitor cells but not in neurons, and those contributing region-specific activity have not been reported.

The *L1* gene encodes a cell adhesion molecule expressed throughout the peripheral and central nervous system both during development and adulthood (Moos et al 1988; Mirsky et al 1986; Faissner et al 1984). The *L1* gene is expressed early in neural development, beginning at about embryonic day 12. A fragment of 5' flanking sequence of the *L1* gene has been characterized in transgenic mice (Kallunki et al 1997). This sequence is sufficient to direct reporter gene expression specifically to the nervous system in a spatio-temporal pattern that mimics that of the endogenous *L1* gene (Kallunki et al 1997). A functional sequence within the *L1* gene promoter has been identified, and corresponds to the previously iidentified 21bp NRSE sequence (TTCAGCACCAGGGACAGCGAA) (Kallunki et al 1997; Schoenherr et al 1996). Deletion of this sequence leads to ectopic expression of an *L1* driven reporter gene in neural and non-neural tissue, as well as precocious expression of the reporter gene during development of the PNS (Kallunki et al

1997). Furthermore, this deletion also leads to a decrease in reporter gene expression in the mature nervous system (Kallunki et al 1998), indicating the NRSE functions both as an inhibitor and repressor depending on context, or that the sequence contains more than one element with distinct functions. In this regard, the NRSE binding factor "REST" has been detected in the adult brain, where the decrease in *L1* transgene expression is observed following deletion of the NRSE.

Additional evidence supporting the bifunctional nature of the NRSE has come from the analysis of the *nicotinic acetylcholine receptor β2 subunit* gene (nAChRβ2) (Bessis et al 1997). The *nAChRβ2* subunit is neuron-specific and is the most widely expressed neuronal nicotinic receptor subunit in the nervous system. Deletion analysis of the 1.2kb *nAChRβ2* gene promoter (Bessis et al 1995) in transgenic mice has revealed that loss of the NRSE sequence results in ectopic expression both within and outside the nervous system (Bessis et al 1997). In addition, loss of this sequence leads to a loss of expression within the normal expression domains of both the endogenous *nAChRβ2* gene and the wildtype transgene. In vitro analyses further suggest that the position of the NRSE sequence within a promoter may affect its activity in the neuronal context. A positively acting E-box and an additional unknown negative regulatory element have also been identified in the *nAChRβ2* gene (Bessis et al 1995), but their relevance to regulation in vivo is unknown.

The NRSE is also located within the *BDNF* gene. Expression of the *BDNF* gene is not restricted to the nervous system, raising the question of whether or not the NRSE is functional in this context, and if so what its function is. The consequences of deleting the NRSE have been analyzed in transgenic mice (Timmusk et al 1999). Deletion of this NRSE does not lead to ectopic reporter gene expression within or outside of the nervous system. The NRSE does however appear to contribute to normal promoter activity within brain, thymus and lung tissue. In the brain, deletion of the NRSE leads to an increase in the basal level of reporter gene expression, suggesting the element normally functions as a repressor within this neural tissue. This is not the first report ascribing repressive activity within the nervous system to the NRSE. The NRSE was originally hypothesized

to function as a repressor of *sodium type-II channel* gene expression in sensory neurons (Kraner et al 1992), thereby contributing to neuronal diversity through gene repression in a subset of neurons.

A 2.4kb fragment of the *choline acetyltransferase* (*ChAT*) gene is sufficient to confer cholinergic neuronal cell type-specific gene expression to a reporter gene and basal promoter in transgenic mice (Lonnerberg et al 1995). The 2.4 kb fragment functions as an enhancer in cholinergic cells and as a repressor in non-cholinergic cells in a position and orientation-independent manner. Among the regions targeted by the promoter are the basal forebrain, pons and the spinal cord, regions that also express endogenous *ChAT*. Furthermore, the 2.4kb *ChAT* promoter fragment responds to spinal cord injury in a manner similar to the endogenous *ChAT* gene. Thus this region is sufficient to specify cell subtype and to mediate a response to injury within those cells. A function for the NRSE has also been reported in the context of the *ChAT* gene (Lonnerberg et al 1996). An NRSE is found within the 2.4kb promoter region and this sequence binds to the REST protein, the identified trans factor thought to mediate NRSE activity. In vitro studies suggest that the NRSE contributes to the neuron-specific activity of the promoter, but does not contribute to its cholinergic-specific activity.

A function for the NRSE has also been described in the context of the *glutamine synthetase* gene (Avisar et al 1999). The *glutamine synthetase* promoter has been identified, and *in vitro* studies reveal that it is responsive to glucocorticoids in neural cells, but not non-neural cells (Avisar et al 1999). An NRSE is located adjacent to a glucocorticoid responsive element within the promoter, and deletion of the NRSE allows the promoter to respond to glucocorticoids in non-neural cells (Avisar et al 1999). Thus the NRSE appears to contribute to the neural specificity of this dynamic regulation *in vitro*. This activity can be conferred to a heterologous glucocorticoid responsive promoter by situating an NRSE appropriately, rendering it refractory to the influence if glucocorticoids in non-neuronal cells *in vitro*. The NRSE may also be involved in the timing of glucocorticoid responsiveness, as the sequence is bound in both non-neuronal and neural progenitor cells, both of which do not respond to glucocorticoid.

The NRSE sequence is also found within the *NMDA receptor subunit NR2C* gene (Suchanek et al 1997). The *NR2C* gene is transiently expressed in cerebellar granule cells, beginning two weeks after birth, and is also expressed in the developing forebrain. Transgenic mouse analysis has revealed that 800bp of the immediately 5' region is sufficient to confer neural specific expression to a reporter gene, while additional sequence is required for neuronal subtype specificity and dynamic regulation as demonstrated by a lack of transgene downregulation in the mature cerebellum (Sasner et al 1996). A segment containing the NRSE has been implicated in the negative regulation of basal promoter activity in non-neural tissue (Suchanek et al 1997).

Limited NRSE analysis has also been performed in the context of the *dopamine beta hydroxylase* gene (*DBH*). The human and rat *DBH* genes harbor NRSE sequences at corresponding positions in the 5' regulatory region (Ishiguro et al 1995). *DBH* promoter activity is restricted to neural tissue in transgenic mice (Mercer et al 1991). *In vitro*, the NRSE binds to protein in non-neuronal nuclear extracts, and exerts repressive activity when fused to a fragment of the DBH promoter lacking the NRSE in non-neuronal cell lines (Ishiguro et al 1995).

Analysis of the *Mash1* gene has revealed the presence of separable CNS and PNS elements (Verma-Kurvari et al 1996) similar to the organization observed in the Drosophila genes deadpan and scratch. An 8kb segment of the gene directs CNS expression in transgenic mice, while an additional 28kb is required for expression in the sympathetic nervous system. However a 36kb segment of the *Mash1* gene was still unable to direct expression to the developing retina and olfactory epithelium, regions that normally express *Mash1*.

The *Pax6* gene product, a "paired" domain transcription factor with pleiotropic functions in the developing nervous system, has been implicated in the regulation of *NCAM* (Holst et al 1997) and *L1* (Meech et al 1999) *in vitro* and *in vivo*. Pax6 consensus elements are located within both of these neural genes, and Pax-6 binds both sequences and activates

both promoters *in vitro*. Mutation of these elements results in a loss of binding and activation by Pax-6 *in vitro*, and correlates with changes in promoter activity *in vivo*. Mutation of the *NCAM* promoter leads to a loss of activity within the dorsal and mediolateral regions of the mantle zone of the embryonic spinal cord. At later stages of development, mutant *NCAM* promoter activity is nearly abolished whereas *NCAM* promoter activity and endogenous *NCAM* expression are observed in the spinal cord. Mutation of the *L1* promoter causes a loss of activity in the developing telencephalon and mesencephalon, where wild type promoter activity and endogenous *L1* expression are normally observed. These results implicate Pax6 or a related factor(s) in the regulation of these two neural genes within subdomains of the developing CNS.

The *hypoxanthine phosphoribosyl transferase* gene (*HPRT*) is constitutively expressed, but displays enhanced expression in the mammalian brain (Stout and Caskey 1985). An *HPRT* promoter has been identified using transgenic mice, and contains within it a segment that is required for enhanced expression within the nervous system but exhibits repressive activity *in vitro* when transferred to a heterologous promoter (Jiralerspong et al 1996). The identity of the responsible sequence(s) and its mechanism of action are not yet known.

Regulatory sequences directing expression of the *Hes5* gene have been analyzed *in vitro* (Takebayashi et al 1995). The *Hes5* gene is neural specific, expressed in progenitor cells of the developing nervous system, and its expression decreases during neural development. *In vitro* studies have identified *Hes5* genomic sequence that directs reporter gene expression to a neural progenitor cell line. This promoter activity is downregulated when progenitor cells are induced to differentiate *in vitro*. Thus, like the *Hes5* gene, the *Hes5* promoter appears to be active in progenitor cells, but is shut down in differentiating neurons. Within the promoter are several repeats of a GC-rich sequence which are required for enhanced promoter activity in the progenitor cell line. Furthermore, binding activity for this element is found in progenitor cells but is absent in differentiated neurons derived from these cells. The authors suggest that this sequence and binding activity may contribute to the progenitor-specific expression of the *Hes5* gene, but the activity of the

GC-rich sequence and of the promoter itself have not been assessed *in vivo*. Their relevance is therefore uncertain.

Analysis of the *olfactory marker protein* (*OMP*) gene has revealed possible roles for the Olf/CoE2 family of transcription factors in neuronal gene expression (Kudrycki et al 1998). *OMP* is specifically expressed in the olfactory epithelium (Danciger et al 1989). The *OMP* promoter contains a putative Olf/CoE2 binding site, and its function has been analyzed in transgenic mice (Kudrycki et al 1998). Interestingly, mutation of this site in the *OMP* promoter does not alter expression within the olfactory epithelium, but rather leads to ectopic expression in other neuronal populations of the CNS. These results suggest that an Olf/CoE2 family member(s) or related binding activity may repress *OMP* expression in discrete neuronal populations throughout the nervous system in order to restrict *OMP* expression specifically to the olfactory epithelium.

Characterization of the mouse *neurofilament light chain* (*NF-L*) gene promoter in transgenic mice has revealed that the initiation of gene expression during development and the maintenance of gene expression in the mature nervous system are genetically separable (Yaworsky et al 1997). A 1.7kb fragment of the mouse *NF-L* gene confers to a reporter gene a pattern of expression that mimics that of the endogenous *NF-L* gene during development After birth, transgene expression is extinguished, though endogenous *NF-L* gene expression persists. In addition, the promoter contains separable muscle and neural expression promoting modules, which also differ in their temporal regulation during development. The muscle-directing activity of the promoter is consistent with the observation of *NF-L* expression in developing muscle.

The murine *Purkinje cell protein-2* (*Pcp-2*) gene is expressed specifically in Purkinje cells and their precursors in the cerebellum. A 700bp fragment of the *Pcp-2* gene serves as a neural-specific basal promoter. (Vandaele et al 1991). This gene fragment directs reporter gene expression ectopically to a wide array of neurons throughout the developing CNS. Addition of further 5' sequence to this 700bp fragment progressively restricts its activity, bringing it closer to the expression domain of the endogenous *Pcp-2* gene. *In*

vitro analysis has suggested that homeodomain transcription factors may participate in the early repression and subsequent induction of *Pcp-2* expression in the developing cerebellum (Sanlioglu et al 1998). In addition, Hes5 may negatively regulate the *Pcp-2* promoter *in vitro* (Akazawa et al 1992).

Analyses of floor plate development have uncovered a number of critical transcription factors and have begun to trace the molecular mechanisms responsible for the cellular interactions between notochord and presumptive floor plate. In so doing, they have revealed an important neural target for the zinc finger transcription factor Gli-1, namely *HNF3β*. *Sonic hedgehog* is expressed in the notochord and is capable of inducing ectopic floor plate development. This effect may be due to sonic hedgehog's ability to induce *HNF3β* in a *Gli-1*-dependent manner. *Gli-1* is a zinc finger transcription factor induced in the neural tube by sonic hedgehog, and is also capable of inducing ectopic floor plate development (Hynes et al 1997). Analysis of the *HNF3β* gene has revealed a floor plate specific enhancer, which contains a putative site for Gli-1 (Sasaki and Hogan 1996). This site is required for the activity of the floor plate enhancer *in vivo*, and acts as a sonic hedgehog responsive element in vitro (Sasaki et al 1997). As *HNF3β* is required for floor plate development, and is capable of inducing ectopic floor plate development in the midbrain/hindbrain region, it may constitute an important target of *sonic hedgehog* by way of *Gli-1*.

Cross-species promoter analysis has yielded some important results. The analysis of human and avian neuronal gene promoters in transgenic mice has often revealed neuron-specific activity in the host (eg. Beaudet et al 1992; Leconte et al 1994; Yazdanbakhsh et al 1993; Daubas et al 1993). The promoters of spatially restricted or subtype-specific human and avian genes have also exhibited similar restricted activity in transgenic mice. These studies reveal evolutionary conservation in the regulation of neuronal cell type and subtype gene expression. Similarly, the proneural activity of murine transcription factors in Xenopus, as well as the neurogenic activity of Xenopus gene products in zebrafish, point to a conservation of neurogenic mechanisms.

The analysis of Hox genes has provided examples of spatial and tissue-specific enhancers as well as repressors, which collectively specify the normal spatio-temporal expression pattern of their respective genes. Tight control over their expression is thought to be important because misexpression and/or loss of expression often leads to neural malformations within the ectopic or normal expression domain respectively. Mechanisms of Hox gene regulation have remained largely undetermined but retinoic acid receptors, the transcription factors Krox-20 and kreisler, and the Hox gene products themselves appear to be involved.

Retinoic acid is required for neural development, with widespread neural tube and neural crest defects resulting from its absence (Dickman et al 1997). Retinoic acid (RA) is also a teratogen which when administered exogenously perturbs many aspects of neural development, including the segmentation of the hindbrain. In this region of the nervous system, the morphological effects of RA have been correlated with its molecular effects on Hox gene expression. Morphologically, in response to RA anterior regions of the hindbrain develop posterior characteristics. Molecularly, in response to RA anterior regions of the hindbrain express Hox genes normally restricted to more posterior domains. In some instances, the ectopic forced expression of posterior Hox genes in anterior domains has been shown to cause a "posteriorization" of these anterior domains, indicating that Hox gene expression is sufficient for the morphological alteration.

Responsiveness to RA in vivo requires the expression of retinoic acid receptors (Iulianella et al 1997; Folberg A et al 1999). A number of Hox genes normally expressed in the developing hindbrain and exhibiting RA responsiveness contain RA-responsive regulatory elements that have been characterized *in vivo* (Studer et al 1998; Gould et al 1998; Packer et al 1998; Zhang et al 1997; Morrison et al 1997; Morrison et al 1996; Folberg et al 1999). These elements appear to contribute to normal embryonic expression as well as mediating responsiveness to exogenous RA. Whether the expression of Hox genes is altered in response to the disruption of retinoic acid receptor genes has not been reported.

Hoxa-4 regulation depends on autoregulation as well as on regulation by retinoic acid receptors (Packer et al 1998). A reporter gene under the control of the murine *Hoxa-4* promoter mimics endogenous *Hoxa-4* expression. Mutation of a retinoic acid response element in the 5' regulatory sequence results in a dramatic decrease or complete absence of reporter gene expression in *Hoxa-4* expressing domains. Reporter gene expression is also drastically reduced in the nervous system of *Hoxa-4* null mice, indicating a dependency of *Hoxa-4* expression on *Hoxa-4* itself.

Analysis of the murine *Hoxa-4*, *Hoxd-4*, and Hoxb4- genes has revealed a similar organization, in so far as the regulatory regions of these genes consist of separable neural and mesodermal enhancers and the neural enhancers contain functional retinoic acid responsive elements (Morrison et al 1997; Morrison et al 1996; Zhang et al 1997; Packer et al 1998; Folberg et al 1999). The human *Hoxd-4* gene has a similar structure containing separable neural and ectodermal elements, as well as a RA-responsive element within its neural enhancer. In vivo this human neural enhancer sequence mimics to a large extent endogenous *Hoxd-4* expression in the hindbrain, and mediates a RA-responsive anterior shift in reporter gene expression also characteristic of the endogenous murine gene.

The murine *Hoxa-1* and *Hoxa-2* genes contain separable rhombomere-specific hindbrain enhancers and at least one general enhancer that contains an RARE (Frasch et al 1995). Mutation of the RARE causes a loss of enhancer activity caudal to rhombomere four in the hindbrain and spinal cord, suggesting it may contribute to the activity of the enhancer in the posterior of the embryo. Mutation of a distinct RARE in the Hoxa1 gene *in situ* changes the anterior expression boundary of *Hoxa-1*, shifting it caudally in the hindbrain (Dupae et al 1997). Molecularly, Hox gene expression in the developing hindbrain is altered. Morphologically, cranial nerve formation is perturbed in a manner reminiscent of *Hoxa-1* nullification. However, *Hoxa-1* is still responsive to retinoic acid in these mutant mice, suggesting alternative elements, including the RARE within the general enhancer, may regulate the retinoic acid response.

(vii) Suppressor of Hairless/Recombination Signal-Binding Protein is Evolutionarily Conserved and Regulates Mammalian Neural Development

Su(H)/RBP-Jk was first identified in genetic screens in Drosophila, where it was found to affect sensory bristle (hair) development (Campos-Ortega et al 1984; Dietrich and Campos-Ortega 1984). *Su(H)/RBP-Jk* is highly conserved and encodes a transcription factor with and apparently unique DNA-binding domain (Matsunami et al 1989; Furukawa et al 1991; Amakawa et al 1993; Christensen et al 1996). Biochemical analysis suggests that *Su(H)/RBP-Jk* may be poorly named, and that *hairless* might be more appropriately called the suppressor of *suppressor of hairless*, as *hairless* appears to function by antagonizing the effects of the *Su(H)/RBP-Jk* transcription factor (Brou et al 1994).

Mutant alleles of *Su(H)/RBP-Jk* interact with mutant alleles of other neurogenic genes (Campos-Ortega et al 1984; Dietrich and Campos-Ortega 1984; Vassin et al 1985; Shepard et al 1989; Brand and Campos-Ortega 1989; Xu et al 1990) and Su(H) molecularly interacts with a conserved and functionally required portion of the Notch intracellular domain in Drosophila. Mutant analyses suggest that *Su(H)/RBP-Jk* functions downstream of *Notch* to promote the inhibitory effects of *Notch* in the developing nervous system of the fly (Fortini et al 1997; Furukawa et al 1997).

Natural targets of Su(H)/RBP-Jk in Drosophila include *E(spl)* genes (LeCourtois and Schweisguth 1995; Bailey et al 1995). Molecular studies have shown that these genes lie directly downstream of *Su(H)/RBP-Jk* in the Notch pathway and have identified a Su(H)/RBP-Jk consensus binding site, CGTGGGAA.

Mammalian Su(H)/RBP-Jk was originally identified as a factor recognizing the VDJ recombination signal sequence within the immunoglobulin light chain gene (Matsunami et al 1989; Hamaguchi et al 1989). It was subsequently found that Su(H)/RBP-Jk did not bind to this sequence, but rather to the same consensus sequence derived from Drosophila

studies (Henkel et al 1994; Tun et al 1994). This was demonstrated in studies of Epstein Barr virus (EBV), which revealed an endogenous transcription factor was recruited by viral proteins following viral infection during B cell transformation (Henkel et al 1994). Su(H)/RBP-Jk was identified as the culprit transcription factor, and a natural viral target site for it was identified.

Thus the Su(H)/RBP-Jk consensus sequence derived from Drosophila targets matches that derived from Epstein Barr virus genes, suggesting the DNA-binding specificity of Su(H)/RBP-Jk has been conserved. This coincides with the high degree of primary sequence conservation observed between orthologs (Matsunami et al 1989; Furukawa et al 1991). The integrity of the Notch pathway has also been conserved in mammals. Notch activation in mammalian cells, either by mutation or ligand binding, induces the transcription of a reporter gene under the control of the *Hes1* promoter (Jarriault S et al 1995; Jarriault et al 1998). The *Hes1* gene, like *E(spl)* genes, contains a Su(H)/RBP-Jk consensus sequence. Notch mediated activation of *Hes1*-reporter gene expression in mammalian cells depends on the Su(H)/RBP-Jk site, just as it depends on that site within *E(spl)* in Drosophila cells (Jarriault et al 1995; Bailey et al 1995; LeCourtois and Schweisguth 1995). The conservation of a Su(H)/RBP-Jk site in *Hes1* (Jarriault et al 1995), its importance for responsiveness to Notch activation (Jarriault et al 1995), and the consequences of *Notch* (Swiatek et al 1994) *Su(H)/RBP-Jk* (de la Pompa et al 1995) and *Hes1* (Ishibashi et al 1995) gene deletion in mice collectively suggest that this pathway has been conserved and plays an important role in the negative regulation of neuronal differentiation in the mammalian nervous system.

Biochemical studies of mammalian Su(H)/RBP-Jk have defined intrinsic transcriptionally repressive activity, as well as DNA binding activity (Henkel et al 1994; Hsieh and Hayward 1995; Dou et al 1994). The DNA binding domain of Su(H)/RBP-Jk is unique, and only one other family member has been identified in mice. *RBP-L* as it is known, is expressed specifically in the developing lung (Minoguchi et al 1997). *Su(H)/RBP-Jk* is expressed ubiquitously (Hamaguchi et al 1992), and is observed in the nervous system as

early embryonic day 8.5. Expression is observed in neural progenitor cells throughout the nervous system and over the course of neural development.

Epstein Barr virus requires RBP-Jk/Su(H) in order to transform B cells. Following the infection of B cells by EBV, RBP-Jk/Su(H) binds to a viral gene through a RBP-Jk/Su(H) consensus sequence in its regulatory region. The gene thus bound is activated rather than repressed, and this is achieved by the binding of an additional viral protein to RBP-Jk/Su(H). The viral protein harbors an activation domain but has no intrinsic DNA-binding ability (Ling et al 1994), and thus exploits the DNA-binding activity of the endogenous factor RBP-Jk/Su(H) in order to activate its target gene (Henkel et al 1994; Hsieh and Hayward 1995). Activation of the target gene occurs by two mechanisms, namely de-repression and activation. The target gene is normally repressed by RBP-Jk/Su(H) alone owing to the intrinsic repressive activity of RBP-Jk/Su(H), and the viral protein masks the repressive activity of RBP-Jk/Su(H) by binding to its repressive sequence (Henkel et al 1994; Hsieh and Hayward 1995). The viral protein also contributes its own activation domain to the DNA bound complex, thereby activating transcription of the target gene (Henkel et al 1994; Hsieh and Hayward 1995).

Gain of function and loss of function studies in Xenopus suggest that *RBP-Jk/Su(H)* represses neural specification and neuronal differentiation, respectively (Wettstein et al 1997). Targeted disruption of the *RBP-Jk/Su(H)* gene in mice is lethal when homozygous (de la Pompa et al 1997), and causes precocious neuronal differentiation prior to embryonic death by E 10.5. A gain of function study has not been reported for *RBP-Jk/Su(H)* in mice, but from results of *Notch-3* and *Hes1* overexpression in neural precursors *in vivo*, such manipulation might be predicted to result in the inhibition of neuronal differentiation, with a concomitant increase in the precursor cell population.

Resolution of the molecular mechanism of *Hes1* gene induction by Notch activation has been aided by studies of Epstein Barr virus action. Ligand binding of Notch results in proteolytic cleavage of the intracellular domain of Notch, which is subsequently found in the nucleus and thought to associate with Su(H)/RBP-Jk (Scroeter et al 1998).

Su(H)/RBP-Jk binds DNA directly (Lecourtois and Scweisguth 1995; Bailey et al 1995; Jarriault et al 1995; Hsieh et al 1996). The induction of *Hes1* is then thought to result from the de-repression and activation of *Hes1* transcription, with an activation domain being contributed by the Notch fragment and the binding of the fragment to RBP-Jk/Su(H) masking the repressive activity of the latter (Hsieh et al 1996).

Biochemical investigations have revealed two potential mechanisms of transcriptional repression by RBP-Jk/Su(H).The first mechanism involves direct contact between RBP-Jk/Su(H) and the basal transcription factors TFIIA and TFIID (Olave et al 1998). This mechanim of repression has been found to be position dependent. Through these interactions, RBP-Jk/Su(H) may inhibit interactions between basal transcription factors that facilitate formation of the pre-initiation complex. The second mechanism involves an interaction with SMRT and HDAC (Kao et al 1998). Due to this molecular interaction, RBP-Jk/Su(H) may direct histone deacetylase activity to genes containing the RBP-Jk/Su(H) binding site and lead to their repression via the modification of chromatin structure. This second mechanism does not appear to be dependent on position. Both mechanisms provide a means of gene derepression and activation upon association of the intracellular fragment of Notch with RBP-Jk/Su(H). In the first mechanism, a fragment of the Notch protein binds to RBP-Jk/Su(H) masking the repressive domain within it, thereby de-repressing target genes. In the second mechanism, a fragment of the Notch protein binds to RBP-Jk/Su(H) preventing the interaction between RBP-Jk/Su(H) and SMRT, thereby preventing recruitment of HDAC to the RBP-Jk/Su(H) target gene.

(viii) Genetic Analyses of Telencephalon Development

Lineage tracing and transplantation studies performed in a variety of vertebrates have been used to follow anterior nervous system development from the neural plate stage (reviewed in Rubenstein et al 1998). These analyses have revealed an organization of morphogenic primordia within the neural plate. Molecular analyses have revealed a gridwork organization within the anterior neural plate, whereby the expression patterns of

several genes encoding transcription factors divides the plate into longitudinal and horizontal domains, and collectively divides the plate into quadrants with unique molecular profiles (Rubenstein et al 1998). A comparison reveals morphogenic primordia often lie within molecular boundaries, and genetic analyses suggest that some of the transcription factors demarcating primordial zones are required for neural development within those zones.

Along these lines, molecular analyses combined with morphogenic analyses have led to the prosomeric hypothesis, which suggests that the developing diencephalon and telencephalon are organized into segments in a manner analogous to the rhombomeric organization of the developing hindbrain (Rubenstein et al 1994). Many of the genes involved in the segmentation of the telencephalon and diencephalon are of the homeobox type and are orthologs of Drosophila genes that play a role in anterior nervous system development.

Genetic studies in Drosophila have led to the identification of a number of genes involved in anterior embryonic development, a process which appears to involve different factors and mechanisms than those involved in body segmentation (Reichert and Boyan 1997). Remarkably, homologs of most of these genes have been identified in mammals where they are expressed in developing brain tissue and are required for normal brain development.

A number of conserved homeobox genes are specifically expressed in the developing brain and have been found to play a role in its development. The expression of these genes is restricted to particular subdomains of the developing brain, often coinciding with prosomeric boundaries and proposed longitudinal divisions. Many of these contribute to the specification and development of neural tissue within their domains of expression. The Drosophila counterparts of these genes also divide the anterior fly nervous system and have been shown to function in brain development. Among these genes are homologs of the Drosophila genes *orthodenticle* (*otd*) (Finkelstein and Perrimon 1990), *empty*

spiracles (*ems*) (Cohen and Jurgens 1990; Waldorf and Gehring 1992) and *distalless* (*dls*) (Cohen et al 1989).

Two otd homolgs have been identified in mammals, namely *Otx1* and *Otx2*, and their expression patterns have been characterized (Boncinelli et al 1993; Acampora and Boncinelli 1999; Simeone et al 1992; Muccielli et al 1996). *Otx1* is first expressed in the developing brain at E8, while *Otx2* is expressed earlier throughout the embryo at E5.5, and becomes restricted to the anterior domain by E7.5. At E9.5, the two genes are expressed in broad domains within the forebrain and midbrain, with the domain of *Otx2* expression engulfing that of *Otx1*. *Otx1* is expressed in both basal and alar plate tissue, with an anterior dorsal limit within the telencephalon and an anterior ventral limit within the diencephalon. Posteriorly, the *Otx1* expression domain terminates at the mesencephalon/metencephalon border. *Otx2* is expressed in nearly all alar and basal plate tissue throughout the developing forebrain and midbrain. Anteriorly, neither *Otx1* nor *Otx2* are expressed in the optic recess.

At E12.5, the *Otx* genes are expressed in stripes of tissue, rather than broad domains of the developing brain (Acampora and Boncinelli 1999; Boncinelli et al 1993; Muccielli et al 1996; Simeone et al 1992). These striped domains are observed at boundaries within the diencephalon, metencephalon, and telencephalon. In the telencephalon, the *Otx* genes are expressed dorsally in a domain with its rostral limit at the boundary between the archicortex and the neocortex, a division separating putative prosomeres 4 and 5. Ventrally, the domain of *Otx* gene expression stops rostrally at the caudal limit of the medial ganglionic eminence. The *Otx* genes have also recently been shown to play an important role in the establishment of the boundary between mesencephalon and metencephalon and the positioning of the isthmus, an important midbrain organizing centre (Joyner 1996).

Disruption of the *Otx1* gene is homozygous lethal in a strain dependent manner (Suda et al 1996). Commonly however, deletion of *Otx1* leads to telencephalic abnormalities that are particularly pronounced in temporal and perirhinal cortex. In addition, eye and inner

ear development are perturbed, and among adults that survive, spontaneous epileptic seizures are observed (Acampora et al 1996). Within the telencephalon, there is a reduction in cortical thickness and cell number, and neuronal layer separation is poor in the temporal and perirhinal areas of the cortex. Proliferation of neuronal precursors is lower in mutant animals, and may account for the cortical hypoplasia observed (Acampora et al 1998).

Otx2 gene disruption is lethal at an early embryonic age, with correlated defects in the primitive streak and prechordal mesendoderm (Matsuo et al 1995; Ang et al 1996). Embryos completely lack head structures anterior to rhombomere 3. The early defects are consistent with the early expression of *Otx2* in the anterior mesendoderm, but obscure the function of *Otx2* within the neuroectoderm. Recently however, chimeric embryos have been used to demonstrate a two stage requirement for *Otx2*, first within anterior mesendoderm for anterior neural plate induction, and second, within the neural plate for the specification of midbrain and forebrain domains and their appropriate patterns of gene expression (Rhinn et al 1998). The targets of both *Otx1* and *Otx2* remain elusive, as do their mechanisms of action in controlling development of the telencephalon.

Two *ems* homologs have been identified to date, namely *Emx1* and *Emx2*, and their expression patterns have been characterized (Boncinelli et al 1993; Fernandez et al 1998; Simeone et al 1992a; Simeone et al 1992b). The expression of these genes is restricted to the developing telencephalon. *Emx2* expression is first detected at E8.5, while expression of *Emx1* is first detected at E9.0. At E10, both genes are expressed in the developing forebrain, with the dorsal caudal limit of *Emx2* at the border between telencephalon and diencephalon, just caudal to the limit of *Emx1* expression. The expression domain of each gene extends into the dorsal telencephalon and terminates within this region, with the limit of *Emx2* rostral to that of *Emx1*. Neither gene is expressed in the roof plate. *Emx1* is not expressed in basal plate tissue, though *Emx2* is expressed in a restricted domain within the diencephalon, corresponding roughly to the presumptive ventral thalamus. The expression domain of *Emx2* thus subsumes that of *Emx1*, and in turn, the expression

domain of *Emx2* is completely subsumed by that of *Otx1* and *Otx2* (reviewed in Rubenstein et al 1998).

Disruption of the *Emx1* gene leads to inexplicable neonatal lethality in approximately 50% of homozygous mutant animals (Qiu et al 1996). Heterozygous mutant animals appear normal. Morphological defects are observed in homozygous null animals and appear to be restricted to the forebrain. The indusium griseum and taenia tecta are always absent, and the corpus callosum and anterior commissure frequently display defective fasciculation. The cerebral hemispheres appear slightly smaller than those from wildtype mice. The cortical plate and white matter from mutant animals is noticeably thinner and often poorly differentiated. In addition, the subplate is often diminished in mutant mice. No defects are observed in the olfactory bulb, and the hippocampus is always present, though occasionally it appears marginally reduced in size.

Disruption of *Emx2* is more detrimental than disruption of *Emx1* (Pellegrini et al 1996). Homozygous mutant mice die postnatally, lacking kidneys and other elements of the urogenital system. This is consistent with the expression of *Emx2* outside of the nervous system in primordia of the urogenital system (Simeone et al 1992a, b). At E18.5 there is a grossly observable reduction in the size of the cerebral hemispheres and olfactory bulbs of mutant animals. Morphological defects appear to be restricted to the dorsal telencephalon. The medial cortex is not formed and the lateral cortex layers are poorly demarcated and disorganized. The cortical plate and white matter appear thin, and the subplate is unidentifiable. Cortical defects are apparent early, as telencephalic vesicles of mutant animals are noticeably smaller at E11.5. Telencephalic commissures appear abnormal at E18.5, with the anterior commissure and corpus callosum both lacking fibres. The hippocampus is severely reduced in size, the dentate gyrus is completely absent, and the fimbria and fornix are greatly reduced at E18.5. In addition, the hippocampal commisure is reduced or absent, and the medial limbic cortex is also severely reduced. Defective hippocampal development is apparent early. At E14.5, *Emx2* is normally expressed in the neuroepithelium giving rise to the hippocampus and in the dentate gyrus anlagen. In mutant animals, the neuroepithelial ventricular zone domain

where precursors of the hippocampus and dentate gyrus reside is noticeably and specifically reduced, and the dentate gyrus anlagen is not distinguishable.

Close comparison of *Emx1* and *Emx2* expression in the caudal limit in the dorsal telencephalon reveals that *Emx2* extends to the telencephalon/diencephalon border while *Emx1* expression terminates rostral to this point (Simeone et al 1992a,b; Boncinelli et al 1993; Shimamura et al 1995; Rubenstein et al 1998). Thus there is a region of dorsal, caudal telencephalon that expresses *Emx2* but not *Emx1* and this region appears to correspond to the primordium of the hippocampus. *Emx2* mutant animals display a great loss of hippocampal tissue, an early expansion of the roof plate, and disorganization of the choroid plexus normally formed at this location (extending ventrally into the third ventricle). It has been proposed that in this region (where Emx1 is absent) Emx2 is required for the specification of hippocampal tissue. In its absence, this tissue may be recruited to a roof plate fate (Rubenstein et al 1998).

Six *dls* homologs have been identified in mammals. Two of these have been disrupted in transgenic mice, and each mutation is homozygous lethal. The disruption of *Dlx1* (Anderson et al 1997) and *Dlx2* (Qiu et al 1995) individually led to postnatal death in homozygous mutant animals and each mutation was associated with craniofacial abnormalities and defects in the enteric nervous system, consistent with the expression of both genes in the cranial and spinal neural crest (Fernandez et al 1998; Robinson and Mahon 1994; Bulfone et al 1993a,b; Price 1993; Porteus et al 1994). However neither mutation alone affects forebrain morphology in a noticeable manner. Within the CNS, the two genes are expressed in largely indistinguishable patterns (Fernandez et al 1998; Robinson and Mahon 1994; Bulfone et al 1993a,b; Price 1993; Porteus et al 1994). At E12.5, the two genes are expressed in the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and the septum. Expression in the LGE terminates before reaching the cortex. Neither gene is expressed in the cortex, nor the olfactory bulbs. The caudal limit of expression lies within the diencephalon, approximately within the ventral thalamus. While the expression domain runs rostrally, these genes show a ventral

restriction and are not expressed in rostral ventral tissues of the alar plate, including the ventral hypothalamus, mammilary area and the infundibulum.

Beginning at E9.5, *Dlx1* and *Dlx2* are expressed in the VZ and SVZ of the developing LGE, which contains precursors of the striatum, and weakly in the mantle zone, which contains postmitotic neurons (Fernandez et al 1998; Robinson and Mahon 1994; Bulfone et al 1993a,b; Price 1993; Porteus et al 1994). Two additional Dlx genes, *Dlx5* and *Dlx6* are expressed mainly in the mantle zone (Liu et al 1997; Eisenstat et al 1999). Disruption of both alleles of *Dlx5* is lethal and causes severe craniofacial and vestibular defects (Acampora et al 1999), though the effects on the developing striatum are unknown. One hypothesis concerning development of the striatum suggests that a number of Dlx genes act successively to specify and promote the differentiation of striatal neurons.

Dlx1/Dlx2 homozygous double mutants exhibit early postnatal lethality, but unlike individual mutants, also display morphological defects in the CNS (Anderson et al 1997). The striatum may be broken down into two components, an early formed striosome component, and a later formed matrix component (Sussel et al 1999). In the mature striatum the two components display different connectivity and biochemical properties, and the two components appear to be formed from distinct precursor populations (Krushel et al 1995; Sussel et al 1999). In double mutant animals, early components of the striatum are formed (striosome), but striatal defects are observed after E12.5 (Anderson et al 1997). These defects include the inappropriate accumulation of partially differentiated cells within a proliferative zone of the LGE (SVZ) and an absence of later born neurons in the striatum (matrix). The expression patterns of several genes are perturbed in the SVZ of double mutant animals, including the *Dlx5* gene which is normally expressed in the SVZ as well as the mantle. In addition, olfactory bulb interneurons known to be born in this area are absent.

Striosome cells may derive from precursors in the VZ, while matrix cells may derive from precursors in the SVZ (Krushel et al 1995; Sussel et al 1999). Thus there may be two segregated populations of precursor cells generating the two distinct components of

the striatum, and one may be sensitive to the loss of *Dlx1* and *Dlx2* function. Both genes are expressed at markedly higher levels in the SVZ than the VZ, and at E12.5 the SVZ becomes the predominant proliferative zone of the LGE. *Dlx1* and *Dlx2* appear to be redundant in the formation of the striatum, but are individually required for development of the neural crest.

An additional transcription factor affecting the development of the basal ganglia is encoded by *Nkx2.1*. The basal ganglia may be divided into two components, a ventral component (the pallidum) and a dorsal component (the striatum) (reviewed in Rubenstein et al 1998). *Nkx2.1* encodes a transcription factor that is restricted to the developing pallidum (Shimamura et al 1995). It is one of the earliest genes known to be expressed within the forebrain, in the hypothalamic primordium. It is found in both precursor cells and neurons, as it is expressed in the VZ, SVZ and mantle of the MGE at E12.5. Expression of *Nkx2.1* is also restricted in an anterior/posterior manner, and expression terminates within the diencephalon (Shimamura et al 1995).

The LGE gives rise to the striatum, while the MGE gives rise to the pallidum (reviewed in Rubenstein et al 1998). Dlx genes are expressed in both regions, while *Nkx2.1* is expressed only in the MGE. It has been suggested that Dlx genes and *Nkx2.1* expression codefine pallidal identity, while Dlx genes alone specify striatal identity (Sussel et al 1999). Disruption of the *Nkx2.1* gene is lethal when homozygous, and results in severe defects within the lungs, thyroid and pituitary (Minoo et al 1999). In addition, the ventral hypothalamus and ventral telencephalon display morphological abnormalities. In the absence of Nkx2.1, pallidal structures (such as the globus pallidus) fail to form (Sussel et al 1999). In addition, separate populations of neurons that are normally formed in the pallidum and then migrate to the striatum (cholinergic) or cortex (GABAergic, calbindin(+) fail to form (Sussel et al 1999). The MGE and LGE mantle zones do not appear distinct in mutants, and the striatum appears to have expanded into pallidal territory (Sussel et al 1999). At E10.5, when the MGE is normally forming, an MGE structure is observed in the mutant animals, and the mantle zone contains differentiating neurons. However gene expression analyses at this and subsequent time points indicate

that the MGE acquires an LGE-like identity in *Nkx2.1* mutant animals (Sussel et al 1999). Finally, the expression of *sonic hedgehog* (*Shh*) an important gene product for the formation of ventral cell types along the entire neuraxis, is specifically absent in the VZ of the basal telencephalon at E10.5 and E11.5 (Sussel et al 1999).

The changes observed as a consequence of *Nkx2.1* gene disruption are consistent with a ventral to dorsal transformation of fate in the region normally expressing the gene. This is consistent with the consequences of disruption of the related gene *Nkx2.2* that is expressed in a similar D/V restricted pattern (Price 1992). Disruption of *Nkx2.2* leads to a similar ventral to dorsal transformation in the spinal cord (Briscoe et al 1999). These results have led to the hypothesis that the Nkx genes are involved in D/V patterning, carving the neural plate and tube into longitudinal segments that give rise to discrete structures at different A/P positions (Shimamura et al 1995). This is a function that appears to have been conserved as an Nkx ortholog in Drosophila is similarly involved in the D/V patterning of the fly CNS (Shimamura et al 1995).

An important transcription factor involved in brain development which does not contain a homeodomain is the winged helix transcription factor brain factor-1 (BF-1). *BF-1* is expressed in the neuroepithelium throughout the developing telencephalon and terminates at the presumptive border of the diencephalon (Hatini et al 1994; Tao and Lai 1992). *BF-1* is first expressed in neural precursors, but is maintained in a number of neuronal populations throughout adulthood (Tao and Lai 1992; Hatini et al 1994). Targeted disruption of *BF-1* in transgenic mice leads to perturbations throughout the developing telencephalon, but is particularly detrimental to the formation of the ventral telencephalon (Xuan et al 1995).

Homozygous *BF-1* mutant mice die at birth and display a dramatic reduction in the size of the cerebral hemispheres, while heterozygous mutant mice appear normal (Xuan et al 1995). Homozygous mutant mice display decreased precursor cell proliferation in both basal and alar plate tissue, and precocious neuronal differentiation in the developing dorsal telencephalon. Formation of the ventral telencephalon is drastically perturbed, both

morphologically and with respect to patterns of gene expression. Importantly, *Shh* expression is not detected in the ventral telencephalon of mutant animals, where it is normally expressed and thought to play an important role in ventral neural tube development. Ventral defects in the optic vesicle are also observed in mutant mice, and may be an indirect consequence of *Shh* alteration. The mechanism of mammalian *BF-1* action remains elusive, but analysis of a Xenopus ortholog of *BF-1* (Mariani et al 1998) suggests the factor may function as both a transcriptional activiator and repressor (Bourguignon et al 1998). A role for *BF-1* in growth control is also suggested by the finding that the avian oncogene *qin* encodes a *BF-1* ortholog (Li and Vogt 1993). In mammalian cells, indications are that BF-1 interacts with the groucho family of transcriptional co-repressor proteins, and may affect the expression of target genes through this association (S Stifani unpublished observations).

The *Hes1* gene encodes a bHLH transcription factor lying in the Notch pathway and is expressed in precursors of the developing telencephalon (Sasai et al 1992). As previously mentioned, disruption of the *Hes1* gene led to precocious neuronal differentiation within the telencephalon, coupled with gross malformations within the region (Ishibashi et al 1995). In many homozygous *Hes1* null animals the anterior neural tube failed to close. The precocious neuronal differentiation observed was hypothesized to deplete the precursor pool within the region and lead to subsequent morphological defects. Combined with findings from *in vitro* loss of function and gain of function experiments (Tomita et al 1996a,b; Castella et al 1999), as well as *in vivo* gain of function experiments (Ishibashi et al 1994), it has been suggested that *Hes1* contributes to the regulation of cortical neurogenesis by repressing cell differentiation, potentiating proliferation, and allowing a sufficient number of precursor cells to be generated in order to generate the neocortex.

The disruption of *Lim-1*, a gene encoding a LIM domain-containing transcription factor, has dramatic consequences similar to those resulting from *Otx2* disruption (Shawlot and Behringer 1995). Homozygous mutant mice lack forebrain, midbrain and anterior hindbrain structures. Similar to *Otx2*, *Lim-1* is expressed early in the anterior

mesendoderm. In *Lim-1* mutants, the morphological malformation appears to be a consequence of an inability of underlying mesoderm and endoderm to induce anterior neural plate structures.

In summary, a number of transcription factor-encoding genes are expressed over the course of telencephalon development in restricted domains. Combined with findings from lineage tracing experiments, the expression patterns of these genes provide insight into the organization of histogenic primordia within the developing brain. The prosomeric hypothesis suggests that the diencephalon and telencephalon are divided into six segments along the A/P axis, which function as morphogenic units. Many of the genes expressed within these boundaries have been evolutionarily conserved and play roles in both fly and mouse head development. Some of these show restrictions in the D/V plane as well. Similarly, a family of conserved genes appears to be expressed within discrete longitudinal domains extending through A/P divisions set forth by the prosomeric hypothesis. These genes function in the D/V specification of precursors and resultant cell fates in both the fly and mouse CNS. Together, these systems appear to impart molecular divisions within the developing telencephalon that are functionally significant and correlate with the discrete morphological development of telencephalic subdomains.

(ix) Heterogeneity Among the Neural Precursor Cell Population

It has been hypothesized that the segment of the *Tα1 α-tubulin* promoter referred to as the NRE may function as a repressor of premature neuronal gene expression within the neural lineage. This suggests that the NRE may function within neural precursor cells. A brief summary of observations on the nature of neural precursor cells is therefore presented.

The neural precursor cell population is an apparently heterogeneous population. This has been concluded from studies focusing on cell behaviour, gene expression, and the consequences of gene disruption. The labeling of a limited number of neural precursor

cells by various techniques and the subsequent monitoring of their movement and differentiation has addressed the question of what these cells normally become *in vivo*. Transplant studies in turn have focused on what these cells can become when challenged by a new environment. *In vitro* studies have similarly addressed the properties of neural precursor cells, asking what they can become and do become under defined or controlled conditions. All of these types of studies have revealed differences in precursor cell behaviour and concluded that the precursor cell population at any point does not consist of a single cell type, but rather a collection of related cell types. Even the most undifferentiated and multipotential precursor cells, the so-called "stem cells", exhibit different requirements for proliferation, survival, and differentiation *in vitro* (Weiss et al 1996). Genetic analyses have led to the same conclusion, that precursor cells are related but distinct.

The heterogeneity observed among neural precursor cells may be due to lineage divergence or the asynchronous nature of neural development throughout the nervous system. Precursor cells progress through many discrete developmental stages (Temple and Xian 1996; Lillien 1998). The difference between two distinct precursor cells may therefore represent the choice of two alternative paths of differentiation, or two different stages of development along the same path. The extent of divergence among precursor cells in the nervous system is not well known, and to what extent the apparent divergence at any point is due to differences in lineage progression is uncertain. Finally, little is known about when and by what mechanisms true divergence is achieved between lineages.

Behavioural mosaicism among neural precursor cells in the VZ and SVZ of the developing mammalian CNS has been reported with respect to cell-type and subtype lineage restrictions (Krushel et al 1993; Fishell et al 1990; Na et al 1998; Alder et al 1996; reviewed in Bayer and Altman 1991), migration patterns (Fishell et al 1993; Neyt et al 1997; Tan et al 1998), as well as laminar fating among progenitors destined to give rise to neurons (Frantz and McConnell 1996). *In vivo*, these results are based on the analysis of descendants of marked precursor cells. It should be noted that relatively recent

evidence regarding the extent of cell death that takes place in the developing ventricular zone and cortical plate of the nervous system (Blaschke et al 1996) calls into question the results of clonal analysis experiments, upon which these conclusions regarding cell heterogeneity are based. It is not known whether such cell death is selective. It is possible that massive cell death may skew the results of such analyses, misguiding interpretations as to what a cell can become or does become *in vivo* and masking the true degree of heterogeneity among precursor cells (Voyvodic 1996).

Cultures derived from the embryonic forebrain and spinal cord (Mayer-Proscel et al 1997; Davis and Stemple 1994; Kilpatrick and Bartlett 1995; Vescovi et al 1993; Williams and Price 1995; Burrows et al 1997; Qian et al 1997) have revealed mixtures of precursor cells with distinct lineage potentials *in vitro*. The normal course of lineage divergence during development is thought to stem from a complex interplay between cell intrinsic and extrinsic cues over time (Temple and Xian 1996; Lillien 1998). The response of precursor cells to identified extrinsic cues has been observed to change with time (reviewed in Lillien 1998), supporting the notion that precursor cells pass through a number of stages en route to neuronal differentiation, and that such a progressive differentiation process contributes to precursor cell heterogeneity.

Molecular mosaicism has been noted with respect to the expression of growth factor/mitogen receptors (reviewed in Lillien 1998). These differences in receptor expression underlie behavioural differences between precursor cells observed *in vitro* and *in vivo*. There are also differences in the complement of transcription factors that precursor cells express (Lillien 1998; Fishell 1997; Rubenstein et al 1998). The expression of different transcription factors in different precursor cells is thought to tailor neuronal development regionally, generate a wide variety of neuronal subtypes at appropriate times and places, and shape the development of the nervous system. Manipulating these transcription factors *in vivo* can often alter morphogenesis, with consequences for discrete neuronal structures (Lillien 1998; Fishell 1997; Rubenstein et al 1998). The consequences of such gene manipulation at the cellular level are less well

understood in most cases, but may involve a direct effect on neuronal specification and differentiation.

New attention has been focused on the character of neural precursor cells since the identification of resident stem cells in the adult mammalian CNS (Reynolds and Weiss 1992). Stem cells have been isolated from the subependymal zone of the adult forebrain near the striatum and hippocampus (Reynolds and Weiss 1992). It was also recently shown that stem cells reside in the ependymal layer as a subpopulation surrounding the lateral ventricles of the adult mammalian brain (Johansson et al 1999). Interestingly, deletion of the NRE in one line of transgenic mice led to ectopic transgene expression in a subset of cells located in the ependymal layer, as well as the subependymal layer surrounding the the lateral ventricles. This expression may reflect ectopic promoter activity in neural stem cells and suggests that the NRE may normally function in neural precursor cells.

The distinction between stem cells and other neural precursor cells, so-called "progenitor cells", is difficult to make. By definition the stem cell is more potent than the progenitor and has a "perpetual" self-renewing capacity which the progenitor cell lacks. Thus a stem cell generates progenitor cells, but the converse is not true. The relationship between stem cells and progenitor cells is beginning to be addressed. *In vivo*, gene expression studies suggest that stem cells and progenitor cells are distinct populations. Proliferative activity and sensitivity to a growth dependent toxin *in vivo* may also distinguish the two populations (Morshead et al 1994). Analysis of stem cell differentiation *in vitro* suggests that progenitor cells are generated by stem cells, continue to proliferate, and progressively differentiate (Torii et al 1999; Mayer-Proschel et al 1997). Whether stem cell differentiation cues are required once at the beginning of the lineage or function continually either as directive or selective agents for subsequent progenitor cells remains unanswered. One type of progenitor cell may be generated from such a directive event, or one subtype of generated progenitor cells might be subsequently selected or specifically expanded by the cue. Alternatively, some stem cells may not give rise to progenitor cells,

and directive cues may operate on these stem cells to provoke neuronal differentiation directly without passing through an intermediate progenitor cell stage.

Analysis of the developing peripheral nervous system has led to some general conclusions about the nature of progenitor cells in the neural crest. The neural crest is a multipotent cell population which can give rise to cell types otherwise restricted to particular germ cell layers (LaBonne and Bronner Fraser 1999; Le Dourain and Dupin 1993, Le Douarin et al 1994; Anderson 1997). These cell types include osteoclasts, chondrocytes, muscle cells, melanocytes, neurons and glia. A general conclusion reached by following neural crest progenitor cells *in vitro* and *in vivo* and challenging them with new environments *in vitro* and *in vivo* is that these cells are heterogeneous at an early stage, directed by environmental factors to assume specific fates, and their potential to respond to such environmental signals changes with time (LaBonne and Bronner Fraser 1999; Le Dourain and Dupin 1993; Le Douarin et al 1994; Anderson 1997). The cells appear to be progressively restricted over the course of development to particular cell fates, prior to neuronal differentiation. That is, they progressively lose their ability to assume other fates.

Heterogeneity appears early in the neural crest, as groups of migrating neural crest progenitor cells can be distinguished molecularly, and neural crest progenitor cells from different regions display different abilities very early in neural development. An example where molecular distinction correlates with fate acquisition differences involves the expression of a transcription factor gene, neurogenin, which is intimately involved in sensory neuron development. The expression of *neurogenin* in migrating neural crest cells prefigures and predicts their incorporation into coalescing sensory ganglia (Perez et al 1999). Furthermore, ectopic expression of *neurogenin* biases migrating neural crest cells to incorporate into sensory ganglia (Perez et al 1999). The enteric nervous system also forms from the neural crest, and is generated by at least two distinct lineages of progenitor cells that are distinguishable based on their developmental dependency on a transcription factor very similar to neurogenin, namely Mash1 (Guillemot et al 1993). More generally, a comparison of early trunk neural crest and anterior neural crest

progenitor cells reveals that they posses different capabilities, as only the head neural crest can generate bone tissue and normally does so in the developing head region (Le Douarin et al 1994).

Exactly how progenitor cells in the neural crest are rendered molecularly "suggestible" or refractory to the influence of environmental signals remains a question, as does the identity of some environmental factors influencing fate decisions in the population (Anderson 1997; LaBonne and Bronner Fraser 1999). Regardless, divergence among neural progenitor cells with respect to some aspects of fate potential appears to occur very early, suggesting that from the earliest stages, the neural crest progenitor cell population is heterogeneous (LaBonne and Bronner Fraser 1999; Le Dourain and Dupin 1993; Le Douarin et al 1994; Anderson 1997).

Progenitor cell diversity is also observed in the ectodermal placodes (Le Dourain 1992). Among the distinct ectodermal placodes that give rise to the various cranial ganglia of the peripheral nervous system, different *neurogenin* genes are selectively expressed (Fode et al 1998; Ma et al 1998). The disruption of a single *neurogenin* gene leads to the selective loss of cells within the placode that normally expresses it (Fode et al 1998; Ma et al 1998).

Thus the analysis of the neural progenitor cell population at present suggests that they are a heterogeneous population of cells. This may reflect heterogeneity between cells at equivalent points in lineage progression, or could partially reflect cells at different states of commitment or points along their lineage (Lillien 1998; Stemple and Xian 1996). Neurogenesis is not a point in time defined by the simultaneous differentiation of all neurons, but rather a window of time over which neurons are generated asynchronously. While the length of the lineage and character of the individual stages for presumptive neurons in different regions of the nervous system is not necessarily the same, some of the heterogeneity observed probably reflects cells at different stages of maturation rather than phenotypically different lineages.

Regardless of the mechanism, at any one point in time, the ventricular zone and subventricular zone of the CNS, as well as the progenitor cells of the ectodermal placodes and the neural crest appear to be composed of phenotypically distinct cell types. We have observed that the loss of the NRE affects only a subset of precursor cells within the developing CNS and PNS, suggesting that it may function at a particular time within a lineage, or that its activity may be restricted to a sublineage(s), or both. In the transgenic line displaying expression in progenitor cells, transgene expression is highly penetrant in differentiating neurons suggesting that partial penetrance likely does not account for the limited number of expressing progenitor cells. However, transgene expression has not been directly correlated with an intrinsic marker of progenitor cell subpopulations and occurs throughout the nervous system in an apparently non-regionally restricted manner.

Finally, it was noted that the NRE had no obvious effect on gene expression in differentiated glial cells. The implications of this finding are unclear however, as this may indicate that the NRE does not function in progenitors of glial cells, or alternatively, any function in glial progenitor cells may not be reflected using our assay, owing to a lack of reporter gene induction.

In conclusion, the nature of the cell(s) that displays β-gal activity as a consequence of the NRE deletion is uncertain. As such, the precise context in which the NRE may normally function is unclear, though it may function in both progenitor and stem cells prior to neuronal differentiation in the neural lineage.

Results

(A) A 66-Nucleotide Sequence in the *Tα1* Promoter Contains a Conserved 10-Nucleotide Sequence Found in Other Neuronal Genes, and is Required for Reporter Gene Repression in Neural Precursor Cells

(i) Identification of the Neuronal Restriction Element

Through sequence analysis and comparison, a conserved sequence in the 1.1kb rat *Tα1* α-tubulin gene promoter was identified. This sequence, *CTCCCAGGTG*, is located in multiple neuronal genes and has been designated the "neuronal restriction element" (NRE). In figure 1, the NRE from the *Tα1* promoter has been aligned with sequences from multiple neuronal genes, including the goldfish *α-1* α-tubulin gene. The NRE occurs either alone or at the core of the previously described "neuronal restriction-silencing element" (NRSE).

(ii) Generation of *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Transgenic Mice

The generation of *Tα1-nlacZ* mice has been described previously (Gloster et al 1994). Briefly, a fragment of the rat *Tα1* gene containing 1028 nucleotides of 5' flanking sequence (*Tα1* promoter fragment), the contiguous 99 nucleotides of 5' untranslated region, and the ATG translation start site was isolated and fused to the E. coli *lacZ* gene which encodes the enzyme β-galactosidase. The *lacZ* gene used was modified and contained an amino-terminal nuclear translocation signal sequence derived from SV40 *T antigen* (Kalderon et al 1984) and the murine *protoamine 1* gene (Peschon et al 1987) from +95 to +625 at the C-terminus of *lacZ*. This *protoamine 1* gene fragment provided an intron and a polyadenylation signal. The resultant "*Tα1-nlacZ*" transgene is shown schematically in figure 2. Transgenic mice were generated by injecting the purified *Tα1-*

nlacZ transgene fragment into the pronuclei of CD1 embryos. Animals from eight distinct founder lines were identified and five of these lines exhibited transgene expression.

Mutagenesis of the 1028-nucleotide *Tα1* promoter fragment was performed to delete a specific 66-nucleotide sequence. This deletion removed the NRE and its flanking sequences from the *Tα1* promoter fragment, and the resultant fragment is referred to as the *ΔNRE* promoter. The deleted sequence included a number of consensus sequences representing potential binding sites for bHLH transcription factors (E-box), retinoic acid receptors (RARE), the estrogen receptor (ERE), the SP1 zinc finger transcription factor (SP1), the conserved transcription factor suppressor of hairless (Su(H)) and a gamma-interferon responsive factor (IRE).

The *ΔNRE* promoter remained upstream of the 5' untranslated *Tα1* gene sequence, the translation start site, and the modified *lacZ* gene carrying an SV40 nuclear localization sequence and the murine *protoamine-1* gene fragment, creating the "*ΔNRE-nlacZ*" transgene. Figure 2 shows schematically how *ΔNRE-nlacZ* was constructed. Transgenic mice were generated by injecting the purified *ΔNRE-nlacZ* transgene fragment into the pronuclei of C3H embryos. Animals from five distinct founder lines were identified and transgene expression was detected in three of these. Two expressing lines were used for subsequent analysis, namely line 9 and line 23.

Site directed mutagenesis was performed a second time to delete a specific 184-nucleotide sequence from the 1028-nucleotide 5' flanking sequence of the *Tα1* gene, generating the *ΔFRE* (forebrain response element) promoter. The deleted sequence included the 66-nucleotide segment that was deleted to generate the *ΔNRE* promoter, as well as 3' flanking sequence. In addition to the consensus sequences common to the 66-nucleotide deletion, the 184-nucleotide deletion also removed a conserved 30-nucleotide sequence that constitutes a tandem repeat of a homeodomain consensus element. This putative homeodomain-binding element is located 41 nucleotides 5' of the 3' end of the deleted 66-nucleotide NRE-containing sequence. Figure 2 shows schematically how *ΔFRE-nlacZ* was constructed. Transgenic mice were generated by injecting the purified

ΔFRE-nlacZ transgene fragment into the pronuclei of C3H embryos. Animals from six distinct founder lines were identified and transgene expression was detected in three of these. Two expressing lines were used for subsequent analysis, namely line 1 and line 17.

(iii) Comparison of *Tα1-nlacZ* and *ΔNRE-nlacZ* Transgenic Mice During Development Reveals Potentially Precocious Reporter Gene Expression in the CNS and PNS in one *ΔNRE-nlacZ* Mouse Line

(a) *ΔNRE-nlacZ* may be Precociously Expressed in Neural Precursor Cells and/or Immature Neurons in the CNS and PNS at E9.5

In a previous study, we demonstrated that embryonic day 9.5 was the earliest time at which β-gal activity could be detected *in vivo* in *Tα1-nlacZ* transgenic mice (Gloster et al 1999). The onset of β-gal activity correlated with previously reported neuronal birthdates in many populations in both the CNS and PNS. At E9.5 β-gal activity was detected in newborn motor neurons of the spinal cord above the thoracic segment, and in a few neurons of the hindbrain, midbrain, and forebrain (Gloster et al 1999). Thin section analysis revealed that only a small number of cells displayed β-gal activity, correlating with the generation of the first postmitotic neurons in these populations (Gloster et al 1999; Angevine 1970; McConnell 1981; Taber Pierce 1973; Nornes and Carry 1978). In X-gal stained whole embryos from *Tα1-nlacZ* line K6 at E 9.5, β-gal activity was apparent in presumed motor neurons of the rostral spinal cord (arrowhead, figure 3b). A similar staining pattern was observed in whole embryos from *ΔNRE-nlacZ* line 9 at E 9.5 (figure 3c), where presumed motor neurons of the rostral spinal cord expressed β-gal (figure 3c, arrowhead). The low level of β-gal activity observed in the rostral spinal cord in line 9 continued rostrally through to the mesencephalic flexure (figure 3c). A completely different staining pattern was observed in *ΔNRE-nlacZ* line 23 at E9.5. At this time point, *ΔNRE-nlacZ* line 23 whole embryos showed more extensive and higher levels of β-gal activity (figure 3a, d). In the CNS, this was observed throughout the spinal cord

(large arrowhead), midbrain and hindbrain suggesting that *ΔNRE-nlacZ* may have been precociously expressed in neural precursor cells within these populations in line 23.

Precocious *ΔNRE-nlacZ* transgene expression, preceding *Tα1-nlacZ* expression and cell cycle exit, may also have occurred in the presumptive trigeminal (V) and geniculate (VII) cranial ganglia in *ΔNRE-nlacZ* line 23. In whole embryos, β-gal activity was apparent in the presumptive Vth and VIIth ganglia in *ΔNRE-nlacZ* line 23 by E9.5 (figure 3a, d, "V" and "VII" respectively), though it was not detectable in *ΔNRE-nlacZ* line 9 (figure 3c) nor in whole embryos from *Tα1-nlacZ* line K6 at this timepoint (figure 3b). However thin section analysis revealed that a small number of cells within the Vth and VIIth ganglia exhibited β-gal activity in both *Tα1-nlacZ* mouse lines (Gloster et al 1999), correlating with the previously reported timing of neuronal birth in the Vth ganglion (Rhoades et al 1991). The large number of β-gal positive cells in *ΔNRE-nlacZ* line 23, the small number of β-gal positive cells in *Tα1-nlacZ* mice, and the fact that cell cycle exit among neural precursors is just beginning in the Vth ganglion at this time point suggests that neural precursor cells and/or immature neurons may have expressed the *ΔNRE-nlacZ* transgene in line 23. The constellations of β-gal positive cells clustered around the presumptive Vth and VIIth ganglia (figure 3a) may have included migrating precursor cells and immature neurons en route to the Vth and VIIth ganglia.

Littermates do not develop synchronously, and embryos from E9.5 staged pregnant females can differ slightly in their developmental stage. None of the *ΔNRE-nlacZ* line 23 embryos taken from E9.5 staged pregnant females showed a β-gal activity pattern similar to that seen in *ΔNRE-nlacZ* line 9 or *Tα1-nlacZ* lines K6 and Q54 at E9.5. Furthermore, examination of both *ΔNRE-nlacZ* line 9 and line 23 at E9.0 revealed no β-gal activity at that time (data not shown). This suggests that *ΔNRE-nlacZ* induction occurred between E9.0 and E9.5, the same time at which *Tα1-nlacZ* was induced. We cannot rule out the possibility that an intermediate stage with a pattern of activity similar to *Tα1-nlacZ* was not observed in *ΔNRE-nlacZ* line 23 due to its potentially short lived nature. This seems unlikely however, given that more than one litter was examined.

In summary, a comparison of *Tα1-nlacZ* and *ΔNRE-nlacZ* transgenic embryos at E9.5, the time at which *Tα1-nlacZ* is first expressed in newborn CNS and PNS neurons (Gloster et al 1999), suggests that in one line of *ΔNRE-nlacZ* mice reporter gene expression may have preceded *Tα1-nlacZ* induction just slightly in populations which were beginning to generate postmitotic neurons.

(b) ΔNRE-nlacZ may be Precociously Expressed in Neural Crest-Derived and Placodally–Derived PNS Primordia at E10-E10.5

The first sensory neurons of the dorsal root ganglia (DRG) are born at E10.0, though neurogenesis continues over several days in the ganglia (Lawson and Briscoe 1979). In *Tα1-nlacZ* mice, reporter gene activity was first detected between E10.0 and E10.5 in DRG's at the thoracic and cervical level (Gloster et al 1999). In whole embryos from *Tα1-nlacZ* line K6 β-gal activity was not detected in DRG's at E10.0, but was detected in DRG's by E10.5 (figure 4a, arrows). Similarly, in *ΔNRE-nlacZ* line 9 β-gal activity in the DRG's was first detected between E10.0 and E10.5 (figure 4d, short arrows). Figure 4b, 4c, and 4d show two *ΔNRE-nlacZ* line 9 littermates from an E10.5 staged pregnant female, revealing the developmental variation that can be observed between littermates. Figure 4b shows an additional *ΔNRE-nlacZ* line 9 embryo at E9.5 for comparison. No β-gal activity was detected in the DRG's of the younger E10.5 littermate (figure 4d, left), while in the slightly older littermate β-gal activity was detected in the newly formed DRG's (figure 4d, right). This suggests that β-gal expression in the DRG's commenced between E10.0 and E10.5 in *ΔNRE-nlacZ* line 9, as it appeared to in *Tα1-nlacZ* mice. In contrast, β-gal activity was clearly detected in the presumptive DRG's in *ΔNRE-nlacZ* line 23 at E10.0 (figure 4a), a half day prior to detection in *ΔNRE-nlacZ* line 9 and *Tα1-nlacZ* lines K6 and Q54 (figure 4a; Gloster et al 1999). This precocious β-gal activity suggests that the *ΔNRE-nlacZ* transgene may have been expressed in neural precursor cells and/or immature neurons derived from the neural crest in *ΔNRE-nlacZ* line 23.

The development of the proximal and distal cranial ganglia depends upon the neural transcription factors *ngn1* and *ngn2* (Fode et al 1998; Ma et al 1998). The cranial ganglia develop asynchronously, with the trigeminal (V) and geniculate (VII) ganglia expressing stage specific markers, including the *ngn's*, earlier than the petrosal (IX) and nodose (X) ganglia (Fode et al 1998; Ma et al 1998). Placode-derived precursors of the presumptive Vth and VIIth ganglia appear to delaminate from their respective ectodermal placodes and commence their development and neuronal differentiation earlier than those of the IXth and Xth ganglia (Fode et al 1998; Ma et al 1998). The development of the Vth and VIIth ganglia versus the IXth and Xth ganglia appears to be offset by between 12 and 24 hours. It is interesting to note that β-gal activity was first detected in the IXth and Xth ganglia at E10.5 in *Tα1-nlacZ* line K6 (figure 5b; Gloster et al 1999), one day later than the first detectable activity in the Vth and VIIth ganglia (Gloster et al 1999). Similarly, the appearance of β-gal activity in the IXth and Xth ganglia may have followed that in the Vth and VIIth ganglia in *ΔNRE-nlacZ* line 9. In the earliest line 9 littermate from E10.5 staged pregnant females, few cells in the IXth and Xth ganglia exhibited β-gal activity whereas activity in the Vth and VIIth ganglia was widespread (figures 4b-d, 5c, d). In contrast, by E10.0 β-gal activity was present in the presumptive IXth and Xth ganglia in *ΔNRE-nlacZ* line 23 (figure 5e), a half day before activity was detected in the same ganglia in *Tα1-nlacZ* mice. The precocious β-gal activity detected in *ΔNRE-nlacZ* line 23 suggests that *ΔNRE-nlacZ* may have been expressed in neural precursor cells and/or immature neurons derived from the ectodermal placode in line 23.

In summary, the appearance of β-gal activity in *ΔNRE-nlacZ* line 23 consistently preceded that in *Tα1-lacZ* mice in both the CNS and PNS, suggesting that *ΔNRE-nlacZ* induction may have preceded *Tα1-nlacZ* induction, the former being induced in placode-derived, neural crest-derived, and CNS-derived neural precursor cells and/or immature neurons. The timing of *ΔNRE-nlacZ* induction in line 23 differed between populations, but consistently appeared to precede *Tα1-nlacZ* induction in cells within those populations. Such precocious transgene expression was not observed in *ΔNRE-nlacZ* line

9, possibly due to a low level of expression imposed by the location of the transgene in the genome (Jaenisch et al 1981; Harebers et al 1981; Palmiter and Brinster 1986; Al-Shawi et al 1990; Pravtcheva et al 1994). We cannot rule out the possibility that the precocious expression observed in *ΔNRE-nlacZ* line 23 was due to a higher expression level in the population rather than to a physiological difference in onset time.

(iv) Cortical Neural Precursor Cells From *ΔNRE-nlacZ* Mice Exhibit Precocious Reporter Gene Expression Prior to Neuronal Differentiation

In order to more accurately assess the timing of *ΔNRE-nlacZ* transgene induction relative to *Tα1-nlacZ* transgene induction and other aspects of neuronal differentiation, cortical neural precursor cells were cultured from *Tα1-nlacZ* and *ΔNRE-nlacZ* transgenic mice, and β-gal expression was followed as the progenitor cells exited the cell cycle and differentiated *in vitro*. *Beta-galactosidase* expression was measured using a mouse monoclonal anti-β-gal antibody for immunolabeling. This culture system and detection method were used previously to demonstrate that *Tα1-nlacZ* expression coincided with or immediately followed cell cycle exit and induction of the neuronal marker *β-III tubulin* (Gloster et al 1999).

The appearance of β-III tubulin in cortical cultures from E12.5 *Tα1-nlacZ* embryos was previously reported to require one day *in vitro*, while *β-gal* expression required 1-2 days *in vitro* (Gloster et al 1999). In these cultures, β-gal was co-localized with β-III tubulin suggesting transgene expression was restricted to newborn neurons (Gloster et al 1999). In addition, most cells (approx. 95%) in these cultures derived from E12.5 embryos appeared to be cycling during their first day *in vitro*, as indicated by BrdU uptake analysis (Gloster et al 1999). The expression of *β-III tubulin* after one day *in vitro* suggested that *β-III tubulin* was induced coincident with or immediately following terminal mitosis, making it a very early neuronal marker.

When cortical neural precursor cells were cultured from *Tα1-nlacZ* transgenic mice at E10.5 and left in culture for one day, few neurons were generated as indicated by the low abundance of *β-III tubulin* expression (figure 6, compare a to c). Cultures derived from E10.5 embryos presumably consisted mainly of immature neural precursor cells that were incapable of differentiating within one day *in vitro*.

Though very few cells derived from E10.5 *Tα1-nlacZ* mice expressed *β-III tubulin* after one day *in vitro*, even fewer expressed *β-gal* (figure 6b). However those that did express *β-gal* co-expressed *β-III tubulin* (figure 6, compare a and b), suggesting that *Tα1-nlacZ* expression was restricted to newborn neurons. This was consistent with observations of E12.5 *Tα1-nlacZ* cultures (Gloster et al 1999).

Though *β-gal* expression was restricted to *β-III tubulin* expressing cells in cultures from *Tα1-nlacZ* mice at E10.5, *β-gal* expression was detected in β-III negative cells derived from *ΔNRE-nlacZ* line 23 transgenic mice at E10.0 (figure 6d, e, f, arrowheads). In later staged cultures, greater numbers of cells expressed *β-III tubulin*, and β-gal was colocalized with β-III tubulin (data not shown), suggesting that *ΔNRE-nlacZ* expression preceded *β-III tubulin* expression within cells. In addition, though not quantitated, *β-gal* was expressed in fewer cells in cultures from *ΔNRE-nlacZ* line 23 than cultures from *Tα1-nlacZ* at E10.5. Further, *β-gal* expression was very low in preliminary cultures derived from *ΔNRE-nlacZ* line 9, and line 9 was not used further for these experiments.

The number of cells expressing *β-III tubulin* after one day *in vitro* in cultures from E10.0 embryos was not dramatically lower than in cultures from E10.5 *Tα1-nlacZ* embryos. But the cells that expressed *β-III tubulin* in E10.0 cultures appeared to be at an earlier stage of development morphologically (figure 6, compare a and d). That is, the *β-III* expressing cells in E10.5 cultures had begun to change shape from rounded to elongated and displayed what might have been immature processes after one day *in vitro* (figure 6a). In contrast the *β-III* expressing cells in E10.0 cultures were rounded and did not have extensions resembling processes after one day *in vitro* (figure 6d). These results suggest

that after one day *in vitro*, cells in E10.0 cultures were less mature than cells in E10.5 cultures, though differentiation in both cultures in absolute terms appeared to be accelerated relative to E12.5 cultures. Culturing earlier than E10.0 proved to be technically unfeasible.

In summary, *β-gal* expression appeared to follow *β-III* tubulin expression in cortical precursor cell cultures from *Tα1-nlacZ* line K6, but precede *β-III* tubulin expression in cortical precursor cell cultures from *ΔNRE-nlacZ* line 23. These results suggest that *ΔNRE-nlacZ* transgene expression preceded the expression of *β-III tubulin* and *Tα1-nlacZ* expression in cortical neural precursor cells. Though not directly demonstrated, it is likely that some of the *ΔNRE-nlacZ* expressing cells had not exited the cell cycle based on previous characterization of these cultures (Gloster et al 1999; Slack et al 1998). Line 23 was the same *ΔNRE-nlacZ* line that appeared to exhibit putative precocious reporter gene expression relative to *Tα1-nlacZ* induction *in vivo* in the developing PNS and CNS.

(v) *ΔNRE-nlacZ* Expression is Detected in a Region of the Adult Brain Where Neural Precursor Cells Reside and *Tα1-nlacZ* Expression is Excluded

The *in vitro* and *in vivo* comparisons of *ΔNRE-nlacZ* and *Tα1-nlacZ* expression during neuronal differentiation suggested that in *ΔNRE-nlacZ* line 23, the *lacZ* reporter gene was precociously expressed in neural precursors and/or immature neurons. We next compared β-gal activity in the adult brain in *Tα1-nlacZ* and *ΔNRE-nlacZ* mice, looking for evidence of precocious reporter gene expression in *ΔNRE-nlacZ* mice. In the mature mouse brain, neural precursor cells and mature neurons coexist but are spatially separated. "Stem cells" have been cultured from the embryonic and adult CNS at various axial levels, including the forebrain and spinal cord (Weiss et al 1996; Kuhn and Svendsen 1999). These stem cells are believed to be the source of progenitor cells that are the precursors of the various neuronal and glial cell subtypes. Stem cells and possibly progenitor cells have been identified in both the ependymal (Johansson et al 1999) and subependymal (Reynolds and Weiss 1992) regions of the mature brain surrounding the

lateral ventricles. Neurons are believed to be excluded from the ependymal layer (Johansson et al 1999).

Coronal brain slices through the rostral forebrain of adult *Tα1-nlacZ* mice were stained with X-gal solution and then sectioned to examine β-gal activity in the neocortex and the ependymal layer surrounding the lateral ventricles where neural precursors are known to reside (Johansson et al 1999). As previously shown (Bamji and Miller 1996), the *Tα1-nlacZ* transgene was expressed in neurons of the mature cortex (figure 7B b). However, β-gal activity was not detected in cells of the ependymal layer surrounding the lateral ventricles in the rostral forebrain of *Tα1-nlacZ* mice (figure 7A a-d, 7B b,d,f,g). In contrast, β-gal activity was detected in ependymal layer cells in *ΔNRE-nlacZ* line 9 (figure 7A e-j) and *ΔNRE-nlacZ* line 23 (figure 7B c, e). Further, β-gal activity was detected in the ependymal layer in *ΔFRE-nlacZ* line 17 (data not shown; expression in *ΔFRE-nlacZ* line 1 was not examined). In *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice, β-gal activity was also detected in the ependymal layer at the level of the third ventricle (figures 7A k, l; , 7B h; data not shown).

In summary, the *ΔNRE-nlacZ* transgene may have been expressed in neural precursor cells residing in the mature brain. The *Tα1-nlacZ* transgene was robustly expressed in mature neurons of the neocortex but appeared not to be expressed in cells of the ependymal layer surrounding the lateral ventricles where neural precursor cells reside. This suggests that *Tα1-nlacZ* expression was restricted to postmitotic neurons. In addition to the two lines of *ΔNRE-nlacZ* mice, putative precocious *β-gal* expression was observed in the adult brain in *ΔFRE-nlacZ* transgenic mice. This suggests that the 66-nucleotide deletion common to the *ΔNRE* and *ΔFRE* promoters was responsible for transgene disinhibition and precocious *β-gal* expression.

(B) ΔNRE-nlacZ and ΔFRE-nlacZ Mice Exhibit Lower Reporter Gene Expression than Tα1-nlacZ Mice in the Developing and Mature Neocortex

(i) Reporter Gene Activity is Reduced in the Developing Neocortex of *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Mice Relative to *Tα1-nlacZ* Mice

A comparison of β-gal activity in *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* embryos at E13.5, a time at which *Tα1-nlacZ* is expressed robustly in the CNS and PNS (Gloster et al 1994), revealed dramatic differences in the neocortex. Beta-galactosidase activity in the neocortex was much lower in *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice than in *Tα1-nlacZ* mice (figure 8). High levels of β-gal activity were detected in the olfactory bulb, midbrain, hindbrain and spinal cord in *ΔNRE-nlacZ* mice (figure 8b, c) as in *Tα1-nlacZ* mice (figure 8a; Gloster et al 1994), suggesting the decrease in β-gal activity was specific to the neocortex.

A greater decrease in neocortical β-gal activity was seen in *ΔFRE-nlacZ* mice. Both *ΔFRE-nlacZ* line 1 and line 17 showed very little β-gal activity in the neocortex at E13.5 (figure 8e, d respectively). As in *ΔNRE-nlacZ* mice, the decrease in β-gal activity was fairly specific to the neocortex, though in contrast to *Tα1-nlacZ* and *ΔNRE-nlacZ* mice a small decrease in activity was observed in the olfactory bulbs. In addition, β-gal activity was dramatically reduced in the region surrounding the isthmus in *ΔFRE-nlacZ* line 1 (figure 8d, arrow).

Previous analyses of *Tα1-nlacZ* mice showed that β-gal activity was first detected in the developing neocortex at E12.5, the time at which cortical plate-forming neurons first appear (Caviness 1982). To determine whether transgene activation was simply delayed

in *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice, we next examined transgene expression in the early postnatal brain.

High levels of β-gal activity were detected in the hippocampus, piriform cortex, and throughout the neocortex in *Tα1-nlacZ* mice at postnatal day 1 and postnatal day 7 (figure 9a, b respectively), suggesting *Tα1-nlacZ* was continuously expressed in the neocortex during development. A much lower level of β-gal activity was observed in the early postnatal neocortex in *ΔNRE-nlacZ* line 23 (figure 9d). The decrease appeared specific to the neocortex, and activity was easily detected in the hippocampus, piriform and entorhinal cortex (figure 9d). An even greater decrease in β-gal activity was seen in the neocortex of *ΔNRE-nlacZ* line 9 at postnatal day 2 (figure 9c). This dramatic decrease in expression appeared to be confined to the neocortex, and β-gal activity was abundant in the hippocampus, piriform and entorhinal cortex (figure 9c). A difference in β-gal expression appeared to form a sharp border at the junction of the piriform cortex and the lateral neocortex, being highly expressed in the former but not in the latter. The brain slice in figure 9c was left in X-gal solution for an extended period of time to accentuate the piriform cortex/neocortex border.

Still further decreases in β-gal activity in the neocortex were observed in *ΔFRE-nlacZ* line 1 and line 17. Beta-galactosidase was detected in very few cells in the early postnatal neocortex of *ΔFRE-nlacZ* mice (figure 9e, f). The decrease in activity appeared fairly specific to the neocortex. Beta-galactosidase was readily detected in the olfactory bulbs (figure 9f, g) but was notably low in the developing hippocampus (figure 9e) and cerebellum (figure 9f) in *ΔFRE-nlacZ* line 1.

In summary, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice consistently displayed less β-gal activity in the developing neocortex than *Tα1-nlacZ* mice. The level of expression in the neocortex appeared to be lower throughout brain development and not simply delayed in *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice. The decrease in expression within the cortex appeared to be specific to the neocortex, as expression in the rest of the pallium including

the hippocampus, piriform cortex, and entorhinal cortex did not appear to be consistently altered.

These results suggest that the 66-nucleotide *Tα1* promoter sequence commonly deleted in the *ΔNRE* and *ΔFRE* promoters contributed to *Tα1* promoter activity in newborn neurons of the developing neocortex. They further suggest that the 118-nucleotide *Tα1* promoter segment immediately 3' to this 66-nucleotide sequence also contributed to *Tα1* promoter activity in newborn neocortical neurons.

(ii) Reporter Gene Activity is Reduced in the Adult Neocortex of *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Mice Relative to *Tα1-nlacZ* Mice

We next compared β-gal activity between adult brains of *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice. In a previous study, *Tα1-nlacZ* expression in transgenic mice was compared to endogenous *Tα1* α-tubulin gene expression in the adult rat brain (Bamji and Miller 1996). With few exceptions, reporter gene expression in distinct populations of the brain was consistently detected in both lines of *Tα1-nlacZ* mice, and the corresponding rat brain populations also expressed *Tα1* mRNA. However, the number of cells exhibiting β-gal activity within a population varied between the two transgenic lines, owing to partial penetrance of the transgene. Beta-galactosidase activity was detected throughout the pallium including the hippocampus, neocortex, entorhinal cortex and piriform cortex, as well as in the diencephalon, pallidum, striatum, amygdaloid nuclei and midbrain of adult *Tα1-nlacZ* mice (figure 10a, b). One population in which expression was not consistently found in the two lines was the Purkinje cell population of the cerebellum. Beta-galactosidase activity was detected in Purkinje cells in *Tα1-nlacZ* line K6, but not in line Q54. Like β-gal in line K6, *Tα1* mRNA was detected in Purkinje cells of the adult rat cerebellum. *Tα1-nlacZ* expression in the Islands of Calleja also appeared to be inconsistent between lines, being expressed in line K6 but not in line Q54. A low level of endogenous *Tα1* expression was detected in these cells in the rat brain. Finally,

disparity between *Tα1-nlacZ* expression and endogenous *Tα1* expression was noted in the superior colliculus, where *Tα1-nlacZ* was expressed at relatively high levels in both lines, though the level of *Tα1* mRNA was relatively low in these cells in the adult rat brain.

The number of cells with detectable β-gal activity was generally lower in every adult neuronal population in *Tα1-nlacZ* line Q54 than in line K6. One noted exception was the CA3 region of the hippocampus, which displayed easily detectable levels of β-gal activity in line Q54 but very low levels of activity in line K6. *Tα1* mRNA appeared evenly distributed throughout the adult rat hippocampus, including the CA3 region.

In *Tα1-nlacZ* line K6, β-gal activity was detected throughout the adult dorsal, lateral and medial neocortex. Activity appeared to be higher in neocortical layers V and III. In the adult neocortex of *Tα1-nlacZ* line Q54 mice, radial columns containing a high density of β-gal positive cells appeared organized and separated by intervening regions of low β-gal expression. The uniform distribution of *Tα1* mRNA throughout the dorsal, lateral and medial neocortex in the adult rat brain was similar to the β-gal activity pattern in line K6, suggesting the pattern in line Q54 was likely a consequence of eipgenetic effects due to the location of the transgene in the genome (Jaenisch et al 1981; Harbers et al 1981; Palmiter and Brinster 1986). In the present study β-gal activity was detected in the neocortex, hippocampus, entorhinal cortex, piriform cortex, hypothalamus, amygdala, globus pallidus, caudate, putamen, thalamus, and pons in *Tα1-nlacZ* line K6 (figure 10a, b), in agreement with previous results.

In *ΔNRE-nlacZ* line 9, some β-gal activity was detected evenly throughout the neocortex of the adult brain, though at a much lower level than in *Tα1-nlacZ* mice. This β-gal activity did not appear to be restricted to a particular layer (figure 14a-c). Activity was detected in the neocortex in caudal (figure 12b), medial (figures 14a-c, 10g, h) and rostral brain slices (figure 11d). Noticeably lower levels of β-gal activity were also observed in the hippocampus, piriform cortex, entorhinal cortex, and globus pallidus of the adult

brain (figure 10g, h). Activity was higher in the piriform and entorhinal cortex than the neocortex in the adult brain. In rostral slices, β-gal activity also appeared reduced in the caudate, putamen, cingulum, amygdala, hypothalamus and septum relative to *Tα1-nlacZ* line K6 (figur 11, compare a and d). In addition, β-gal expression was not detected in the Islands of Calleja in line 9, unlike *ΔNRE-nlacZ* line 23 (figure 11e) and *Tα1-nlacZ* line K6 (Bamji and Miller 1996).

The β-gal activity pattern in *ΔNRE-nlacZ* line 9 mice also differed from that of *Tα1-nlacZ* mice in the cerebellum, where expression in Purkinje cells and granule cells appeared to be diminished in line 9 relative to *Tα1-nlacZ* mice (figure 13, compare a and b). In contrast, activity in the superior colliculus was higher in *ΔNRE-nlacZ* line 9 than it was in *Tα1-nlacZ* mice (figure 12 compare a and b). Figure 12b shows a dorsal view of a line 9 adult brain segment, and reveals the sharp contrast between the high activity in the superior colliculus and the low activity in the cerebellum. Finally, ectopic β-gal activity was detected in the kidney in *ΔNRE-nlacZ* line 9 (figure 15b). This ectopic activity was unique to line 9, but was consistently detected within the line.

Beta-galactosidase activity was higher in the adult neocortex in *ΔNRE-nlacZ* line 23 than it was in line 9, but lower than it was in *Tα1-nlacZ* mice (figures 7B a, b, 10a, b, f, g, h 11a, d, e). This expression level relationship was consistent with that observed at E13.5 (figure 8), postnatal day 2 (figure 9), and in cultures of cortical neural precursor cells (data not shown). Beta-galactosidase activity was detected in the piriform and entorhinal cortex, hippocampus, globus pallidus, caudate, putamen, hypothalamus, thalamus and amygdala in line 23 (figure 10j, f). As in line 9, β-gal activity in the neocortex did not appear to be restricted to a particular layer (figure 14d, e), and was higher in the piriform and entorhinal cortex than the neocortex (figures 10j, f; 11 e). In the rostral forebrain, β-gal activity also appeared to be low in the septum, piriform cortex, caudate and putamen relative to *Tα1-nlacZ* mice (figure 11 e). In caudal brain slices, robust β-gal activity was detected in Purkinje cells (figure 13c, d arrows) and granule cells of the cerebellum

(figure 13c, d). Activity was also apparent in the midbrain much like that observed in *Tα1-nlacZ* K6 (figure 12a, data not shown).

Several differences were noted between *Tα1-nlacZ* and *ΔFRE-nlacZ* line 17 adult mice. Very few β-gal positive cells were detected in the neocortex in line 17, as had been seen at postnatal day 2 (figures 10c, d; 11c; 14f, g). However, one specific region of the medial neocortex consistently appeared to be unaffected. Beta-galactosidase was detected in bilateral stripes of medial cortex corresponding to the retrosplenial granular cortex (figure 10c, d "rsg"). In addition, β-gal activity was notably decreased in the globus pallidus and amygdala. Activity was also lower in the piriform and entorhinal cortex, though it remained higher than the level in the neocortex (figures 10c, d; 14f, g). A dramatic and unique decrease in activity was also observed in hypothalamic and thalamic nuclei in line 17 (figure 10c, d).

Beta-galactosidase positive cells did not appear to be restricted to a particular layer of the neocortex in *ΔFRE-nlacZ* line 17 mice (figure 14f, g). In rostral brain slices, β-gal activity was apparent but very limited in the neocortex (figure 11c). Activity appeared to be low in all structures at this level, including the septum, piriform cortex, amygdala, cingulum, caudate and putamen. In caudal brain slices, activity was found to be extremely high in the cerebellum, in both Purkinje cells (figure 13f, arrow) and granule cells (figure 13e, arrow). In contrast, activity was diminished in the superior colliculus (figure 12c), giving a pattern of expression opposite to that of *ΔNRE-nlacZ* line 9 in the midbrain and cerebellum (figure 12, compare b, c). Interestingly, the low level of expression in the midbrain of the adult was not prefigured by a low level of expression in the midbrain at postnatal day 2 (figure 9g).

In *ΔFRE-nlacZ* line 1 adult mice, a low level of β-gal activity was detected in the neocortex and piriform cortex (figures 10e, i; 13 g, h), as observed at postnatal day 2 (figure 9f). In contrast to line 17, robust β-gal activity was detected in hypothalamic nuclei, but not in the hippocampus (figure 10, compare e, i to c, d). The lack of activity in the adult hippocampus was prefigured by a lack of activity in the developing

hippocampus at postnatal day 2 (figure 9e). In addition, β-gal activity was notably diminished in the caudate, putamen and globus pallidus (figure 10e), while high levels of activity were observed in the diencephalon, amygdala, and cingulum in the adult brain (figure 10e,i).

Cells exhibiting β-gal activity did not appear to be evenly distributed throughout the neocortex in *ΔFRE-nlacZ* line 1 (figure 14h, i). Beta-galactosidase activity was primarily detected in cells in the superior layers of the neocortex. A high density of β-gal positive cells in the cingulum stood in sharp contrast to the low density of β-gal-positive cells in the inferior neocortex (figure 14h, i). In the rostral telencephalon, activity was detectable but low in the neocortex (figure 11b), and was generally low in all structures at his level. In the cerebellum, a very low level of activity was detected in Purkinje cells (figure 13g, h, short arrows), and activity was barely detectable in granule cells (figure 13g, h), in contrast to the high level of activity detected ventrally in pontine nuclei (figure 13h "p"). This low level of activity in the adult cerebellum was prefigured by a low level of activity in the developing cerebellum at postnatal day 2 (figure 9f). In addition, activity was detected in the superior colliculus in line 1 similar to *Tα1-nlacZ* line K6 (figure 12a, data not shown), yielding a pattern of expression roughly complementary to that of line 17 in the caudal region of the brain.

Comparing *ΔNRE-nlacZ* lines 9 and 23, β-gal activity was consistently decreased in the adult neocortex relative to *Tα1-nlacZ* line K6 mice and compared to the relative expression of endogenous *Tα1* mRNA between regions of the adult rat brain (Bamji and Miller 1996). Beta-galactosidase activity was consistently higher in the piriform and entorhinal cortex than it was in the neocortex. Activity varied quantitatively in the globus pallidus, caudate, putamen, superior colliculus, cerebellum, Islands of Caleja and hippocampus with line 23 displaying greater β-gal activity in all but the superior colliculus. Notably, β-gal activity in the caudate, putamen and globus pallidus was generally low but varied quantitatively between *Tα1-nlacZ* lines, and the level of *Tα1* α-tubulin mRNA in these regions approached background levels in the adult rat brain. Further, the level of *Tα1-nlacZ* expression in the hippocampus and superior colliculus

appeared to vary between lines, and expression in the cerebellum and Islands of Caleja was inconsistent between *Tα1-nlacZ* transgenic lines.

Similarly, comparing *ΔFRE-nlacZ* lines 1 and 17, there was a consistent and dramatic decrease in β-gal activity in the adult neocortex relative to *Tα1-nlacZ* line K6. The magnitude of the decrease was greater than that observed in *ΔNRE-nlacZ* mice. But similar to *ΔNRE-nlacZ* mice, β-gal activity was more extensive in the piriform cortex and entorhinal cortex than it was in the neocortex. Activity in the hippocampus varied quantitatively, being robust in line 17 and barely detectable in line 1. In addition, a unique decrease in activity was noted in the hypothalamus and thalamus in line 17, while β-gal activity was notably high in the dorsal endopiriform nucleus of line 1. Notably,β-gal activity also differed quantitatively in the thalamus and hypothalamus between *Tα1-nlacZ* transgenic lines.

Overall, the relative expression of *ΔNRE-nlacZ* and *ΔFRE-nlacZ* appeared to be consistently lower in the neocortex than the relative expression of *Tα1-nlacZ* and the endogenous *Tα1* gene. The decrease in reporter gene activity observed in the neocortex was greater in *ΔFRE-nlacZ* mice than it was in *ΔNRE-nlacZ* mice at all stages examined, though the decrease in *ΔNRE-nlacZ* mice was dramatic at each stage. This consistent decrease was specific to the neocortex and was observed from the time the cortical plate began to develop through to adulthood. In addition, expression appeared to be consistently higher in the piriform and entorhinal cortex than it was in the neocortex in these promoter mutant mice.

(C) Su(H)/RBP-Jk Binds the NRE of the *Tα1* Promoter *in vitro*

(i) Nuclear Extracts from Developing Neurons and Neural Precursor Cells Form Multiple Complexes with the *Tα1* NRE Sequence, Some of Which May be Enriched in Neural Tissue, Developmentally Regulated and Co-Migrate with RBP-Jk Consensus Sequence-Binding Complexes

To identify NRE-binding proteins in various tissues, and within nervous tissue at various developmental stages, a double stranded ^{32}P end-labeled DNA "probe", corresponding to 30 nucleotides of sequence from the *Tα1* promoter, was used in electrophoretic mobility shift assays (EMSA's) with nuclear protein extracts from different tissues and at different developmental stages. The 10-nucleotide NRE was located in the centre of the probe. A schematic of the EMSA procedure is shown in figure 16, and the sequence of the NRE probe is found in the legend.

Equal amounts of probe and equal amounts of different nuclear extracts were used in EMSA's to compare the relative abundance of similarly migrating complexes between extracts. However, complexes from different extracts that migrate similar distances in EMSA's need not contain the same binding protein(s). Co-migration does not imply a common identity.

A number of complexes were formed when NRE probe was incubated with E13.5 nuclear extract (figure 17). This extract presumably contained the nuclear contents of developing neurons and neural precursor cells. Based on co-migration, a number of these complexes appeared to be enriched in nervous tissue and some appeared to be developmentally regulated. Three complexes were focussed on initially (figure 17 "A", "B", "C") and one of these was further examined (figure 17 "C").

The NRE-binding complex "A" was formed with E13.5 nuclear extract ("A" figure 18). A similar band appeared with P0 nuclear extract, though at a higher intensity. A similar band appeared with adult brain extract as well, though it was less intense than that

observed in E13.5 and P0 nuclear extracts. A very low intensity band at the same position appeared with liver nuclear extract. If the complexes co-migrating with complex "A" were the same, which cannot be concluded from co-migration alone, it appears that the complex was enriched in nervous tissue and developmentally regulated.

A more intense band appeared with E13.5 extract ("B" figure 18). This complex was more abundant than either complex "A" or "C" in E13.5 extract. As with complex "A", complex "B" was potentially enriched in nervous tissue as indicated by the presence of an abundant co-migrating complex in both P0 and adult brain extract, and the low level of such a co-migrating complex in liver extract. Complex "B" may also have been developmentally regulated within the nervous system, with levels decreasing from E13.5 to adulthood.

Complex "C" in E13.5 extract may also have been enriched in nervous tissue given the presence of the co-migrating band in adult brain and its near absence in liver. This complex may have been dramatically developmentally regulated, being present in E13.5 extract, nearly absent in extract from the P0 brain, and abundant in the adult brain.

As the NRE closely resembles the RBP-Jk consensus sequence, a double stranded ^{32}P end-labeled DNA "probe", corresponding to 29 nucleotides of sequence from the adenovirus *pIX* gene was used to examine RBP-Jk/Su(H) binding activity in nuclear extracts. The *pIX* gene has been characterized and is a natural target of mammalian RBP-Jk/Su(H). It contains a consensus RBP-Jk/Su(H) binding sequence also found in the Drosophila *m8* gene, a natural target of RBP-Jk/Su(H) in the Drosophila *E(spl)* complex. RBP-Jk binds the *pIX* sequence *in vitro* and *in vivo*.

RBP-Jk/Su(H)-binding complexes that co-migrated with "A", "B" and "C" formed with E13.5 nuclear extract. These are referred to as "A-prime", "B-prime", and "C-prime" respectively (figure 17). Complex "A-prime" shows a distribution similar to "A", being enriched in nervous tissue, most abundant in P0 brain, and more abundant in E13.5 extract than adult brain extract. It should be stressed that the complexes formed with

different extracts need not contain the same proteins in order to co-migrate. Co-migration is only suggestive that common complexes may be present in different extracts.

Like complex "B", complex "B-prime" appeared to be enriched in neural tissue. The developmental regulation of complex "B-prime" appeared to differ from that of complex "B", increasing from E13.5 to P0 rather than decreasing. Complex "C-prime" appeared to be more abundant than complex "C" in all extracts examined. In addition, the developmental regulation of complex "C-prime" differed from that of complex "C", the former being most abundant in E13.5 extract and decreasing to adulthood. Complex "C-prime" was also detected in liver extract, while complex "C" was not.

(ii) Cross Competition Suggests Common Complexes Bind the NRE and RBPJk/Su(H) Consensus Site DNA Sequences

To see if complexes binding the NRE and RBP-Jk probes were related, we performed competition experiments looking at the ability of RBP-Jk consensus sequence to antagonize binding to the NRE sequence and vice versa.

Unlabeled NRE oligonucleotide was able to antagonize the formation of most E10.0 complexes with the NRE probe (figure 18 a). Among the complexes that could be antagonized by unlabeled oligonucleotide was complex "C". Complex "C" increased in abundance with increasing amounts of E10.0 nuclear extract, and did not form in the presence of 100 and 200 fold molar excess unlabeled NRE oligonucleotide (figure 18 a). In a control experiment, the addition of unlabeled mutated NRE oligonucleotide, a sequence identical to the NRE probe except for five point mutations within the NRE and putative RBP-Jk/Su(H) binding site, was unable to antagonize formation of complex "C" even at 300 fold molar excess, (figure 18 b) suggesting that the binding of complex "C" to the NRE was sequence-specific and dependent on the putative RBP-Jk/Su(H) binding sequence.

In contrast to mutated NRE oligonucleotide, unlabeled RBP-Jk oligonucleotide identical in sequence to the RBP-Jk probe was able to antagonize the formation of complex "C". In the presence of a 100 fold molar excess of unlabeled RBP-Jk oligonucleotide, no complex "C" formation was detected with NRE probe and E10.0 nuclear extract (figure 19 c, b). This suggested that the sequence-specific binding complex "C" also had affinity for the RBP-Jk consensus sequence, and may have bound to the NRE probe via the NRE sequence and its core RBP-Jk/Su(H) consensus sequence.

In the converse experiment, formation of complex "C-prime" between E10 nuclear extract and the RBP-Jk probe was antagonized by incubation with unlabeled NRE oligonucleotide. Increasing the molar excess of NRE oligonucleotide from 100 to 300 fold decreased the amount of complex "C-prime" formed (figure 18 b, d). This competition appeared to be sequence specific and depend upon the putative RBP-Jk/Su(H) site in the NRE, as the mutated NRE oligonucleotide was unable to compete with RBP-Jk probe even at 300 fold molar excess (figure 18 d).

These results suggest that complex "C" and complex "C-prime" can both bind the NRE and RBP-Jk probes with similar sequence requirements. This does not imply that complex "C" and complex "C-prime" consist of the same protein(s). They may or may not share specific protein(s).

(iii) *In Vitro* Translated RBP-Jk/Su(H) Binds the NRE and RBP-Jk Consensus Sequences, Forming Complexes that Co-Migrate with those Formed by Neural Tissue Nuclear Extracts

In vitro translated (IVT) RBP-Jk was used in EMSA's to see if it could bind the NRE sequence, to confirm the RBP-Jk probe was in fact an RBP-Jk/Su(H) binding sequence, and to compare the migration of any complexes so formed with those found in tissue extracts.

A comparison of *in vitro* translation reaction products incubated with the NRE probe and the RBP-Jk probe revealed the formation of specific complexes in the reactions charged with RBP-Jk cDNA (figure 19 a). *In vitro* translated RBP-Jk appeared to bind the NRE probe yielding complex "i" which was not observed when the NRE probe was incubated with the products of the luciferase cDNA-charged reaction, the Mash-1 cDNA-charged reaction, nor the uncharged reticulocyte lysate. The formation of complex "i" was not antagonized by incubation with a 100 fold molar excess of unlabeled mutated NRE oligonucleotide (figure 19 b), suggesting it was a sequence specific interaction. This suggested that IVT-RBP-Jk could bind the NRE probe, and that it likely did so through the putative RBP-Jk/Su(H) binding site.

In vitro translated RBP-Jk also appeared to bind the RBP-Jk probe forming complex "i-prime" (figure 19 a), confirming that the *pIX* gene sequence used as the RBP-Jk probe was in fact an RBP-Jk binding sequence. Furthermore, the formation of complex "i-prime'" was not antagonized by a 300 fold molar excess of unlabeled mutated NRE oligonucleotide (figure 19 b), suggesting the interaction was sequence specific.

Complexes co-migrating with "C" were formed with extracts from adult brain, E10.0 embryo, cultured cortical precursor cells at one day *in vitro* ("cort precursors"), and cultured cortical precursor cells at four days *in vitro* ("cort neurons") (figure 20). Previous studies have demonstrated that after one day *in vitro*, these cultures consist largely of undifferentiated precursor cells, while after four days *in vitro*, they consist largely of differentiating neurons (Gloster et al 1999). Complex "C" was more abundant in the adult brain than it was in the developing embryo (figure 20), as had been observed previously (figure 17). Interestingly, complex "C" appeared to be more abundant in cultured neural precursor cells than in newborn neurons (figure 20), similar to previous results showing a decrease from E13.5 to P0 brain (figure 17). When IVT-RBP-Jk ("CBF-1") was incubated with the NRE probe and run alongside these samples, complex "i" formed and co-migrated with complex "C" (figure 20). *In vitro* translated RBP-Jk ("CBF-1") was also incubated with the RBP-Jk probe and run on the same gel. Complex "i-prime" was formed and co-migrated with complex "C-prime". Interestingly, a complex

co-migrating with complex "C-prime" and complex "i-prime" was detected in extract from both cultured cortical precursor cells and neurons derived from these precursors (figure 20). As with the NRE probe, complex formation with the RBP-Jk probe was more abundant in precursor cells than in neurons.

Together these results suggested complex "C-prime" and the co-migrating complex "C" may have been RBP-Jk bound to the RBP-Jk probe and the NRE probe respectively (figure 20). However, this did not prove the identity of complexes "C" and "C-prime".

(iv) Anti-RBP-Jk Antibody Shifts Co-Migrating NRE Complexes Formed with *In Vitro* Translated RBP-Jk and Neural Tissue Nuclear Extract

To see if complex "C" contained RBP-Jk, we used a ploYclonal antibody raised against an RBP-Jk-GST fusion protein in supershift EMSA's. The anti-RBP-Jk antibody was pre-incubated with E13.5 nuclear extract before adding NRE or RBP-Jk probe. If RBP-Jk was a component of complex "C", the inclusion of the antibody would be predicted to disturb complex formation specifically and/or to cause formation of a modified complex "C" with the antibody attached. The formation of such a complex generally increases the size and weight of a complex, causing its migration to be retarded. This is often referred to as a "supershift".

Complex "C" was readily formed with E13.5 nuclear extract and the NRE probe (figure 21 a). Formation of complex "C" was antagonized by a 100 fold molar excess of either unlabeled NRE or RBP-Jk oligonucleotide, but was not antagonized by a 300 fold molar excess of mutated NRE oligonucleotide (figure 21 a). Similarly, complex "C-prime" was readily formed with E13.5 nuclear extract and the RBP-Jk probe. Formation of complex "C-prime" was antagonized by a 100 fold molar excess of either unlabeled NRE or RBP-Jk oligonucleotide, but was not antagonized by a 300 fold molar excess of mutated NRE oligonucleotide (figure 21 a, b). The inability of unlabeled NRE oligonucleotide to

completely eliminate complex "C-prime" formation suggested that complex "C-prime" had a higher affinity for the RBP-Jk probe than the NRE probe.

Pre-incubation of E13.5 nuclear extract with anti-RBP-Jk antibody led to a supershift of complex "C" and complex "C-prime" (figure 21 c). In comparison to control serum, anti-RBP-Jk serum selectively eliminated complex "C" formation and a higher intensity band was observed running slower than complex "C". This suggested that complex "C" contained RBP-Jk or an immunologically related factor that could also bind the RBP-Jk probe. Similarly, in comparison to control serum, anti-RBP-Jk serum appeared to cause a shift in complex "C-prime" (figure 21 c). This suggested that complexes "C" and "C-prime" both contained RBP-Jk protein or an immunologically related factor that could bind both sequences.

To confirm that complex "C" contained RBP-Jk, anti-RBP-Jk antibody was used in a supershift assay with E13.5 nuclear extract and IVT-RBP-Jk run side by side. *In vitro* translation reactions were charged with RBP-Jk cDNA. In one reaction, ^{35}S-labeled methionine was used to label the protein product, and a portion of the reaction was run on an SDS-containing denaturing polyacrylamide gel. The *in vitro* translation reaction produced one major product of approximately 52kD (figure 22 a, arrowhead), the molecular weight of IVT-RBP-Jk (Hsieh et al 1996) and of endogenous RBP-Jk in mammalian tissue extracts (Shirakata et al 1996). Unlabeled IVT-RBP-Jk synthesized in a parallel reaction formed complex "i" with the NRE probe (figure 22 b, c). Complex "i" formation was antagonized by incubation with a 100 fold excess of unlabeled RBP-Jk oligonucleotide, but was not antagonized by a 300 fold molar excess of unlabeled mutated NRE oligonucleotide (figure 22 b).

Complex "i", formed between the NRE probe and IVT-RBP-Jk, co-migrated with complex "C", formed between the NRE probe and E13.5 nuclear extract. Both complexes were shifted to a similar position, and gave a more intense band, in response to pre-incubation with the anti-RBP-Jk antibody (figure 22 c). These results suggested that complex "C" was related to complex "i" and that complex "C" likely contained RBP-Jk.

(v) *In Vitro* Translated NRSE-binding Protein "REST" does not Bind the NRE

The 10-nucleotide NRE has been found in a number of neuronal genes, occurring either alone or at the core of the previously identified 24–nucleotide neuronal restriction silencing element (NRSE) (figure 1). An NRSE binding factor has been identified and is hypothesized to be responsible for the function of the NRSE. To see whether this factor, named the "RE1 silencing transcription factor" or "REST", could interact with the NRE in addition to the NRSE, IVT-REST was used in EMSA's with the NRE probe.

An *in vitro* translation reaction charged with REST cDNA and ^{35}S-labeled methionine produced two major protein products. The larger of these ran at a molecular weight of 116 kD (figure 23), the molecular weight of recombinant and endogenous REST protein (Tapia-Ramirez et al 1997). The identity of the smaller product was not determined, but may have been a major REST breakdown product. *In vitro* translated REST bound to a probe comprising the NRSE of the *type-II sodium channel* gene (figure 23), as previously demonstrated (Chong et al 1995). The REST cDNA-charged reaction gave a unique complex with the NRSE probe (figure 23 b, arrow). This complex was antagonized by unlabeled NRSE oligonucleotide (figure 23 b, RE1) but was not antagonized by either unlabeled NRE or RBP-Jk oligonucleotide (figure 23 b). In contrast, IVT-REST did not produce a unique complex with the NRE probe (figure 23 b). The complexes that formed were also observed in the luciferase-charged reaction, and were not antagonized by excess unlabeled NRSE oligonucleotide. These results suggest that IVT-REST did not bind the NRE, and that the NRE itself at the core of the NRSE is not sufficient for the REST-NRSE interaction.

Figure 1: Alignment of Neuronal Gene Sequences Reveals Common "NRE"

Several mammalian neuronal genes possess the 10-nucleotide "NRE" sequence found in the *Tα1* gene. These sequences occur alone or within the larger 24-nucleotide NRSE (Schoenherr et al 1996). In addition the NRE is conserved in the goldfish *α-1* α-tubulin gene (Hieber et al 1998). Within the NRE of *Tα1* is a sequence resembling the consensus binding sequence of RBP-Jk/Su(H), which is found in the natural target genes *m8* (Drosophila, *E(spl)* complex gene) and *Hes-1* (mammalian *E(spl)* homolog) (Jarriault et al 1995).

Figure 1

Alignment of Neuronal Gene Sequences Reveals Common "NRE"

NRE:	C A C C T G G G A G
rat Tα1:	C A C C T G G G A G
goldfish α-1:	T G C C T G G G G T
SCG-10:	C A C C A C G G A G
Sodium channel type II:	A A C C A C G G A G
GAP-43:	T G C C G G A G
peripherin:	C T C C T G G G C G
HES-1:	T G G G A A
m8 (E(spl)):	C A C_TG T G G G A A
synapsin:	C A C C A G G G A C
L1:	C A C C A G G G A C
BDNF:	C A C C T T G G A C
nAChRβ2:	C A C C A C G G A C
NMDA-R1:	C A C C T C G G A C
synaptophysin:	C A C C G T G G A C
synaptotagmin:	C A C C T C G G A C
calbindin:	C A C C G C G G A C
nAChRα7:	C G C C G C G G C C
HES-3:	C A C C A C G G A C
AMPA-R:	C A C C A C G G A C
glycine-R:	C A C C T C A G A C
VGF:	C A C G C T G G A C
proenkephalin:	C A C A C C G G A C
RBP-Jk consensus:	C G T G G G A A

Figure 2: Construction of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Transgenes

Construction of the *Tα1-nlacZ* transgene has been described previously (Gloster et al 1994), and is shown schematically. A fragment of the rat *Tα1* α-tubulin gene containing a 1028-nucleotide segment immediately upstream of the transcription start site (promoter) and the contiguous 99-nucleotides of 5' untranslated sequence was fused to a modified E.coli *lacZ* gene. The *lacZ* gene had an N-terminal nuclear localization signal sequence (NLS) from the SV40 *large T antigen* (Kalderon et al 1984) placed in frame with the β-galactosidase coding sequence. In addition, a segment of the mouse *protoamine 1* gene, from +95 to +625 (Peschon et al 1987), was attached to the 3' end of the *lacZ* gene providing an intron and a poly-A tail.

The *ΔNRE-nlacZ* transgene was identitical to the *Tα1-nlacZ* transgene except for a 66-nucleotide segment of the *Tα1* promoter sequence, from –674 to –609 inclusive, which was absent in *ΔNRE-nlacZ*. The *ΔFRE-nlacZ* transgene was identitical to the *Tα1-nlacZ* transgene except for a 184-nucleotide segment of the *Tα1* promoter sequence, from –674 to –491 inclusive, which was absent in *ΔFRE-nlacZ*.

Figure 2

Construction of Tα1-nlacZ, ΔNRE-nlacZ, and ΔFRE-nlacZ Transgenes

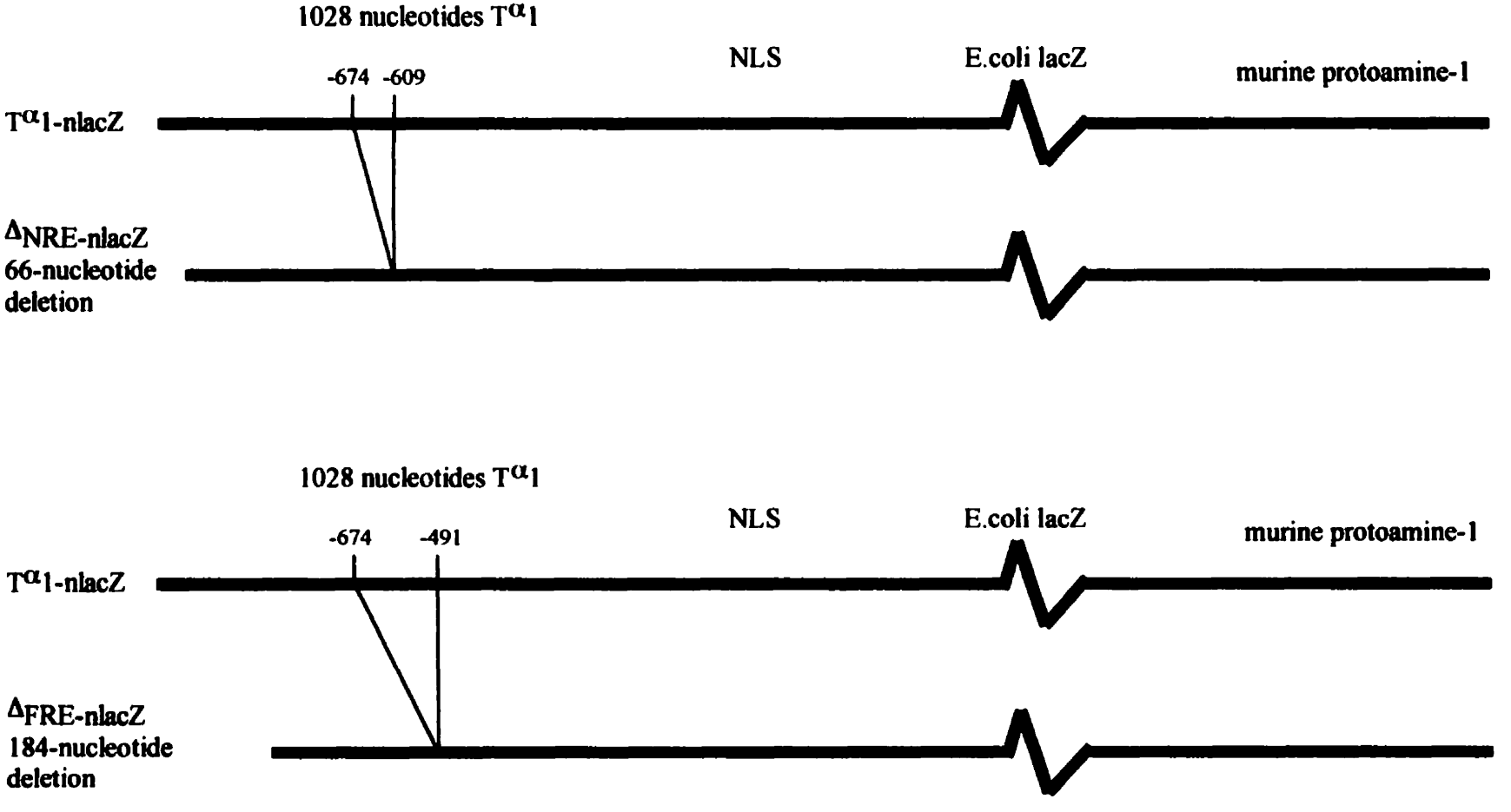


Figure 3: Comparison of *Tα1-nlacZ* and *ΔNRE-nlacZ* Mice at E9.5

Embryos obtained from pregnant females at E9.5 were stained overnight in Xgal solution to visualize β-gal activity, and subsequently fixed in 4% paraformaldehyde. The morning a plug was observed was taken to be embryonic day 0.5.

Figures a, d, *ΔNRE-nlacZ* line 23; figure b, *Tα1-nlacZ* line K6; figure c, *ΔNRE-nlacZ* line 9. Precocious β-gal activity was observed in cells within the trigeminal (a, d "V") and geniculate (a, d, "VII") cranial ganglia. In addition, more β-gal activity was observed in the developing spinal cord (a-d arrowhead) of *ΔNRE-nlacZ* line 23 (a, d) than in *Tα1-nlacZ* line K6 (b) and *ΔNRE-nlacZ* line 9 (c).

Figure 3

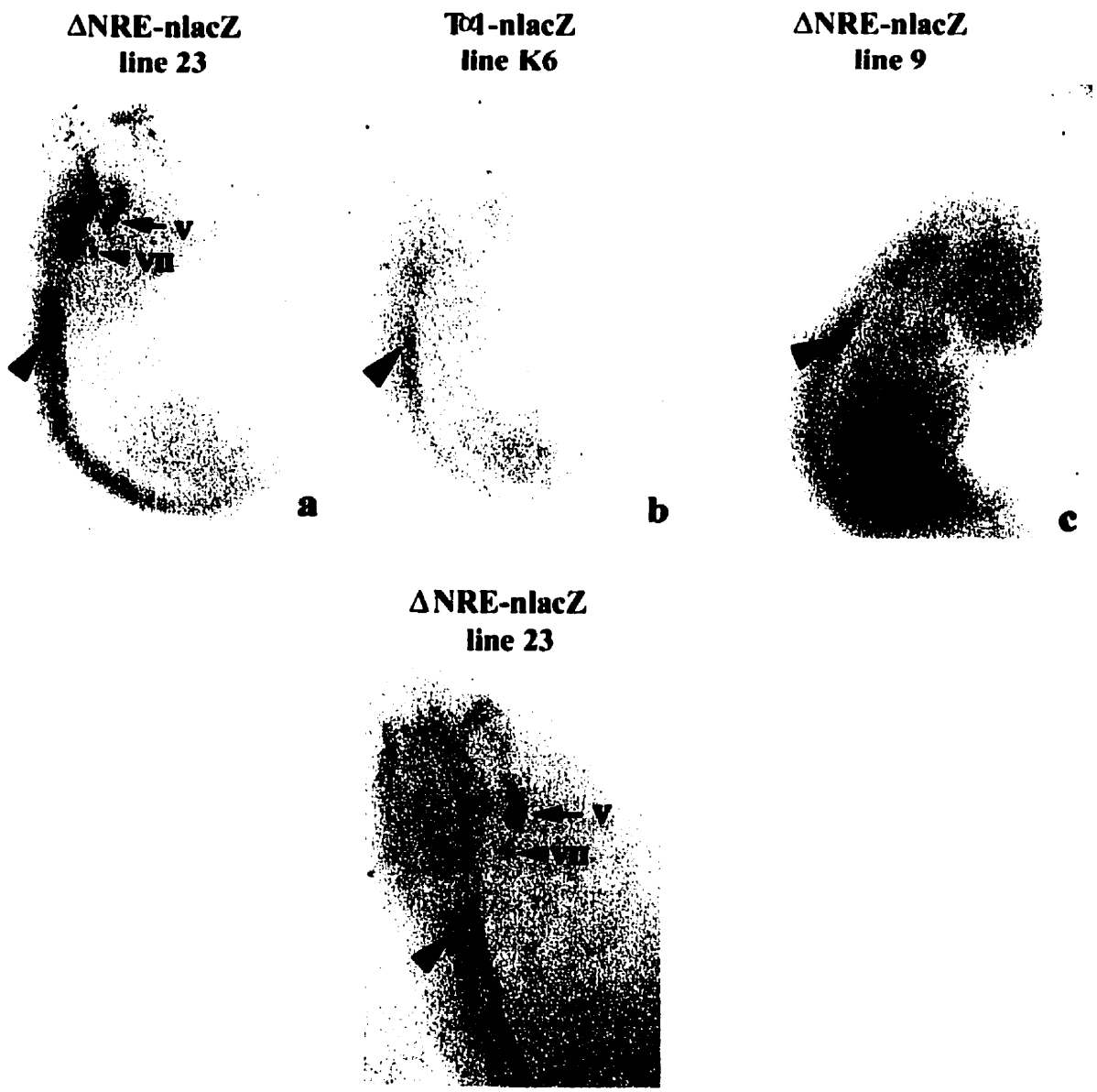

ΔNRE-nlacZ
line 23
V
VII
a
Tα1-nlacZ
line K6
b
ΔNRE-nlacZ
line 9
c
ΔNRE-nlacZ
line 23
V
VII

Figure 4: Comparison of *Tα1-nlacZ* and *ΔNRE-nlacZ* Mice at E10.0-10.5

Embryos obtained from pregnant females at E10.0 or E10.5 were stained overnight in Xgal solution to visualize β-gal activity, and fixed in 4% paraformaldehyde. The morning a plug was observed was taken to be embryonic day 0.5.

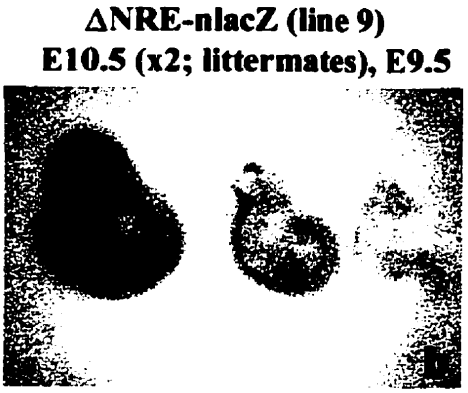
In figure 4a, β-gal activity is observed in the presumptive DRG's (4a, left, arrowheads) of *ΔNRE-nlacZ* line 23 at E10.0, but not in the presumptive DRG's of *Tα1-nlacZ* line K6 at this same timepoint (4a, centre). Beta-galactosidase activity is however observed in the DRG's of *Tα1-nlacZ* line K6 at E10.5 (4a, right, arrowheads). The first appearance of β-gal activity at E10.5 in *Tα1-nlacZ* mice is in agreement with previous thin section analysis (Gloster et al 1999).

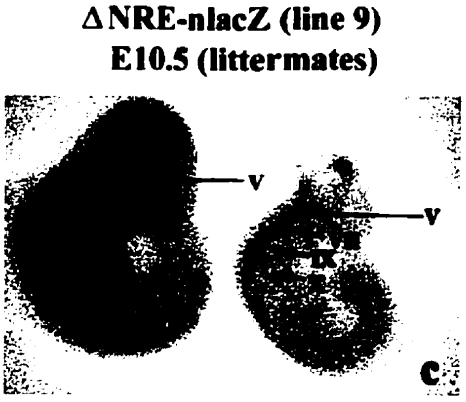
Figure 4b shows two *ΔNRE-nlacZ* line 9 littermates obtained from a pregnant female at E9.5 (4b, left and centre), demonstrating development within a litter is not perfectly synchronous, and that the extent of β-gal activity changes rapidly during development. Figure 4b, right, shows a *ΔNRE-nlacZ* line 9 E9.5 embryo for comparison, revealing a much lower level of β-gal activity and the extent to which embryos increase in size over one day. There is an increase in β-gal activity in the trigeminal (4c, "V"), geniculate (4c, "VII"), petrosal (4c, "IX") and nodose (4c, "X") ganglia from E10.0-10.5 in *ΔNRE-nlacZ* line 9. Figure 4c shows two littermates from an E10.5-staged pregnant female at slightly different developmental stages.

Figure 4d gives a dorsal view of these two *ΔNRE-nlacZ* line9 littermates, revealing that β-gal activity first appears in the DRG's of whole embryos (4d, short arrows) between E10.0 and E10.5. This timing is also observed in *Tα1-nlacZ* line K6 (4a, centre and right), but β-gal activity appears 12 hours earlier in the presumptive DRG's of *ΔNRE-nlacZ* line 23 (4a left).

Figure 4


ΔNRE-nlacZ
line 23, E10.0
Tbx1-nlacZ
line K6, E10.0
Tbx1-nlacZ
line K6, E10.5
a


ΔNRE-nlacZ (line 9)
E10.5 (x2; littermates), E9.5


ΔNRE-nlacZ (line 9)
E10.5 (littermates)
V
V
IX
c

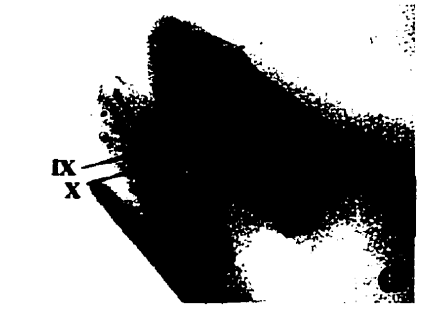

ΔNRE-nlacZ (line 9)
E10.5 (littermates)
IX
X

Figure 5: Comparison of *Tα1-nlacZ* and *ΔNRE-nlacZ* Mice at E10.0-10.5 : The Cranial Ganglia

Embryos obtained from pregnant females at E10.0 or E10.5 were stained overnight in Xgal solution to visualize β-gal activity, and fixed in 4% paraformaldehyde. The morning a plug was observed was taken to be embryonic day 0.5.

Beta-galactosidase activity was not observed in the petrosal and nodose ganglia of *Tα1-nlacZ* whole embryos at E10.0 (5a, arrows) but was faintly visible by E10.5 (5b, "IX" and "X" respectively). In contrast, β-gal activity was observed in the petrosal and nodose ganglia at E10.0 in *ΔNRE-nlacZ* line 23 (5 e, "IX", "X" respectively). Beta-galactosidase activity was detected in the petrosal and nodose ganglia in *ΔNRE-nlacZ* line 9 at E10.5 (5c, d, "IX" , "X" respectively), and appeared to commence between E10.0-10.5 (4c, d). ("V" trigeminal ganglion, "VII" geniculate ganglion)

Figure 5

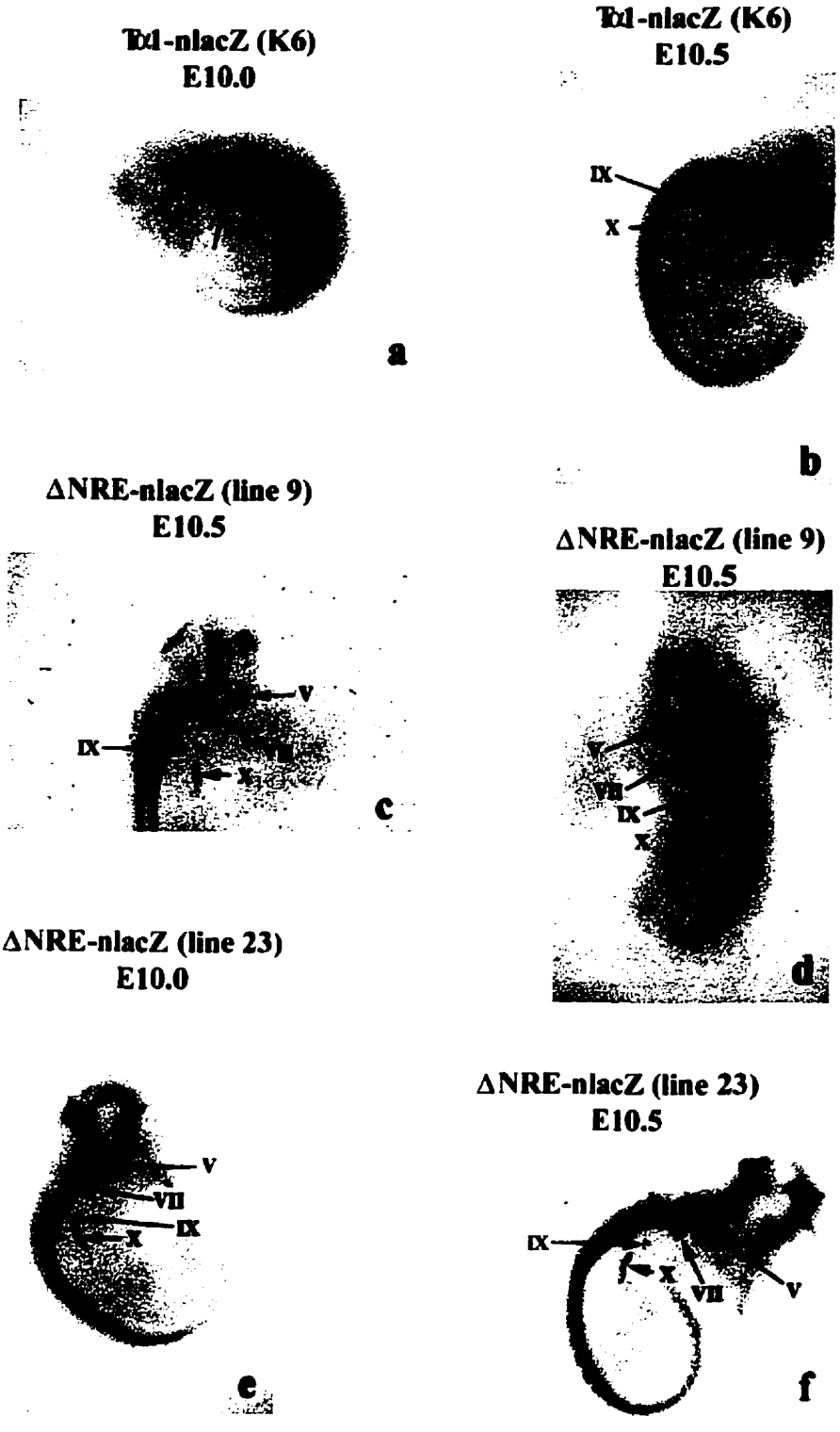

Tx1-nlacZ (K6)
E10.0
a
Tx1-nlacZ (K6)
E10.5
IX
X
b
ΔNRE-nlacZ (line 9)
E10.5
V
IX
X
c
ΔNRE-nlacZ (line 9)
E10.5
V
VII
IX
X
d
ΔNRE-nlacZ (line 23)
E10.0
V
VII
X
IX
e
ΔNRE-nlacZ (line 23)
E10.5
IX
X
VII
V
f

Figure 6: Comparison of Cortical Precursor Cells Cultured from *Tα1-nlacZ* and *ΔNRE-nlacZ* Mice

The preparation of cortical precursor cells from mouse embryos was based on the method described by Ghosh et al. (1995) for rat cultures. The dorsal aspect of the telencephalic vesicle was collected from E10.5 *Tα1-nlacZ* line K6 or E10.0 *ΔNRE-nlacZ* line 23 mouse embryos and triturated with a fire polished Pasteur pipette. Small clusters of cells were plated into chamber slides and cultures were maintained at 37°C in a 5% CO_2 incubator for one day.

After one day *in vitro*, cortical precursor cells grown on chamber slides were fixed and immunostained with anti-β-galactosidase (rabbit polyclonal IgG; 5 Prime 3 Prime; Boulder, CO; 1:500) (6b, e), and mouse monoclonal anti β-III tubulin (TUJ1; Dr. A. Frankfurter; 1:300) (6 a, d). Cells were subsequently incubated for 1 hour with buffer containing both CY3-conjugated goat-anti-rabbit (1:200) (Jackson) (6 b, e) and CY2-conjugated goat-anti-mouse (IgG) (1:200) (Jackson) (6 a, d) secondary antibodies. Beta-III tubulin positive cells were observed in E10.5 cultures from *Tα1-nlacZ* after one day *in vitro* (6a), as they were in E10.0 cultures from *ΔNRE-nlacZ* line 23 after one day *in vitro* (6d). The β-III positive cells from E10.0 embryos appeared rounded and less developed morphologically than those derived from E10.5 embryos (compare 6d to a). *Beta-galactosidase* expression was detected in the same fields in both cultures and β-gal appeared to be localized to the nucleus by comparison with Hoechst staining (6 b, c, e, f). In the *Tα1-nlacZ* culture, β-gal appeared to be strictly colocalized with β-III tubulin, but not all β-III positive cells expressed *β-gal*. In contrast, in *ΔNRE-nlacZ* cultures, β-gal positive cells that did not express *β-III tubulin* were observed (6 d, e, f arrowhead). Though not quantitated, it also appeared that fewer of the *ΔNRE-nlacZ* cells expressed *β-gal* compared to *Tα1-nlacZ*.
scale bars a-f, 50μm

Figure 6

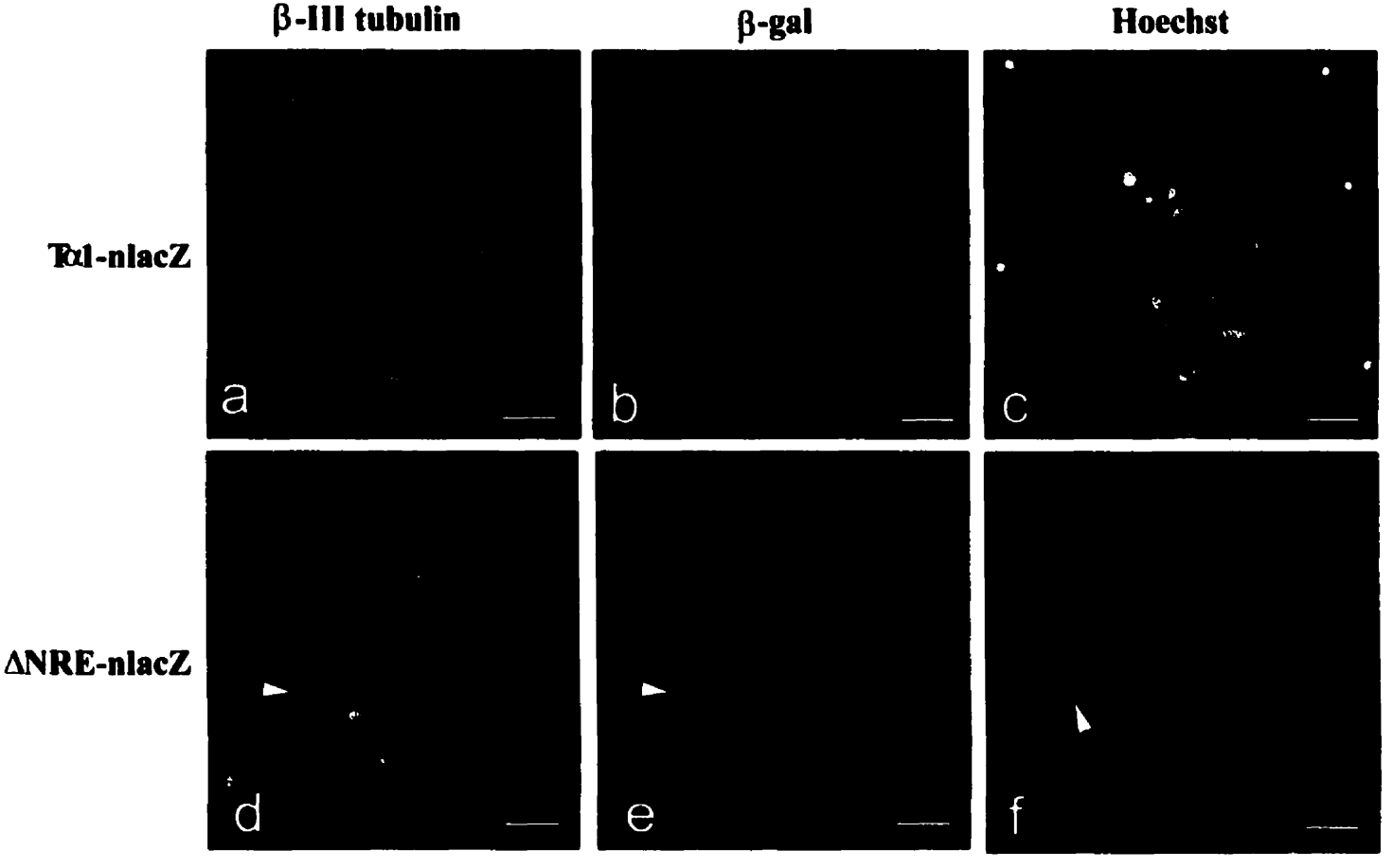


Figure 7: *ΔNRE-nlacZ* and *ΔFRE-nlacZ* are Expressed in the Ependymal Layer of the Adult Brain Where *Tα1-nlacZ* Expression is Excluded

Figure 7A: Thin coronal slices of adult brain were stained in X-gal solution to visualize β-gal activity. Slices were frozen, sectioned on a cryostat at 14μm, and counterstained with eosin. Slices were taken from the rostral forebrain (7A a-d, i, j) and more medially passing through the hippocampus (7A e-h, k, l). Beta-galactosidase activity was detected in cells of the ependymal layer surrounding the lateral ventricles in the rostral forebrain in *ΔNRE-nlacZ* line 9 (7A i, j, arrows). Activity was also observed in the ependymal layer surrounding the lateral ventricles at more caudal positions in *ΔNRE-nlacZ* line 9 mice (7A e-h, arrows). The activity pattern in *ΔNRE-nlacZ* mice differed from that of *Tα1-nlacZ* mice. Beta-galactosidase activity was not detected in the ependymal layer surrounding the lateral ventricles in *Tα1-nlacZ* mice (7A a-d). The β-gal activity observed in the ependymal layer in *ΔNRE-nlacZ* mice was not restricted to the region surrounding the lateral ventricles, but was also observed in the region surrounding the third ventricle (7A k, l).
(LV, lateral ventricle; 3V, third ventricle; cg, cingulum)
scale bars: a, b, 400μm; c, d, e, g, 200μm; f, h-l, 100μm

Figure 7A

Tα1-nlacZ
line K6
ΔNRE-nlacZ
line 9
ΔNRE-nlacZ
line 9
LV
LV
LV
LV
LV
LV
3V
3V

Figure 7B: Tα1-nlacZ tissue 7B b, d, f, g. ΔNRE-nlacZ line 23 adult brain tissue 7B a, c, e, h. Coronal brain slices (7B a, b; high magnification c, d) from which thin sections were taken (7B e-g) are shown, revealing the axial level of the section. Beta-galactosidase activity was observed around the lateral ventricles in the rostral forebrain in ΔNRE-nlacZ line 23 mice (7B a, c, arrowheads) but not in Tα1-nlacZ mice (7B b, d, arrowheads). In thin sections from these slices, β-gal activity was detected in the ependymal layer surrounding the lateral ventricle (LV) in ΔNRE-nlacZ line 23 mice (7B e, arrowhead), but not in Tα1-nlacZ mice (7B f, g). In addition, β-gal activity was detected in the ependymal layer surrounding the third ventricle (3V) in ΔNRE-nlacZ mice in more caudal brain sections (7B m, arrowhead).
scale bars: e-g, 200μm; h, 100μm

Figure 7B

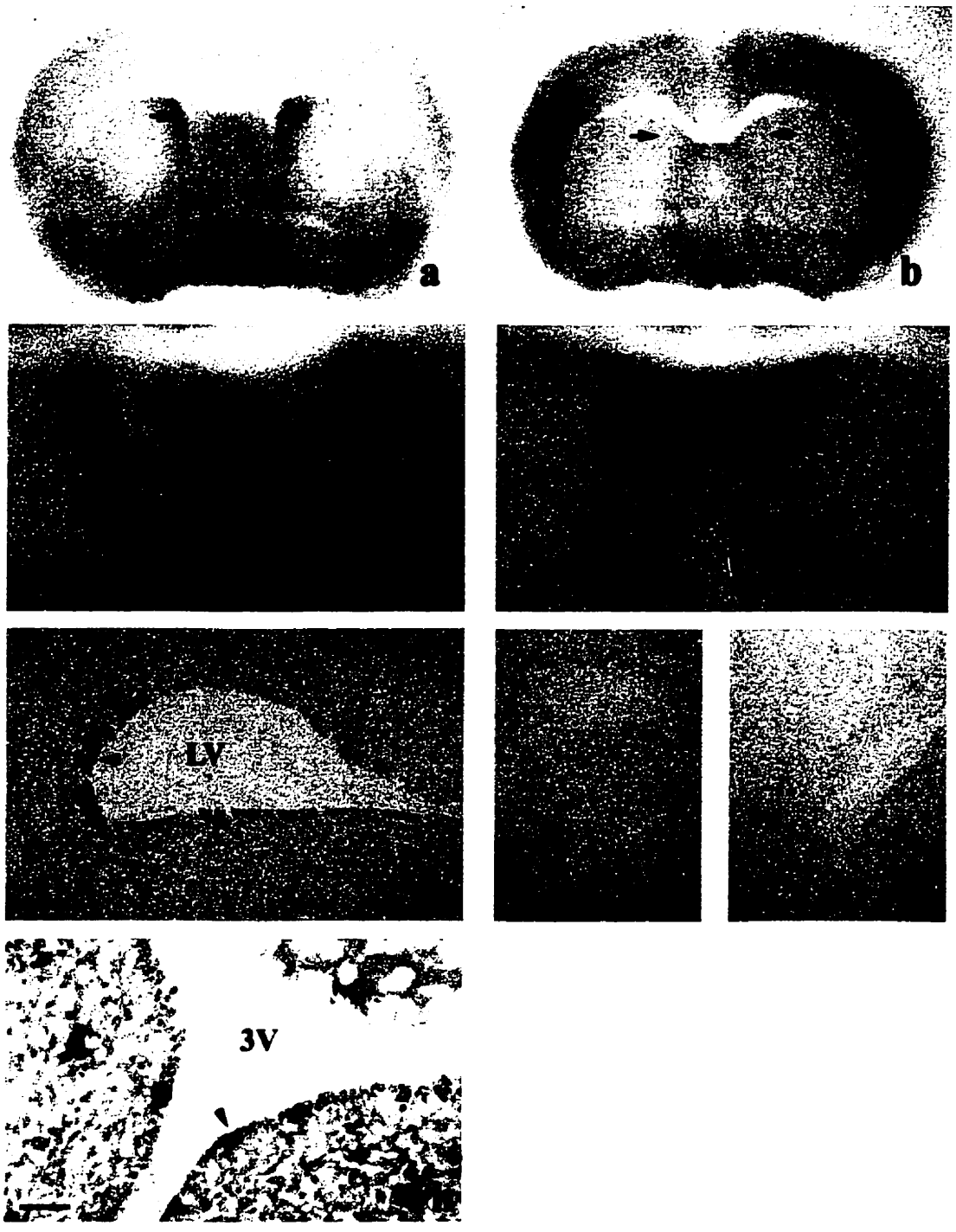


Figure 8: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Mice at E13.5 Reveals Differences in the Forebrain

Embryos obtained from pregnant females at E13.5 were drop fixed for 2 minutes in paraformaldehyde, stained overnight in Xgal solution to visualize β-gal activity, and post-fixed in paraformaldehyde. The morning a plug was observed was taken to be embryonic day 0.5.

Abundant β-gal activity was detected in the dorsal and lateral neocortex of *Tα1-nlacZ* mice at E13.5 (8a, black arrowhead and white arrowhead respectively). In contrast, little activity was detected in the neocortex of ΔNRE-nlacZ line 9 (8b) and line 23 (8c) (arrowheads). *ΔFRE-nlacZ* lines 17 and 1 (8d, e respectively) exhibited less neocortical β-gal activity than the *ΔNRE-nlacZ* mice at E13.5 (arrowheads). In addition, *ΔFRE-nlacZ* line 17 lacked expression in the region surrounding the isthmus.

Figure 8

Td-nlacZ (line K6)
ΔNRE-nlacZ (line 9)
ΔNRE-nlacZ (line 23)
a
b
c
ΔFRE-nlacZ (line 17)
ΔFRE-nlacZ (line 1)
d
e

Figure 9: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Expression in the Early Postnatal Brain

Coronal brain slices from newborn mice were stained in X-gal solution to visualize β-gal activity. In *Tα1-nlacZ* line K6, β-gal activity was detected in the neocortex (nctx), hippocampus (hi), piriform and entorhinal cortex (pi/en) at postantal day 1 and postnatal day 7 (9a, b respectively). A much lower level of β-gal activity was observed in the neocortex (nctx) of *ΔNRE-nlacZ* line 23 and line 9 mice (9 c, d respectively). Activity was still observed in the piriform and entorhinal cortex (pi/en) of these mice, as well as the hippocampus (hi). The low level of activity in the neocortex combined with the high level of activity in the piriform and entorhinal cortex created a sharp border between the neocortex and paleocortex in *ΔNRE-nlacZ* mice (9c, d arrowhead).

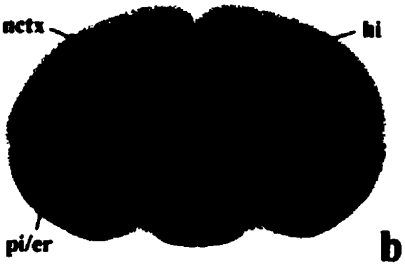
Beta-galactosidase activity was nearly absent in the neocortex in *ΔFRE-nlacZ* mice. Both line 17 and line 1 showed little activity in the neocortex (9e, f, g "nctx"), and line 1 also showed very little expression in the hippocampus (9e, "hi"). Activity was detected in the cerebellum (9g, "cer") and superior colliculus (9g, "sc") in line 17, but was not detected in the cerebellum in line 1 (8f, "cer").

Figure 9

Tα1-nlacZ (line K6)
P1

a

Tα1-nlacZ (line K6)
P7



b

ΔNRE-nlacZ (line 9)
P2

c

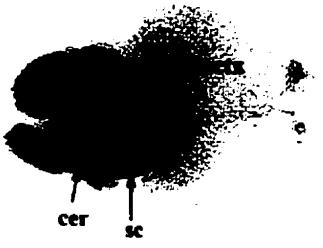
ΔNRE-nlacZ (line 23)
P4

d

ΔFRE-nlacZ (line 1)
P2

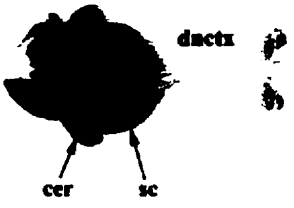
e

ΔFRE-nlacZ (line 1)
P2



f

ΔFRE-nlacZ (line 17)
P2



g

Figure 10: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Expression in the Adult Brain

Coronal slices of adult brain were stained overnight in X-gal solution to visualize β-gal activity. In *Tα1-nlacZ* mice, activity was detected throughout the pallium including the neocortex, hippocampus, entorhinal cortex, piriform cortex, and amygdala (10 a, b). Activity was also detected in the diencephalon, including the hypothalamus, and to a lesser extent the ventrolateral thalamus. A low level of activity was detected in the globus pallidus (10b). This pattern of activity conformed to the findings of a previous study (Bamji and Miller 1996).

In sharp contrast, β-gal activity was very low in the neocortex of *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice (10c-j). The loss of activity appeared to be greatest in *ΔFRE-nlacZ* mice. In *ΔFRE-nlacZ* line 17, few scattered cells throughout the neocortex displayed β-gal activity. One peculiar exception to this absence of neocortical activity was a region of medial neocortex corresponding to the retrosplenial granular cortex (10 c, d). Activity was apparent in the hippocampus in line 17. In addition, activity was detected in the amygdala, and piriform cortex (10 c, d). A low level of activity was detected in the ventrolateral thalamus (10d), and very little activity was observed in the globus pallidus and hypothalamus (10d) in contrast to *Tα1-nlacZ* line K6 (10b) and *ΔFRE-nlacZ* line 1. In line 1, a higher level of activity was observed in the ventrolateral thalamus as well as the hypothalamus, amygdala, and piriform cortex (10 e). Activity was also evident in the cingulum and superioir layers of the neocortex, but to a much lesser extent in the intervening layers of the neocortex. Notably high levels of activity were found in the dorsal endopiriform nucleus (10 e). Activity was hardly detected in the hippocampus (10 e, i).

Beta-galactosidase activity was detected in the hippocampus in *ΔNRE-nlacZ* lines 23 and 9, though it was largely restricted to the dentate gyrus in line 9 (10 f, g, h, j). In both

lines activity was observed in the ventrolateral thalamus, amygdala, and hypothalamus. Activity in the piriform cortex, globus pallidus, neocortex, dorsal endopiriform nucleus, caudate and putamen was greater in line 23 than in line 9 (10 f, g, h,j). Activity in the caudate and putamen was greater in line 23 than in *Tα1-nlacZ* line K6.
(hi, hippocampus; er, entorhinal cortex; pn, pontine nuclei; nctx, neocortex; am, amygdala; gp, globus pallidus; vp, ventrolateral thalamus; rsg, retrosplenial granular cortex; den, dorsal endopiriform nucleus)

Figure 10

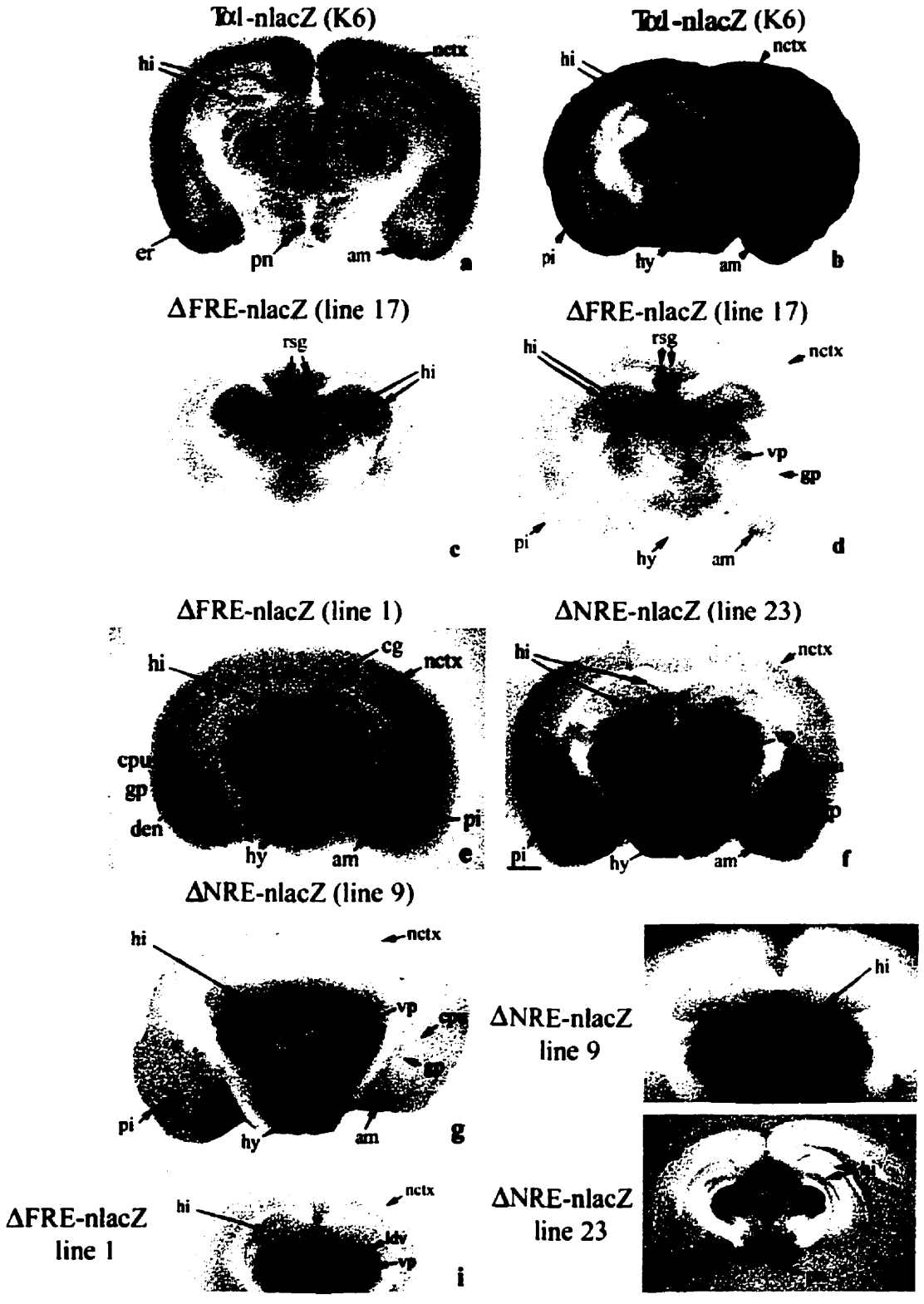

Tcl-nlacZ (K6)
hi
nctx
er
pn
am
a
Tcl-nlacZ (K6)
hi
nctx
pi
hy
am
b
ΔFRE-nlacZ (line 17)
rsg
hi
c
ΔFRE-nlacZ (line 17)
hi
rsg
nctx
vp
gp
pi
hy
am
d
ΔFRE-nlacZ (line 1)
hi
cg
nctx
cpu
gp
den
pi
hy
am
e
ΔNRE-nlacZ (line 23)
hi
nctx
pi
hy
am
f
ΔNRE-nlacZ (line 9)
hi
nctx
vp
pi
hy
am
g
ΔNRE-nlacZ
line 9
hi
ΔFRE-nlacZ
line 1
hi
nctx
ldv
vp
i
ΔNRE-nlacZ
line 23

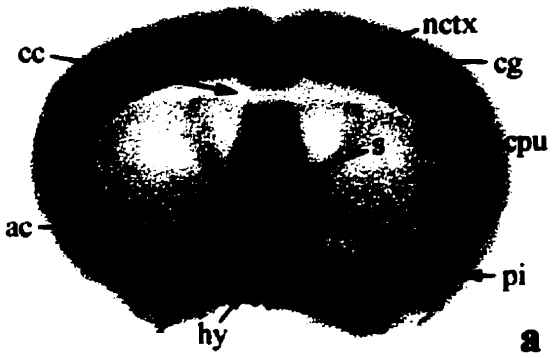
Figure 11: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ* and *ΔFRE-nlacZ* Expression in the Rostral Brain

Coronal slices thorugh the rostral forebrain were stained in X-gal solution overnight to visualize β-gal activity. In *Tα1-nlacZ* mice, activity was evident throughout the pallium including the neocortex, the piriform cortex, septum and the cingulum. In the diencephalon, activity was apparent in the hypothalamus. This activity pattern conformed to results from a previous study (Bamji and Miller 1996).

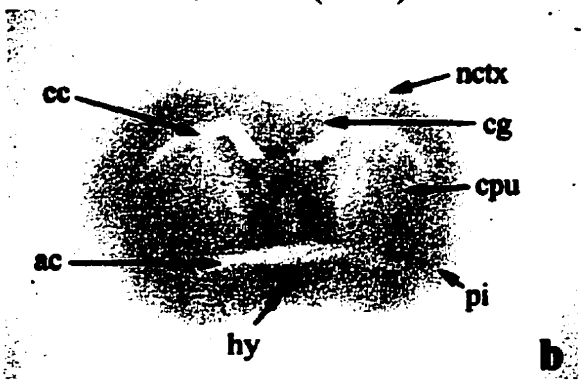
Much lower levels of activity were observed in the rostral forebrain in *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice. *ΔFRE-nlacZ* mice showed very little activity in the pallium including the neocortex, piriform cortex, septum, amygdala, and cingulum (11b, c). In addition, low activity levels were observed in the hypothalamus, caudate and putamen (11b, c). In *ΔNRE-nlacZ* lines 23 and 9, a low level of activity was observed in the neocortex and cingulum. Line 23 exhibited a higher level of activity in the septum, piriform cortex, Islands of Calleja, caudate and putamen than line 9. (cg, cingulum; nctx, neocortex; s, septum; ac, anterior commissure; pi, piriform cortex; am, amygdala; hy, hypothalamus; lv, lateral ventricles; cc, corpus callosum; cpu, caudate and putamen; Icj, Islands of Calleja)

Figure 11

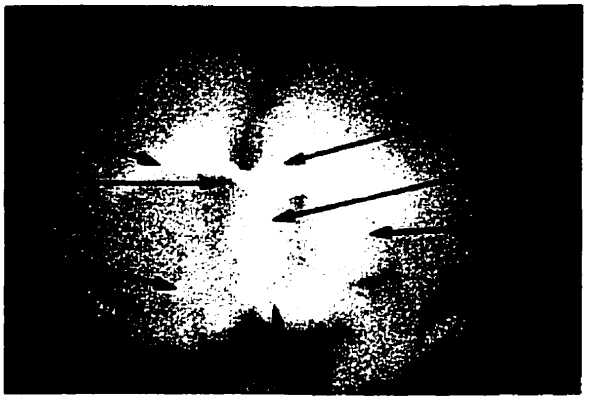
Tα1-nlacZ (K6)



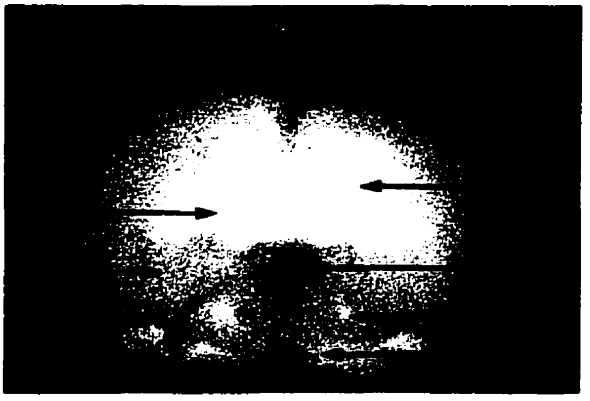
ΔFRE-nlacZ (line 1)



ΔFRE-nlacZ (line 17)



ΔNRE-nlacZ (line 9)



ΔNRE-nlacZ (line 23)

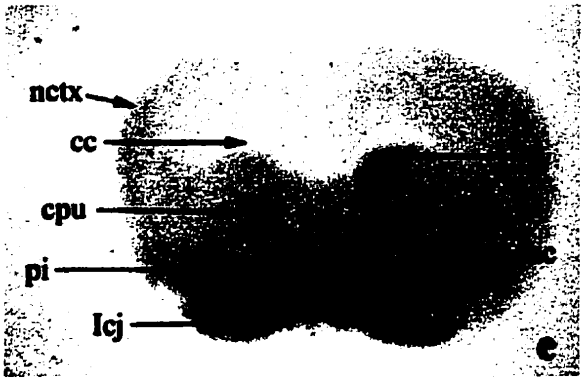


Figure 12: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Expression in the Midbrain

Coronal slices thorugh the midbrain were stained in X-gal solution overnight to visualize β-gal activity. Figure 12 displays dorsal views of X-gal stained midbrain slices. *Tα1-nlacZ* line K6 mice exhibited robust activity in the superior colliculus, and a lower level of activity in the cerebellum (12a). *ΔFRE-nlacZ* line 17 and *ΔNRE-nlacZ* line 9 displayed different patterns of activity. *ΔFRE-nlacZ* line 17 mice exhibited a pattern of activity reciprocal to that of *Tα1-nlacZ* and *ΔFRE-nlacZ* line 1, showing very little activity in the superior colliculus, and robust activity in the cerebellum. *ΔNRE-nlacZ* line 9 exhibited very little activity in the neocortex and cerebellum, but robust activity in the superior colliculus (12b).
(dnctx, dorsal neocortex; cer, cerebellum; sc, superior colliculus)

Figure 12

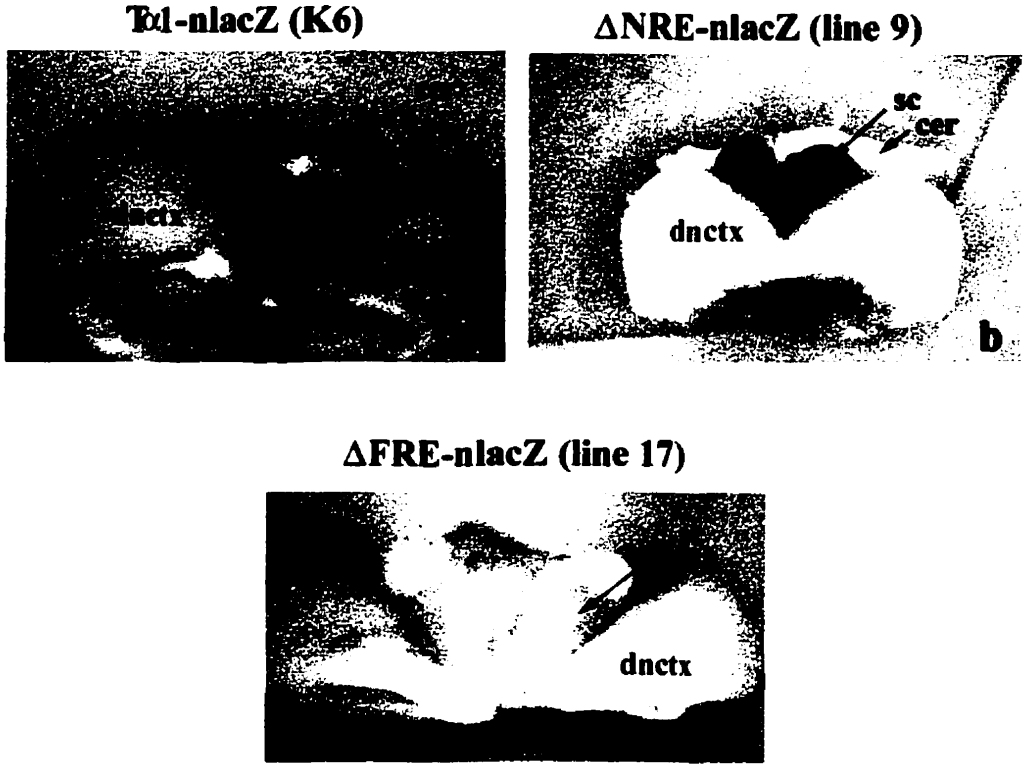

Tα1-nlacZ (K6)
ΔNRE-nlacZ (line 9)
sc
cer
dnctx
b
ΔFRE-nlacZ (line 17)
dnctx

Figure 13: Comparison of *Tα1-nlacZ*, *ΔNRE-nlacZ*, and *ΔFRE-nlacZ* Expression in the Cerebellum

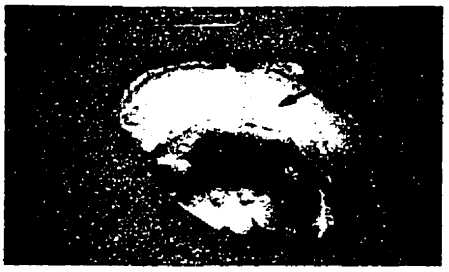
Coronal slices through the adult cerebellum were stained in X-gal solution to visualize β-gal activity. In *Tα1-nlacZ* line K6, activity was observed in Purkinje cells (13a, arrow) and granule cells of the cerebellum (13a), consistent with previous results (Bamji and Miller 1996). A very low level of activity was observed in Purkinje cells and activity was barely detecteable in granule cells in *ΔNRE-nlacZ* line 9 (13 b, arrows) (13b; shown also in figure 12b). In *ΔNRE-nlacZ* line 23, activity was apparent in Purkinje cells (13 c, d arrows) and granule cells. A high level of activity was observed in the granule cells (13 e arrow) and Purkinje cells (13f, arrow) of *ΔFRE-nlacZ* line 17 mice. Figure 13f shows a saggital slice through the centre of the coronal slice of cerebellum. As in *ΔNRE-nlacZ* line 9, very low levels of activity were detected in Purkinje cells in *ΔFRE-nlacZ* line 1, and activity was barely detectable in granule cells (13 g, h). A high level of activity was observed in the pons (p) in *ΔFRE-nlacZ* line 1.

Figure 13

Tα1-nlacZ (K6)

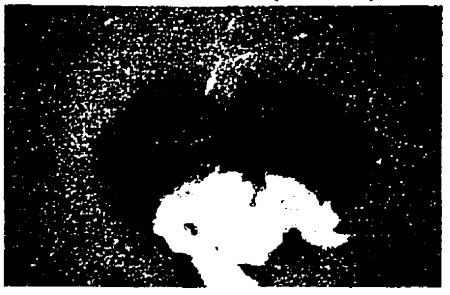
ΔNRE-nlacZ (line 9)

ΔNRE-nlacZ (line 23)



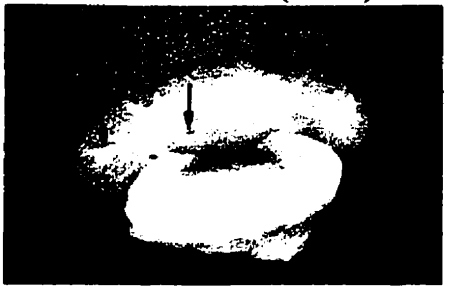
ΔNRE-nlacZ (line 23)

ΔFRE-nlacZ (line 17)



ΔFRE-nlacZ (line 17)

ΔFRE-nlacZ (line 1)



ΔFRE-nlacZ (line 1)

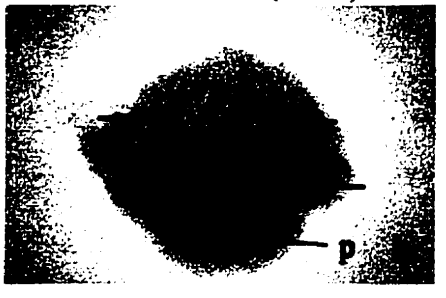
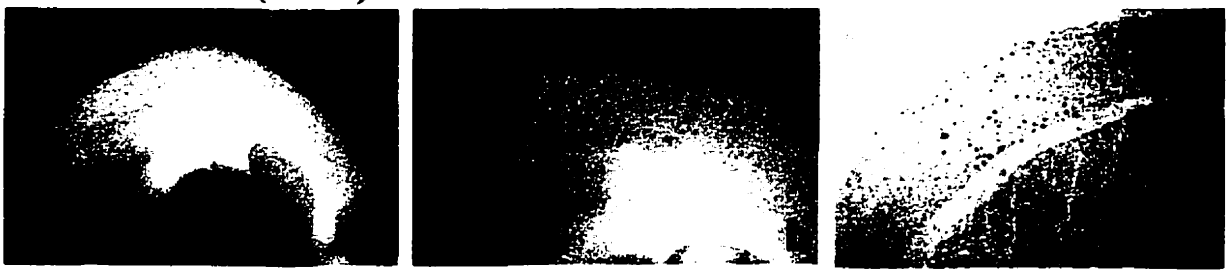


Figure 14: Distribution of Limited β-gal Activity in the Neocortex of *ΔFRE-nlacZ* and *ΔNRE-nlacZ* Mice

Coronal brain slices were stained in X-gal solution to visualize β-gal activity. *In ΔNRE-nlacZ* line 9, activity was apparent throughout the neocortical layers, and did not appear to be enriched in particular layers (14 a-c). Figure 14c shows a thin coronal section (14μm) through the neocortex of *ΔNRE-nlacZ* line 9. Similarly, in *ΔNRE-nlacZ* line 23 cells exhibiting β-gal activity appeared to be dispersed throughout the neocortex (14d, e). Many fewer neocortical cells exhibited β-gal activity in *ΔFRE-nlacZ* line 17, but those that did exhibit β-gal activity were distributed throughout the neocortical layers (14f, g). In contrast, the few neocortical cells that did exhibit β-gal activity in *ΔFRE-nlacZ* line 1 were largely concentrated in the superior layers of the neocortex. There was a much lower density of cells exhibiting β-gal activityin the inferior neocortical layers, between the cingulum (cg) and the superior layers of the neocortex (nctx) (14 h, i).

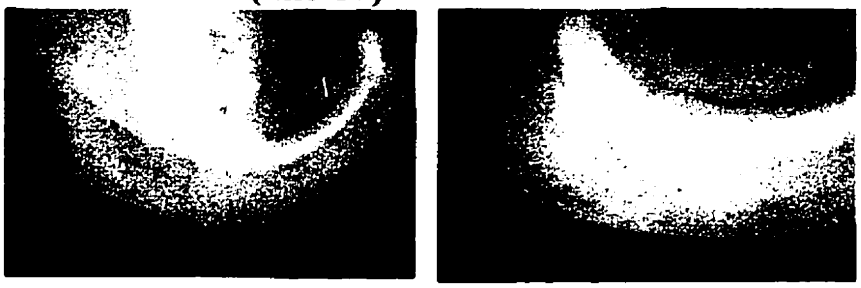
Figure 14

ΔNRE-nlacZ (line 9)



ΔNRE-nlacZ (line 23)

ΔFRE-nlacZ (line 17)



ΔFRE-nlacZ (line 1)

Figure 15: Ectopic β-gal Expression is Detected in the Kidney in *ΔNRE-nlacZ* Line 9

A number of non-neural tissues were analyzed for ectopic β-gal expression in *ΔNRE-nlacZ* and *ΔFRE-nlacZ* mice. Tissues examined included muscle, lung, kidney, skin, liver and heart. In *ΔNRE-nlacZ* line 9, β-gal expression was consistently observed in the kidney (15 b). Ectopic activity was not detected in any of these tissues in *ΔNRE-nlacZ* line 23, nor in *Tα1-nlacZ* and *ΔFRE-nlacZ* mice. Figure 15 a (*Tα1-nlacZ* line K6), b *(ΔNRE-nlacZ* line 9) are cross sections through adult kidneys that have been stained in X-gal solution to visulaize β-gal activity.

Figure 15

Tcl-nlacZ (K6)

ΔNRE-nlacZ (line 9)

Figure 16: Electrophoretic Mobility Shift Assays

Nuclear protein extracts from embryonic tissues and cultures of cortical precursor cells were used in electrophoretic mobility shift assays (EMSA's). *In vitro* translated protein was also used for EMSA's. Double-stranded oligonucleotides were end-labeled with ^{32}P ATP using T4 polynucleotide kinase (T4 PNK). The labeled oligonucleotides (probes) were incubated with nuclear extract or *in vitro* translated protein for 15 minutes and then run on a tris-glycine buffered 5% acrylamide gel. In competition experiments, unlabeled oligonucleotide was pre-incubated with nuclear extract or *in vitro* translated protein for 15 minutes prior to inclusion of the probe. In supershift EMSA's, a rabbit poly IgG anti-RBP-Jk-GST antibody (Dr. J. Coligan, Bethesda, Shirakata et al 1996) was pre-incubated with nuclear extract or *in vitro* translated protein for 15 minutes prior to inclusion of the probe.

In each reaction, approximately 10pg of labeled probe was added to 2μg of nuclear extract or 2-5μl of *in vitro* translated protein. The gel buffer pH was 8.0.

Sequences of probes and competitive oligonucleotides:

NRE:	C T G C C T C T G C C T C C C A G G T G C T G T T G A G G G
Mutated NRE:	C T G C C T C T G C C **G T T A C** G G T G C T G T T G A G G G
RBP-Jk:	G G A A A C A C G C C G T G G G A A A A A A T T T G G C
RE1:	G G G T T T C A G A A C C A C G G A C A G C A C C A G

The NRE probe was identical to the *Tα1* promoter sequence from -647 to –618 inclusive (Gloster et al 1994). The ΔNRE probe was mutated at five positions within the putative RBP-Jk/Su(H) binding site.

Figure 16

Electrophoretic Mobility Shift Assays

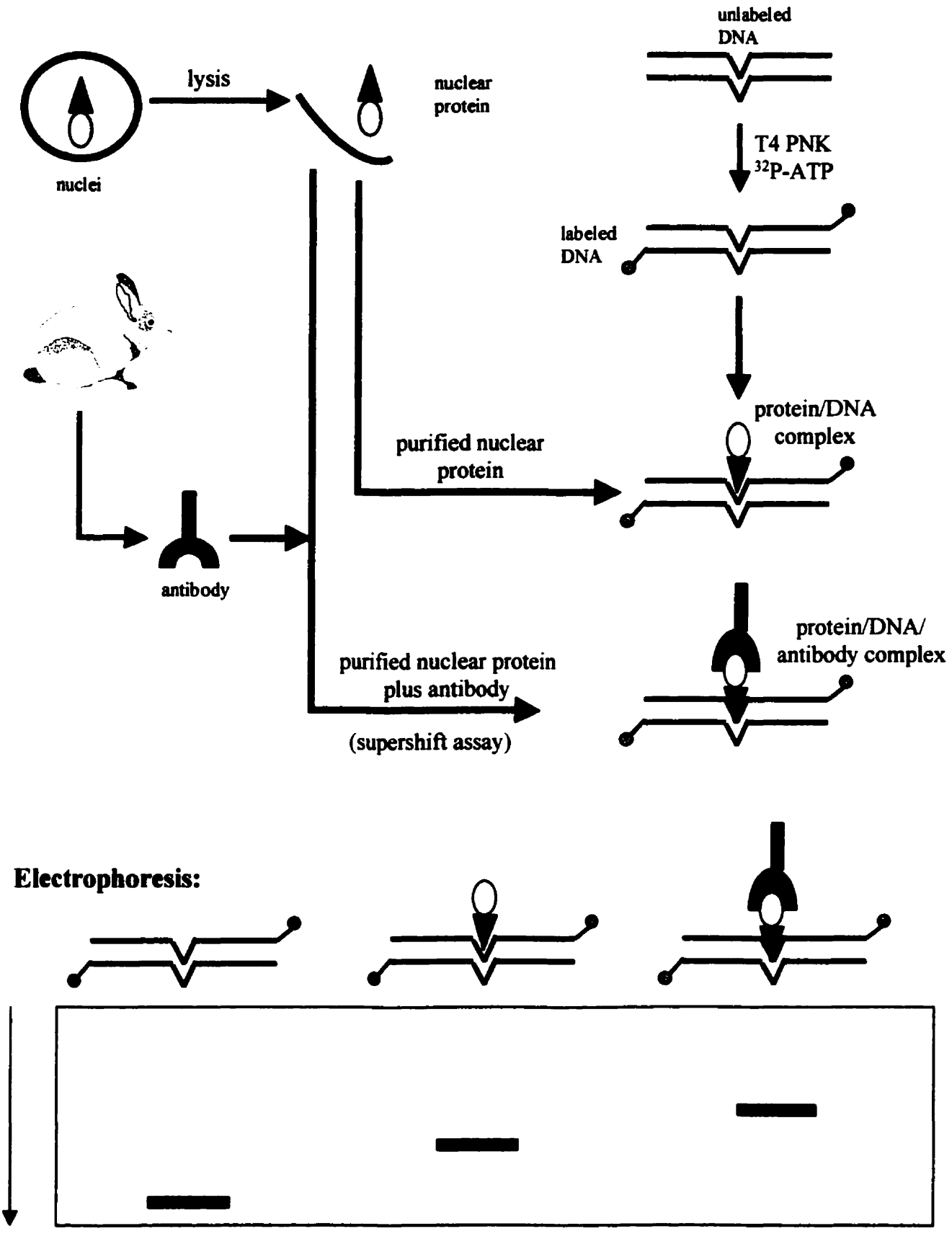


Figure 17: Developmental Distribution and Tissue Distribution of NRE and RBP-Jk Consensus Sequence Binding Complexes

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the RBP-Jk consensus region of the adenovirus *pIX* gene were used as probes for NRE-binding and RBP-Jk-binding complexes in different tissues. Nuclear protein fractions were collected from different tissues, and end-labeled oligonucleotides were incubated with 2μg of nuclear extract. The probe/protein mix was then run on a 5% polyacrylamide gel. Unbound probe was run off of the bottom of the gel.

A number of NRE-binding complexes were observed in the extracts examined. In nuclear extract from E13.5 embryos (17, "E13.5"), newborn brain (17, "P0") and adult brain (17, "adult brain"), three similarly migrating complexes were observed (17, "A", "B", "C"). Similarly migrating complexes were not abundant in liver nuclear extract (17, "liver") suggesting these complexes, though not necessarily identical, might have been enriched in neural tissue. In addition, three similarly migrating complexes were formed with the RBP-Jk oligonucleotide using the same nuclear extracts ("A-prime", "B-prime" and "C-prime"). These three RBP-Jk consensus sequence-binding complexes also appeared to be enriched in neural tissue. The individual complexes varied in abundance between neural extracts. Complex C and C-prime, formed with the NRE probe and the RBP-Jk probe respectively, did not show the same relative distribution between different neural extracts.

The sequence of the NRE probe was identical to *Tα1* promoter sequence from -646 to -617 inclusive. The sequence was as follows:

C T G C C T C T G C C T C C C A G G T G C T G T T G A G G G

The sequence of the RBP-Jk probe was identical to a 28-nucleotide segment of the adenovirus *pIX* gene. The sequence was as follows (the RBP-Jk consensus sequence is italicized):

G G A A A C A C G C *C G T G G G A A* A A A A T T T G G C

Figure 17

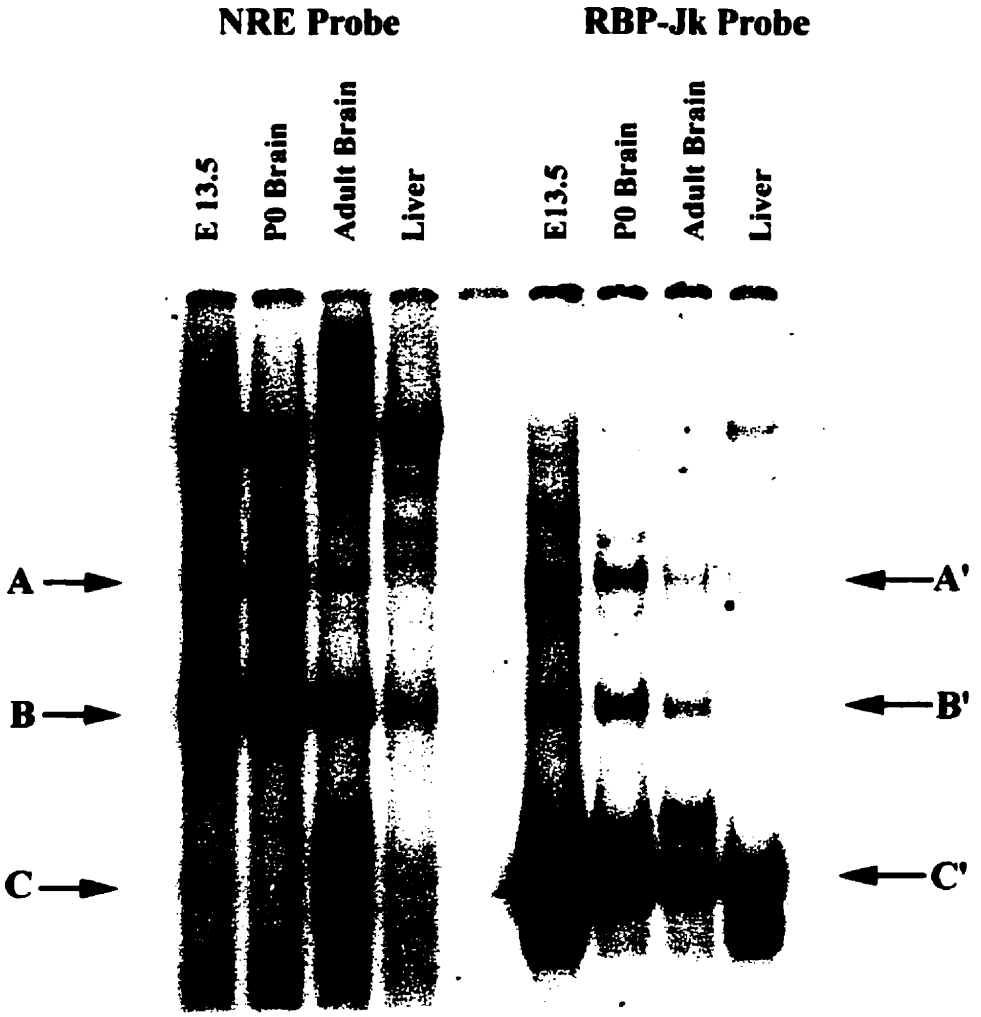

NRE Probe
RBP-Jk Probe
E 13.5
P0 Brain
Adult Brain
Liver
E13.5
P0 Brain
Adult Brain
Liver
A
B
C
A'
B'
C'

Figure 18: Cross Competition Suggests the NRE and RBP-Jk Consensus Sequence Binding Complexes are Related

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the RBP-Jk consensus region of the adenovirus virus *pIX* gene were end-labeled with ^{32}P and used as probes. Unlabeled oligonucleotides were used in excess in competition experiments.

The abundance of a number of NRE-binding complexes, including complex "C", decreased with decreasing amounts of E10 nuclear extract in the presence of a constant amount of probe (18 a, first 3 lanes, "C"). In addition, the pre-incubation of nuclear extract with 100 and 200-fold molar excess of unlabeled NRE oligonucleotide antagonized the formation of several complexes, including complex C (18 a, last 2 lanes).

Similarly, a 100, 200, and 300-fold molar excess of unlabeld RBP-Jk consensus oligonulceotide also antagonized formation of complex "C" (18b, first 4 lanes, "C"; 18c, first 4 lanes, "C"). In the converse experiment, a 100, 200, and 300-fold molar excess of unlabeled NRE oligonucleotide increasingly antagonized formation of complex "C-prime" in a dose responsive manner (18b, last 4 lanes, "C-prime"; 18d, lanes 5-8, "C-prime"). In contrast, 300-fold molar excess of unlabeled NRE oligonucleotide with 5 point mutations in the putative RBP-Jk binding sequence(" mutated NRE") did not antagonize formation of either the RBP-Jk binding complex "C-prime" (18d, last 4 lanes) nor the co-migrating NRE binding complex "C" (18c, last 4 lanes). p= free probe

The sequence of the mutated NRE and NRE oligonucleotides was as follows:

Mutated NRE: C T G C C T C T G C C **G T T A C** G G T G C T G T T G A G G G

NRE : C T G C C T C T G C C T C C C A G G T G C T G T T G A G G G

Figure 18

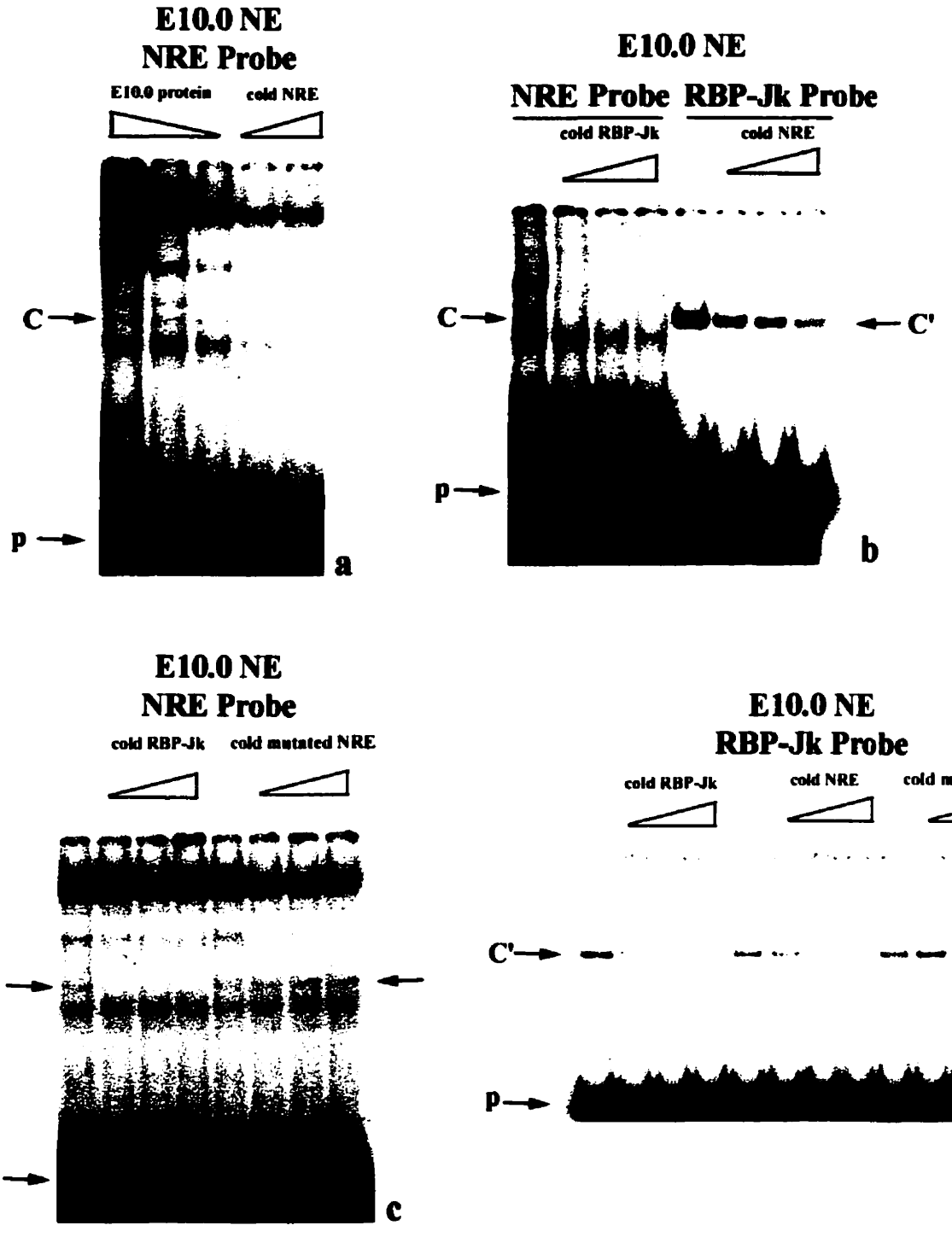

E10.0 NE
NRE Probe
E10.0 protein
cold NRE
C
p
a
E10.0 NE
NRE Probe
RBP-Jk Probe
cold RBP-Jk
cold NRE
C
C'
p
b
E10.0 NE
NRE Probe
cold RBP-Jk
cold mutated NRE
C
p
c
E10.0 NE
RBP-Jk Probe
cold RBP-Jk
cold NRE
cold mutated NRE
C'
p
d

Figure 19: *In Vitro* Translated RBP-Jk Binds the NRE

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the RBP-Jk consensus region of the adenovirus *plX* gene were end-labeled with ^{32}P and used as probes. *In vitro* translated RBP-Jk was synthesized using RBP-Jk cDNA (Dr. S. Hayward, Bethesda, Hsieh et al 1996), T7 RNA polymerase, and reticulocyte extract. Control reactions were charged with cDNA encoding luciferase.

A portion of the *in vitro* translation reaction product was incubated with either the NRE or RBP-Jk probe for 15 minutes prior to running the mixutre on a 5% polyacrylamide gel. Product of the reaction charged with RBP-Jk cDNA formed a unique complex with the NRE probe (19 a, "i"). This complex did not form with product from the *in vitro* translation reaction reaction charged with luciferase cDNA, nor with reticulocyte lysate alone (19a, "IVT luciferase", "Retic lysate" respectively). A co-migrating complex was formed with the RBP-Jk cDNA charged reaction product and the RBP-Jk probe (19a, "i-prime"). This complex did not form with the product of the luciferase charged reaction, nor with reticulocyte lysate alone (19a, "IVT luciferase", "Retic lysate" respectively").

The formation of complex "I" with the NRE probe was not antagonized by a 300-fold molar excess of unlabeled mutated NRE oligonucleotide (19b). Similarly, formation of complex "i-prime" was not antagonized by a 300-fold molar excess of unlabeled mutated NRE oligonucleotide.

Figure 19

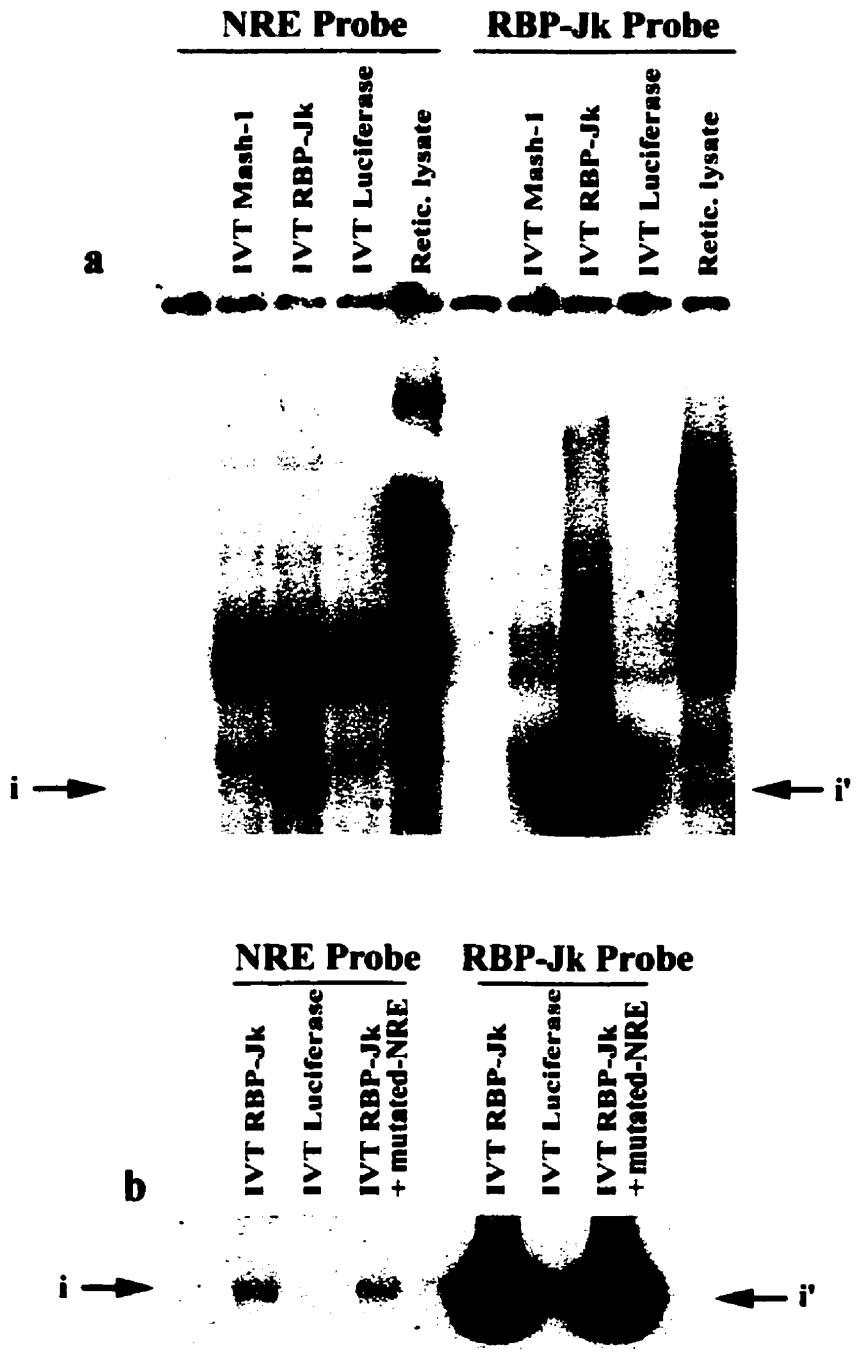

NRE Probe
RBP-Jk Probe
a
IVT Mash-1
IVT RBP-Jk
IVT Luciferase
Retic. lysate
IVT Mash-1
IVT RBP-Jk
IVT Luciferase
Retic. lysate
i
i'
NRE Probe
RBP-Jk Probe
b
IVT RBP-Jk
IVT Luciferase
IVT RBP-Jk + mutated-NRE
IVT RBP-Jk
IVT Luciferase
IVT RBP-Jk + mutated-NRE
i
i'

Figure 20: NRE-Binding Complex in E10.0 and Cultured Cortical Precursor Extracts Co-Migrates with *In Vitro* Translated RBP-Jk/NRE Complex

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the RBP-Jk consensus region of the adenovirus *pIX* gene were end-labeled with ^{32}P and used as probes. Complexes "A", "B", and "C" were formed with the NRE probe and neural tissue extracts (20, "adult brain", "E10", lanes 2 and 3 respectively). In addition, complex "I", formed with *in vitro* translated RBP-Jk protein and the NRE probe, co-migrated with the NRE complex C (20, lane 6 "IVT CBF-1"). A co-migrating complex was also observed in nuclear extract from cultured cortical precursor cells (20, "cort. progenitors"), but a very faint signal was observed at that position with nuclear extract from more mature neuronal cultures (20, "cort. neurons").

Complex "C-prime" was formed with the RBP-Jk probe and neural tissue extract (20, "adult brain", "E10", lanes 8 and 9 respectively). In addition, complex "i-prime", formed with *in vitro* translated RBP-Jk and the RBP-Jk probe, co-migrated with complex "C-prime" (20, "IVT CBF-1", lane 12). A co-migrating complex was observed in nuclear extract from cultured cortical precursor cells (20, "cort. progenitors" lane 10), and a less intense signal was observed in the same position with nuclear extract from more mature cultures (20, "cort neurons" lane 11).
P= free probe

Figure 20

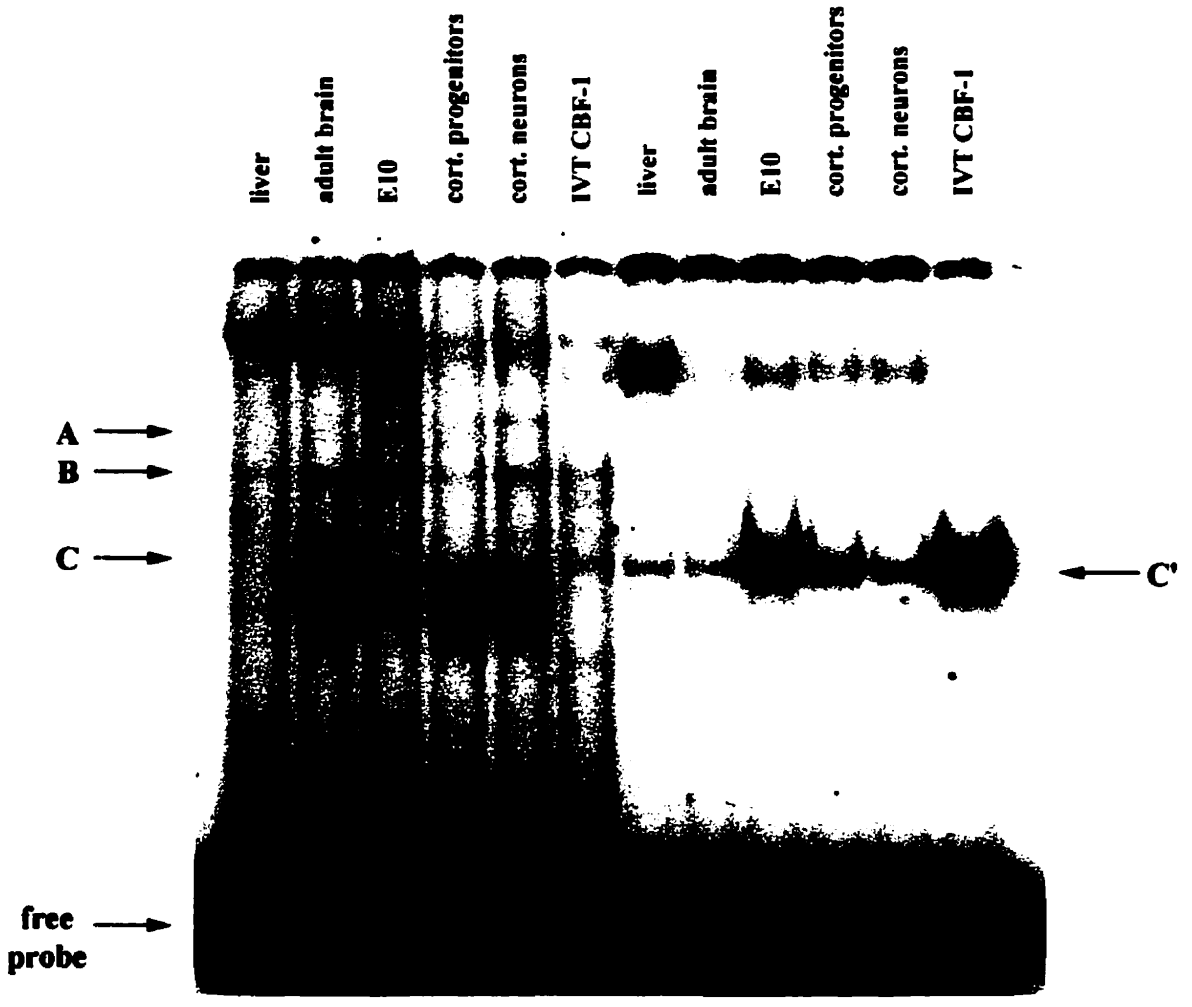

liver
adult brain
E10
cort. progenitors
cort. neurons
IVT CBF-1
liver
adult brain
E10
cort. progenitors
cort. neurons
IVT CBF-1
A
B
C
C'
free probe
NRE
RBP-Jk

Figure 21: Anti-RBP-Jk Antibody Shifts E13.5 NRE-Binding Complex

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the RBP-Jk consensus region of the adenovirus *pIX* gene were end-labeled with ^{32}P and used as probes.

The NRE-binding complex "C" was formed with E13.5 nuclear extract (21a, c, lanes 2 and 2). The formation of this complex was antagonized by pre-incubation with 100-fold molar excess of unlabeled NRE or RBP-Jk consensus sequence oligonucleotide, but not with 300 fold molar excess unlabeled mutated NRE oligonucleotide (21a, lanes 3-5). Complex "C" was shifted (C shift) as a result of pre-incubation of E13.5 nuclear extract with anti-RBP-Jk antibody, prior to inclusion of the NRE probe (21c, lane 3). Pre-incubation with control serum did not shift complex "C" (21c, lane 4).

Similarly, the RBP-Jk consensus sequence binding complex "C-prime" formed with E13.5 nuclear extract (2la, lane 7; 21b, lane 1). The formation of this complex was partially antagonized by pre-incubation with 100-fold molar excess of unlabeled NRE oligonucleotide, completely antagonized by 100 fold molar excess RBP-Jk consensus sequence oligonucleotide, but not with 300 fold molar excess unlabeled mutated NRE oligonucleotide (21a, lanes 8-10; 21b, lanes 2-4). Complex "C-prime" was similarly shifted (C-prime shift) as a result of pre-incubation of E13.5 nuclear extract with anti-RBP-Jk antibody, prior to inclusion of the RBP-Jk probe (21c, lane 7). Pre-incubation with control serum did not shift complex "C" (21c, lane 8).

(rabbit ploYclonal IgG anti-RBP-Jk-GST serum kindly provided by Dr. J. Coligan, Bethesda, Shirakata et al 1996).

Figure 21

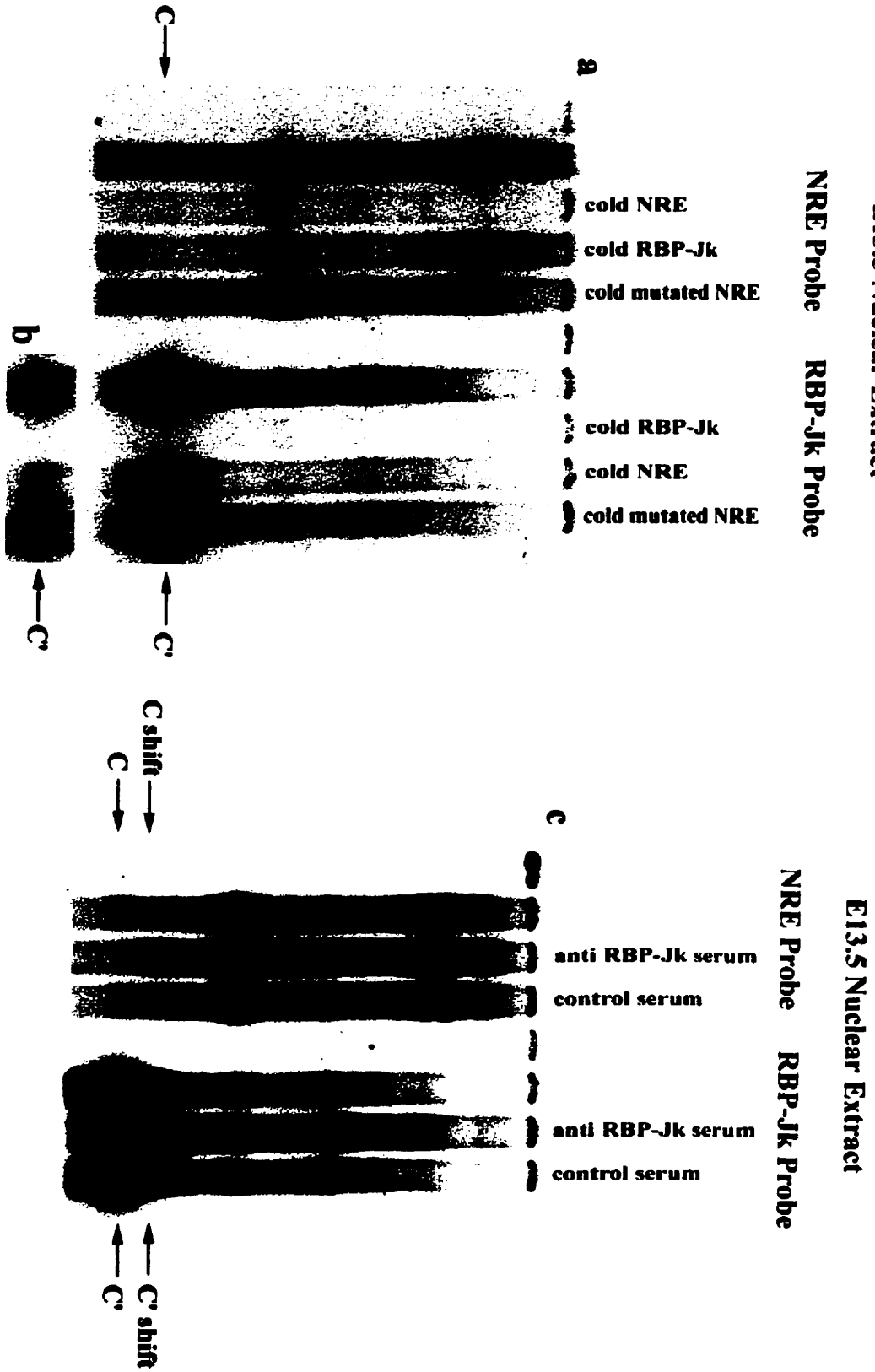

E13.5 Nuclear Extract
NRE Probe
RBP-Jk Probe
a
b
C
C'
cold NRE
cold RBP-Jk
cold mutated NRE
cold RBP-Jk
cold NRE
cold mutated NRE
E13.5 Nuclear Extract
NRE Probe
RBP-Jk Probe
C shift
C
C'
C' shift
c
anti RBP-Jk serum
control serum
anti RBP-Jk serum
control serum

Figure 22: *In Vitro* Translated RBP-Jk/NRE Complex Co-Migrates with E13.5 NRE-Binding Complex and is Similarly Supershifted by Anti-RBP-Jk Antibody

In vitro translation of RBP-Jk was carried out using ^{35}S labeled methionine to check production of the protein product. A portion of the *in vitro* translation reaction was run on a denaturing SDS-containing polyacrylamide gel (22a), revealing a single major synthesis product at ~52kD, the molecular weight of RBP-Jk (Shirakata et al 1996).

Oligonucleotides identical in sequence to the NRE region of the *Tαl* promoter and the RBP-Jk consensus region of the adenovirus *pIX* gene were end-labeled with ^{32}P and used as probes. The NRE-binding complex "I" formed with *in vitro* translated RBP-Jk (22b, lane 1; 22c, lane 2). The formation of complex "I" was antagonized by 100-fold molar excess of unlabeled NRE or RBP-Jk consensus sequence oligonucleotide, but not a 300 fold molar excess of mutated NRE oligonucleotide. The complex did not form with the product of a luciferase cDNA charged *in vitro* translation reaction (22b, lane 5). Complex "I" was shifted by pre-incubation of *in vitro* translated RBP-Jk with anti-RBP-Jk antibody, prior to inclusion of the NRE probe (22c, lane 3).

The NRE-binding complex "C" was formed with E13.5 nuclear extract (22c, lane 6) and co-migrated with complex "i". Complex "C" was shifted by pre-incubation of E13.5 nuclear extract with anti-RBP-Jk antibody, prior to inclusion of the NRE probe (22c, lane 7). Neither complex "i" nor complex "C" were shifted by pre-incubation with control serum.

Figure 22

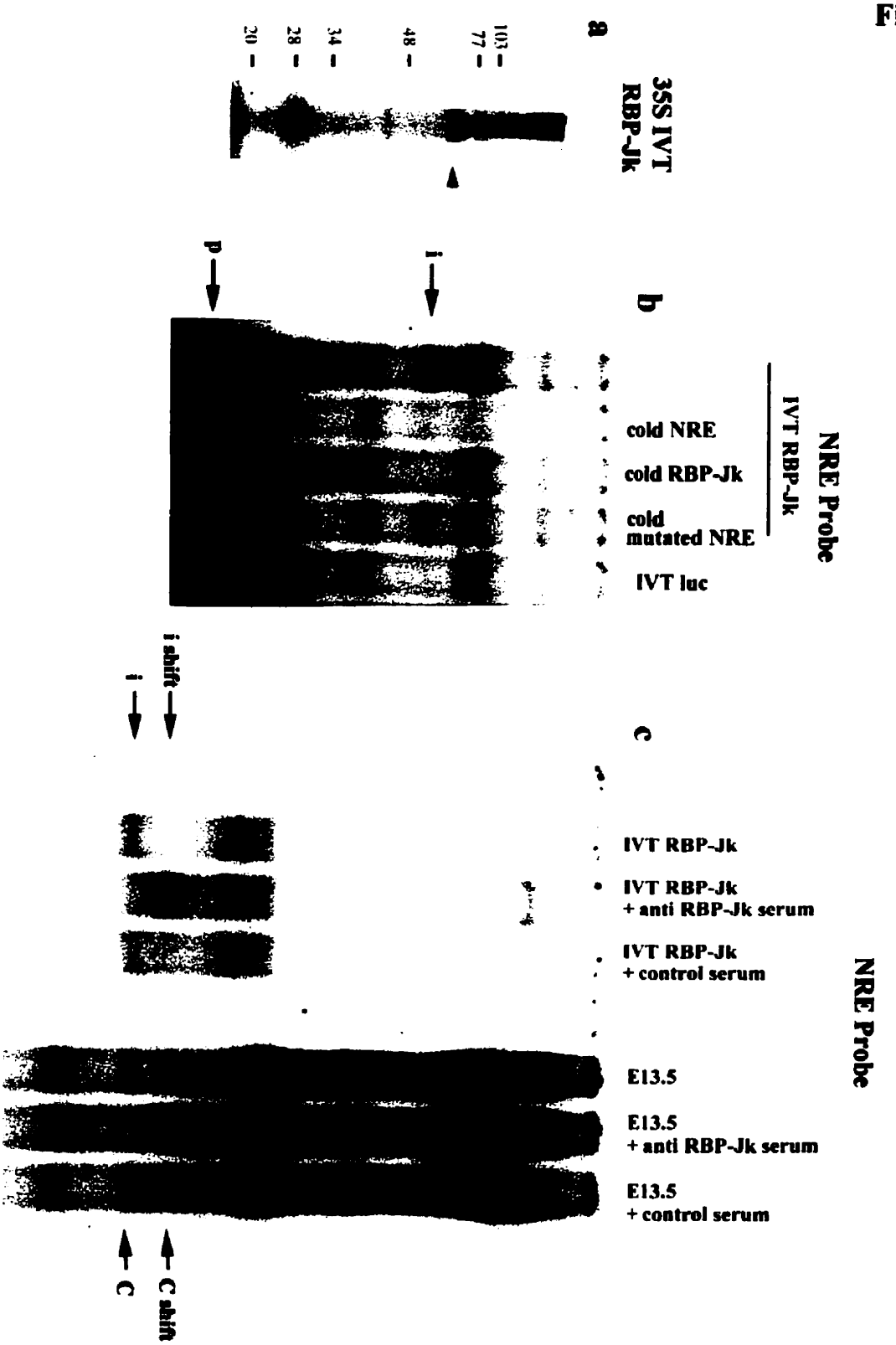

a
35S IVT RBP-Jk
103
77
48
34
28
20
b
p
i
NRE Probe
IVT RBP-Jk
cold NRE
cold RBP-Jk
cold mutated NRE
IVT luc
c
i
i shift
IVT RBP-Jk
IVT RBP-Jk + anti RBP-Jk serum
IVT RBP-Jk + control serum
NRE Probe
E13.5
E13.5 + anti RBP-Jk serum
E13.5 + control serum
C
C shift

Figure 23: *In Vitro* Translated REST does not Bind the NRE

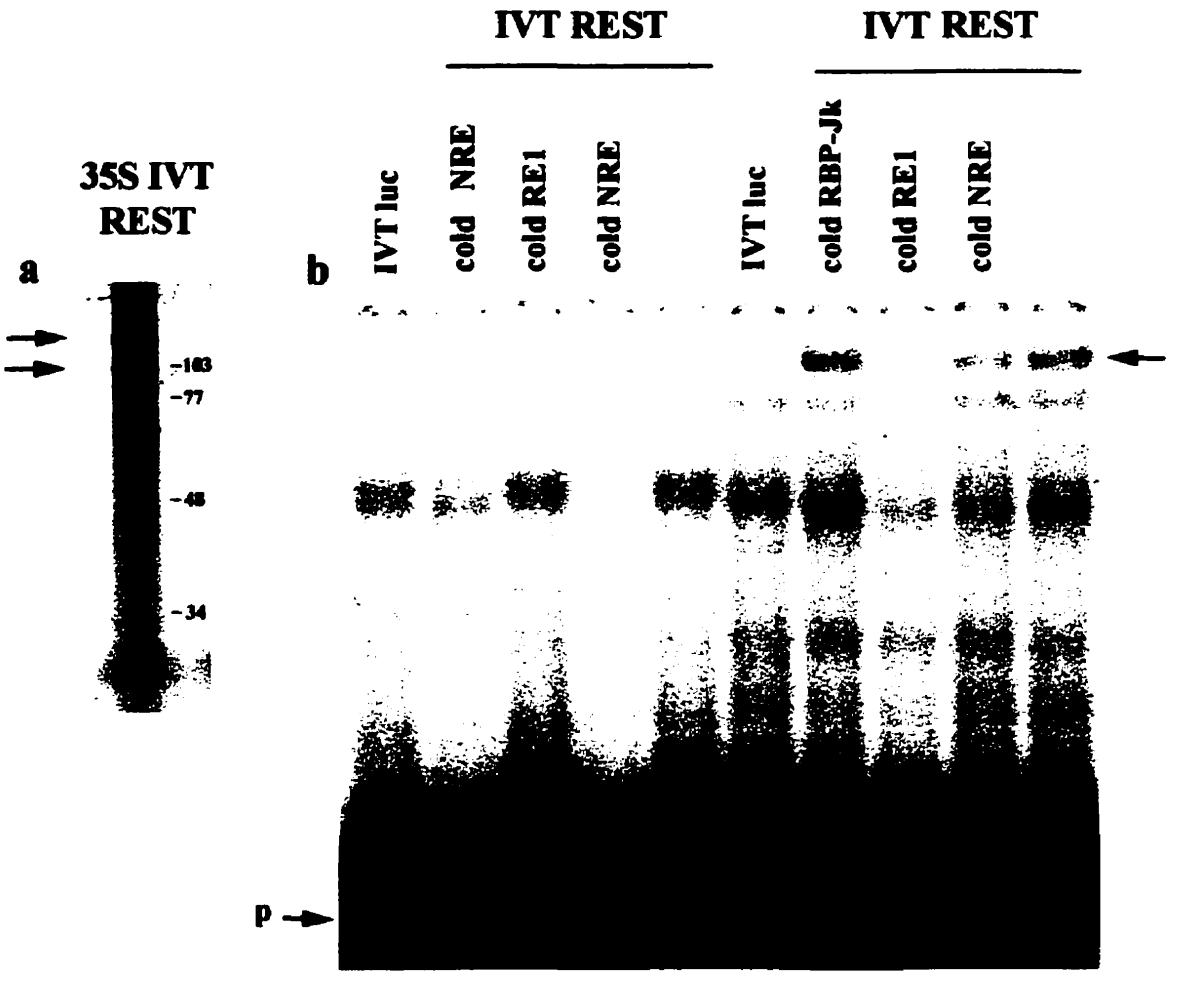
In vitro translation of REST was carried out using ^{35}S labeled methionine to check production of the protein product. A portion of the *in vitro* translation reaction was run on a denaturing SDS-containing polyacrylamide gel (23a), revealing 2 major synthesis products. The heavier product ran at 116kD, the molecular weight of REST (Chong et al 1995). The identity of the second product is unknown, but may be a major REST breakdown product.

Oligonucleotides identical in sequence to the NRE region of the *Tα1* promoter and the REST consensus region (NRSE) of the rat *type II sodium channel* gene (RE1) were end-labeled with ^{32}P and used as probes. The product of the REST cDNA charged *in vitro* translation reaction formed a complex with the RE1 probe (23b, lane 10, arrow). The formation of this complex was antagonized by 100-fold molar excess unlabeled RE1 oligonucleotide, but not by 300 fold molar excess of NRE nor RBP-Jk consensus sequence oligonucleotides. In addition, this complex was not formed with the product of a luciferase cDNA charged *in vitro* translation reaction (23b, lane 6).

The REST cDNA charged *in vitro* translation reaction product did not form a unique complex with the NRE probe (22b, lane 5).

The sequence of the RE1 probe was as follows:
G G G T T T C A G A A C C A C G G A C A G C A C C A G
(*Rest* cDNA and *type II sodium channel* genomic sequence were kindly provided by Dr. G Mandel, New York, Chong et al 1996)

Figure 23


a
35S IVT REST
-103
-77
-48
-34
b
IVT REST
IVT REST
IVT luc
cold NRE
cold RE1
cold NRE
IVT luc
cold RBP-Jk
cold RE1
cold NRE
P

Discussion

(i) The Rat *Tα1* Gene Provides a Tool for Studying Neuronal Differentiation Mechanisms

During development, control over the timing, location and extent of neuronal differentiation is critical for the coordinated assembly of the integrated nervous system. The rat *Tα1* α-tubulin gene promoter has been used to examine intrinsic genetic mechanisms regulating neuronal differentiation in the nervous system.

The rat *Tα1* gene encodes an isoform of α-tubulin and is expressed in a neuron-specific and pan-neuronal manner. The expression of this neuron-specific isoform of α-tubulin appears to be regulated as a function of neuronal growth. *Tα1* is expressed at high levels during neuronal growth, when microtubules composed of α-tubulin and β-tubulin are extended, and is expressed at low levels after neurite extension ceases (Miller et al 1987). *Tα1* is therefore highly expressed in the developing nervous system, and downregulated in neurons of the mature nervous system (Miller et al 1987). In the peripheral nervous system, induction of *Tα1* expression in mature neurons can be triggered by axotomy, with expression remaining high, as in developing neurons, until neurite outgrowth (regeneration) ceases following target contact (Miller et al 1989). *Tα1* mRNA production can also be stimulated in mature neurons of the CNS with axotomy, where it remains at a high level for some time despite the lack of neurite regrowth in these cells (Tetzlaff et al 1991).

The *Tα1* expression pattern is regulated at the transcriptional level (Miller et al 1991), and 1100-nucleotides of the gene's immediately upstream sequence is sufficient to direct expression of a reporter gene in a very similar manner in transgenic mice (Gloster et al 1994; Bamji and Miller 1996; Gloster et al 1999). Using a modified *lacZ* reporter gene (*nlacZ*) engineered to produce an isoform of β-galactosidase that is translocated to the nucleus, we have previously demonstrated that the 1100-nucleotide *Tα1* gene fragment

directs a neuron-specific and pan-neuronal pattern of expression (Gloster et al 1994; Bamji and Miller 1996; Gloster et al 1999). Moreover, this 1100-nucleotide sequence regulates expression as a function of neuronal growth (Bamji and Miller 1996; Wu et al 1997; Gloster et al 1999).

Further examination *in vitro* and *in vivo* has revealed that the 1100-nucleotide *Tα1* gene fragment induces expression soon after the first signs of neuronal differentiation can be detected (Gloster et al 1999). Though β-gal activity first appears in different neuronal populations at different times in *Tα1-nlacZ* transgenic mice, in the neuronal populations examined, the first appearance of β-gal activity coincides with or immediately follows the commencement of cell cycle exit and early neuronal gene expression (Gloster et al 1999). In addition, cortical precursor cells cultured from Tα1-nlacZ transgenic mice do not express β-gal initially, but begin to express it coincident with or immediately following the commencement of cell cycle exit and the induction of the early neuronal gene *β-III tubulin* (Gloster et al 1999). These results suggest that *Tα1* expression is tightly controlled by transcriptional mechanisms, and that *Tα1* is expressed at a specific and early stage in the process of neuronal differentiation.

This early neuron-specific and pan-neuronal expression can be directed by an 1100-nucleotide fragment of the *Tα1* gene, suggesting the sequence might reveal transcriptional mechanisms regulating neuronal differentiation.

(ii) The NRE may Regulate the Timing of Neuronal Differentiation

The 1100-nucleotide promoter of the *Tα1* gene contains potential binding sites for several previously described transcription factors (Gloster et al 1994). In addition, some of the rat gene's promoter sequence has apparently been conserved and is found in the goldfish ortholog *α-1 α-tubulin* (Hieber et al 1998). Of particular interest is a region that has been conserved and is found in other neural genes. The sequence *CTCCCAGGTG* is

found in several neural genes, where it occurs either alone or at the core of the previously described 24-nucleotide "neuronal restriction silencing element" (NRSE) (Schoenherr et al 1996). The NRSE, including the core 10-nucleotide sequence, has also been conserved in several of these neural genes. The *Tα1* gene does not contain the full NRSE sequence, and is not unique among neuronal genes in this respect, suggesting that the NRSE may contain a smaller and separable sub-element that is also conserved.

(a) A 66-Nucleotide Deletion Leads to Potentially Precocious *Tα1* Promoter Activity in the Developing CNS and PNS

The first appearance of β-gal activity in a number of CNS and PNS populations was similar in both *ΔNRE-nlacZ* and *Tα1-nlacZ* transgenic mice. One exception was the dorsal root ganglia, in which β-gal activity was detected precociously at E10.0 in *ΔNRE-nlacZ* line 23. This was 12 hours before activity was first detected in DRG's in *ΔNRE-nlacZ* line 9 and *Tα1-nlacZ* lines K6 and Q54. Apart from this, quantitative differences in β-gal activity were seen within neuronal populations. Greater numbers of cells appeared to express β-gal in *ΔNRE-nlacZ* line 23 than in line 9 or *Tα1-nlacZ* line K6 between E9.5-E10.5. This was observed throughout the developing spinal cord, hindbrain and midbrain, as well as in the developing trigeminal, geniculate, petrosal, and nodose ganglia. These results suggested that on a cellular basis the onset of *ΔNRE-nlacZ* expression in line 23 may have preceded *ΔNRE-nlacZ* induction in line 9 and *Tα1-nlacZ* induction in lines Q54 and K6. Alternatively, β-gal may have been expressed in a similar number of cells in all lines, but at a higher level in line 23. The level of expression in some cells in ΔNRE-nlacZ line 9 and in Tα1-nlacZ lines K6 and Q54 could have been below the threshold for detection of β-gal activity, and a greater average cellular level of expression in line 23 may have rendered a greater number of lacZ expressing cells positive for β-gal activity.

ΔNRE-nlacZ line 9 displayed a pattern of expression similar to that of two previously characterized *Tα1-nlacZ* lines at E9.5. The distinct expression pattern of line 23 therefore appeared to be a consequence of its incorporation site in the genome, a phenomenon that has been previously observed (Jaenisch et al 1981, Harbers et al 1981, Palmiter and Brinster 1986, Al-Shai et al 1990, Pravtcheva et al 1994, Mercer et al 1991). However, the induction of *Tα1-nlacZ* appeared to be tightly controlled and to coincide with the commencement of terminal mitosis and the induction of early neuronal gene expression in two lines of *Tα1-nlacZ* transgene mice. At E9.5, activity in both of these lines was very low, but the location of β-gal positive cells and the timing of their appearance was consistent. In addition, the level of expression in *ΔNRE-nlacZ* line 23 was generally lower than that of *Tα1-nlacZ* line K6 over time, suggesting the precocious β-gal activity in line 23 was not a consequence of higher reporter gene expression generally. An alternative explanation is that the β-gal activity in *ΔNRE-nlacZ* line 23 was a result of transgene disinhibition, and that the reporter gene was then expressed in neural precursor cells and/or immature neurons that would not yet express *Tα1-nlacZ*. The disinhibition may have taken place in *ΔNRE-nlacZ* line 9 but may have been masked by a low level of expression influenced by genomic location of the transgene. The *β-gal* expression level in *ΔNRE-nlacZ* line 9, the line which did not display precocious reporter gene expression, appeared to be generally lower than that of *Tα1-nlacZ* line K6 and *ΔNRE-nlacZ* line 23 over time.

(b) A 66-Nucleotide Deletion Leads to Precocious *Tα1* Promoter Activity in Cultured Cortical Precursor Cells

Neural precursor cells were cultured from transgenic embryos to monitor promoter activity as cells underwent terminal mitosis and began to express early neuronal genes *in vitro*. In neural precursor cell cultures, *Tα1-nlacZ* induction appeared to follow the expression of *β-III tubulin*, while *ΔNRE-nlacZ* induction preceded *β-III tubulin* expression in at least some cells. Beta-galactosidase and β-III tubulin were co-localized

in more mature cultures from both *Tα1-nlacZ* and *ΔNRE-nlacZ* embryos in which neuronal differentiation had progressed (Gloster et al 1999, data not shown). Combined with previous analyses that demonstrated β-III tubulin was a very early neuronal marker and that most precursor cells were dividing during the first day *in vitro* (Gloster et al 1999, Slack et al 1998), these results suggest that *ΔNRE-nlacZ* was induced in neural precursor cells and/or immature neurons in line 23. These results are in agreement with those from embryo analysis which suggest the pattern of β-gal activity in line 23 embryos from E9.5 to E10.5 may have been due in part to expression in neural precursor cells and/or immature neurons prior to *Tα1-nlacZ* expression.

(c) A 66-Nucleotide Deletion Leads to Potentially Precocious *Tα1* Promoter Activity In Adult Neural Precursor Cells

Between E9.5 and E10.5, β-gal activity was more extensive in *ΔNRE-nlacZ* line 23 than in *ΔNRE-nlacZ* line 9 and *Tα1-nlacZ* lines K6 and Q54. To determine if this reflected a quantitative difference due to a greater level of *β-gal* expression in *ΔNRE-nlacZ* line 23 or a qualitative difference due to precocious induction of *ΔNRE-nlacZ* in line 23 in neural precursor cells and/or immature neurons, transgene expression was examined in the adult brain. Specifically, we examined a region of the adult brain where neural precursor cells reside but that is devoid of neurons, namely the ependymal layer surrounding the lateral ventricles. Neural "stem cells' have been isolated from the ependymal layer surrounding the lateral ventricles of the adult rat brain (Johansson et al 1999). Though not all ependymal layer cells surrounding the lateral ventricle are neural precursor cells, differentiated neurons do not reside in the ependymal layer. Beta-galactosidase activity was present in ependymal cells in *ΔNRE-nlacZ* lines 23 and 9, but absent in such cells in *Tα1-nlacZ* line K6 despite a higher level of expression throughout the adult cortex generally. This suggested that neural precursor cells may have expressed *ΔNRE-nlacZ* but not *Tα1-nlacZ*. Further, β-gal activity was also observed in similar ependymal layer cells

in *ΔFRE-nlacZ* adult mice, suggesting the 66-nucleotide deletion common to *ΔNRE* and *ΔFRE* promoters was responsible for the change in β-gal activity.

The expression of *ΔNRE-nlacZ* in putative neural precursor cells of the adult brain in both ΔNRE-nlacZ transgenic mouse lines contrasts with its expression during development, where putative precocious expression was observed only in line 23. The results of adult brain analysis suggest the difference between *ΔNRE-nlacZ* lines 23 and 9 from E9.5 to E10.5 may have been due to a low level of expression by line 9. Thus, disinhibition from E9.5 to E10.5 may have occurred in both *ΔNRE-nlacZ* lines as a consequence of the 66-nucleotide promoter deletion. This disinhibition may have permitted precocious reporter gene expression, as observed in line 23. However the early level of expression may have been influenced by the genomic location of the transgene, and such positional influence may have inhibited (or not activated) precocious expression in line 9. We cannot rule out the possibility that inhibition in neural precursor cells residing in the adult nervous system may be governed differently than inhibition in neural precursor cells residing in the embryonic nervous system, and may therefore be molecularly distinguishable. Such a distinction may have been reflected by the expression patterns in *ΔNRE-nlacZ* line 9.

Interestingly, β-gal activity was also consistently observed in ependymal layer cells surrounding the third ventricle in *ΔNRE-nlacZ* mice, though such activity was absent in *Tα1-nlacZ* line K6. The presence of "stem cells" in the ependymal layer in this region of the adult brain was not previously reported, but may be indicated by the expression of β-gal in *ΔNRE-nlacZ and ΔFRE-nlacZ* transgenic mice.

(d) The NRE is Implicated in the Timing of *Tα1* Induction and Neuronal Differentiation

Deletion analysis suggested that the 66-nucleotide *Tα1* sequence housed a repressive element(s) that was required to prevent precocious neuronal gene expression within the neural lineage. Based on the conservation of the NRE, its potential function as a repressor in the goldfish *α-1 α-tubulin* gene (Hieber et al 1998), and the finding that mutation of the NRE in the context of the *L1* promoter led to precocious activity in neural precursor cells in transgenic mice (Kallunki et al 1997), we propose that the NRE mediates the repressive activity ascribed to the 66-nucleotide sequence. Since the NRE is found in a number of neural genes (Schoenherr et al 1996; figure 1) we further suggest that the NRE-mediated mechanism may be involved in the timing of neuronal differentiation.

The repression of precocious neuronal gene expression is not a new activity, but the proposed NRE-mediated mechanism is novel. Moreover, none of the previously described mechanisms appear to be responsible for the activity ascribed to the 66-nucleotide *Tα1* sequence. A GC-rich sequence in the *Hes5* gene has been purported to be a repressor of precocious neuronal gene expression based on culture experiments with a neural cell line (Takebayashi et al 1995). The *in vivo* relevance, the binding proteins responsible, and the applicablility of this mechanism to other neuronal genes are all unknown. The *GAP-43* gene, which encodes a developmentally regulated protein that is enriched in developing neurites, contains a unique regulatory sequence (SNOG element) that is required for repression in non-neural cell lines (Weber and Skeene 1997). The physiological significance of this *in vitro* activity and the binding proteins responsible are also unknown. The NRSE has also been proposed to act as an inhibitor of precocious neural gene expression during development. This is based on several factors. First, an NRSE-binding factor, named REST, has been detected in the nervous system prior to widespread neuronal differentiation (Chong et al 1995; Schoenherr and Anderson 1995). Second, REST is capable of directly repressing neuronal gene expression in an NRSE-dependent manner *in vitro* (Chong et al 1995) Third, many neuronal genes contain the NRSE, and in some of these the sequence has been evolutionarily conserved (Schoenherr

et al 1996). Fourth, these NRSE-containing neuronal genes do not appear to be expressed in neural precursor cells (Schoenherr et al, references therein). Fifth and finally, mutation of the NRSE in the neural *L1* gene promoter led to precocious promoter activity in transgenic mice (Kallunki et al 1997). However disruption of the REST gene in transgenic mice did not lead to precocious expression of the *L1* gene nor other neuronal genes, including those known to contain the NRSE (Chen et al 1998), leaving the factor responsible for *L1*-NRSE function unidentified. In addition, we have shown that REST does not bind the NRE *in vitro*, suggesting it is not responsible for the NRE's activity *in vivo*.

Though not conclusive, the results presented in this report suggest the NRE may function within the neural lineage to repress precocious neuronal gene expression, providing a prospective mechanism for regulating the timing of neuronal differentiation. Such a mechanism would make neuronal differentiation, to some extent, a process of disinhibition, as neural induction has been found to be.

(iii) ΔNRE and ΔFRE Deletions Decrease Neocortical Activity of the *Tα1* Promoter

Deletion of a 66-nucleotide segment and an engulfing 184-nucleotide segment of the *Tα1* promoter consistently led to a loss of activity specifically in the neocortex. In addition, preliminary results suggested that a 30-nucleotide sequence in the 118 nucleotides unique to the 184-nucleotide deletion also contributed to the neocortical activity of the *Tα1* promoter (F.D. Miller,unpublished observations). Together, these results suggest a model for *Tα1* promoter activity regulation in the neocortex.

The full 184-nucleotide sequence may constitute a bipartite neocortex-specific transcriptional activation module, with one component contributed by the 66-nulceotide segment, and another contributed by the remaining 118-nucleotide (possibly the 30-nucleotide sequence). In this model, each component interacts with a regulatory factor,

but the two display synergy with respect to *Tα1* promoter activation. This synergy may occur at the level of DNA-binding, transcriptional activation, or both. Either factor alone produces only a low level of promoter activation in the neocortex, while the absence of both factors causes a near loss of activation in this region.

Consensus sequences within the 184-nucleotide sequence suggest possible mechanisms for such regulation. *Emx1* and *Emx2* (Simeone et al 1992) are orthologs of the Drosophila gene *empty spiracles* (Cohen and Jurgens 1990; Waldorf and Gehring 1992) that is required for development of the anterior nervous system in Drosophila. *Emx1* and *Emx2* are expressed throughout the developing neocortex and are required for its development (Boncinelli et al 1993; Fernandez et al 1998; Simeone et al 1992a; Simeone et al 1992b; Qiu et al 1996; Pellegrini et al 1996). These factors may interact with the 30-nucleotide sequence, interact with factors binding the 66-nucleotide segment, and synergistically activate transcription in neocortical neurons. It is interesting to note that homeodomain-containing transcription factors have been found to associate with other transcription factors, including bHLH factors capable of binding E-boxes, such as the one located in the 66-nucleotide segment (Johnson et al 1997; Barbaric et al 1996). Whether Emx1 and Emx2 share these properties is unknown.

The regulation of a pan-neuronal gene might be expected to depend upon a mechanism of induction common to all neurons. That pan-neuronal expression may be the sum of neocortical and non-neocortical mechanisms of regulation is somewhat surprising. It appears that induction of the pan-neuronal gene *Tα1* may not follow directly as a consequence of cell type, but may depend on cell subtype or spatial aspects as well. It is interesting that regulation in the neocortex involves a distinct mechanism. Development of the anterior nervous system in Drosophila has been reported to involve regulatory mechanisms distinct from those involved in segmentation and development in the rest of the nervous system (Hirth and Reichert 1999). These specific mechanisms involve a number of spatially restricted homeodomain transcription factors. The same appears to be true of mice, where homologs of these transcription factors have been identified and are restricted to subdomains of the developing anterior nervous system. These transcription

factors, many of which are essential for development of the telencephalon, may regulate *Tα1* promoter activity in neurons of the telencephalon. Why neocortical neurons are selectively sensitive to the promoter deletions described is unknown, but may have to do with the restricted expression of activating factors within the telencephalon (such as the *emx* genes), and distinct mechanisms of Tα1 gene induction.

Regardless of mechanism, deletion analysis suggested Tα1 promoter activity is differentially regulated in different neuronal subtypes and is not induced by a mechanism common to all types of neurons. Whether the promoter activity is further divisible, or whether this division is unique to the neocortex is unknown.

(iv) RBP-Jk/Su(H) is an NRE-Binding Factor

(a) RBP-Jk/Su(H) Binds the NRE *In Vitro*

A number of complexes were formed between the *Tα1* NRE sequence and protein extracts containing the nuclear contents of neural precursor cells, immature neurons, and mature neurons. If co-migration reflects common complexes in different extracts, then some of these complexes were enriched in nervous tissue and were developmentally regulated.

The 10-nucleotide NRE sequence at the core of the 30-nucleotide NRE probe resembled the Su(H)/RBP-Jk consensus sequence. We used a portion of the adenovirus *pIX* gene, a natural target of RBP-Jk, as a probe for RBP-Jk binding activity in extracts. The NRE probe and the RBP-Jk probe were similar only in the core NRE sequence and the RBP-Jk consensus sequence, respectively. Outside of these sequences, the two probes were unrelated.

Some of the NRE complexes co-migrated with those forming with the RBP-Jk probe. Three of these NRE complexes were enriched in neural tissue. One of these three

complexes, namely complex "C", was present in embryonic tissue, diminished in the postnatal brain, and highly abundant in the adult brain. This pattern countered the profile of *Tα1-nlacZ* and endogenous *Tα1* gene expression, which were absent in neural precursors, high in newborn differentiating neurons, and low in mature adult neurons. This pattern suggested that the complex may have been involved in repression of *Tα1* promoter activity.

Complex "C", formed between E13.5 extract and the NRE probe, co-migrated with complex "C-prime", formed between E13.5 extract and the RBP-Jk probe. Cross competition assays demonstrated that the RBP-Jk consensus sequence could compete with the NRE probe for complex "C", and that the NRE sequence could compete with the RBP-Jk probe for complex "C-prime". The NRE sequence was a less effective competitor, but competed in a sequence-specific manner. Thus it appeared that complexes "C" and "C-prime" might have been the same complexes with different affinities for the RBP-Jk and NRE sequences.

In vitro translated RBP-Jk protein bound the NRE and RBP-Jk probes, forming complexes that co-migrated with "C" and "C-prime" respectively. The E13.5 nuclear extract NRE probe complex "C" and the co-migrating IVT-RBP-Jk NRE complex "I" were shifted in parallel on the same gel to the same position with an anti-RBP-Jk antibody. This antibody also shifted the E13.5 nuclear extract RBP-Jk probe complex "C-prime", which co-migrated with complexes "C", "i" and "i-prime". The NRE sequence at the core of the NRE probe resembled the RBP-Jk consensus sequence and was necessary for the formation of complex "C". Further, mutations in this region rendered the oligonucleotide incapable of competing with RBP-Jk probe for complex "C-prime".

These results suggest that complex "C", from embryonic extract, contained RBP-Jk or an immunlogically related molecule capable of binding both the NRE and RBP-Jk probes dependent on the core NRE/RBP-Jk consensus sequence, and capable of forming a complex with each probe similar in size to that formed by IVT-RBP-Jk alone. The

simplest explanation is that the NRE complex "C" and the RBP-Jk complex "C-prime" were in fact RBP-Jk bound to the respective probes.

The distribution of complexes "C" and "C-prime" does not immediately conform to this proposal. Complex "C-prime" was detected in liver extract, while complex "C" was not. If these complexes are formed by the same protein, why does the complex form with the RBP-Jk probe but not the NRE probe? It should first be noted that the anti-RBP-Jk supershifts were performed with E13.5 nuclear extract alone, leaving the possibility that the complexes co-migrating with "C" and "C-prime" in liver extract do not contain RBP-Jk and are distinct. If this is the case, the comparison is moot. Alternatively, the liver complexes may contain RBP-Jk. The differential selectivity of distinct DNA sequences for RBP-Jk binding activity in specific tissues has been described previously (Shirakata et al, 1996). A sequence in the *major histocompatability gene Ia* (*MHC Ia*) binds RBP-Jk-in nuclear extract from the thymus, but not with extracts from other tissues despite the ubiquitous expression of RBP-Jk and the ability to form complexes with other RBP-Jk sequences. There may be a unique isoform of RBP-Jk or unique RBP-Jk complex in thymic tissue that is selectively capable of binding this *MHC class Ia* sequence. Alternatively, sequences elsewhere in the MHC class Ia gene segment used may confer this specificity through interactions with other differentially distributed proteins that affect RBP-Jk binding directly or indirectly. Similarly, the NRE sequence in the *Tα1* promoter could represent an RBP-Jk binding sequence selective for a neural-specific isoform or molecular complex of RBP-Jk, or one that confers specificity by virtue of its sequence and association with other factors that affect RBP-Jk binding. Such selectivity may explain the lack of complex "C" formation by liver extract, despite the abundance of complex "C-prime" and the presence of RBP-Jk protein in the tissue.

In addition to the difference between complex "C" and "C-prime" abundance in liver, the developmental distribution of complexes "C" and "C-prime" was also distinct. Complex "C" appeared to increase in the adult brain, while complex "C-prime" appeared to decrease progressively from E10 to adulthood. Again it is possible that the complexes co-migrating with "C" and "C-prime" in adult brain extract do not contain RBP-Jk and are

distinct. If this is the case, the comparison is moot. Alternatively, the adult complexes may contain RBP-Jk. In this case, just as the interactions between different DNA sequences and RBP-Jk species from different tissues may vary inconsistently, the interactions between different DNA sequences and RBP-Jk species from the same tissue (neural) at different times could conceivably vary inconsistently.

The NRE complex "C" appeared to be less abundant in E13.5 extract than RBP-Jk complex "C-prime". This suggested the presumed common complex, containing RBP-Jk, had a higher affinity for the RNP-Jk probe than the NRE-probe. Such a relationship was supported by the findings with IVT-RBP-Jk, which appeared to have a lower affinity for the NRE probe than the RBP-Jk probe. In addition, the NRE probe sequence was found to be a worse competitor for the presumed common complex than the RBP-Jk probe sequence. It is interesting to note that in the supershift experiments in which anti-RBP-Jk antibody was incubated with extract and NRE probe, the shifted complex appeared to be more stable than the original complex. That is the shifted complex appeared to be more abundant than the original complex "C". The same phenomenon was observed with IVT-RBP-Jk. *In vitro* translated RBP-Jk bound the NRE probe with a lower affinity than the RBP-Jk probe, and in supershift assays, the shifted complex was more abundant than the original complex "i". This suggested that the anti-RBP-Jk antibody stabilized the binding of endogenous RBP-Jk as well as *in vitro* translated RBP-Jk to the NRE sequence. The NRE thus appeared to be a relatively low affinity RBP-Jk binding site, susceptible to stabilization by protein association.

The low affinity RBP-Jk/NRE interaction normally observed may have been an artifact of our system and may not normally occur *in vivo*. The binding of RBP-Jk to the NRE may normally be stabilized by factors binding outside of the NRE segment, or by DNA conformations induced by outlying *Tα1* gene sequence. Alternatively, the *Tα1* NRE may truly constitute a relatively low affinity site that is selective for an RBP-Jk-containing complex in neural extracts. This selectivity may be based on unique isoforms and/or associations of neural RBP-Jk, or on the differential distribution of auxillary factors and their interaction with the NRE probe sequence. What form might these unique RBP-Jk

complexes take and how might they differ between tissues and over time? The co-migration of complex "C", between E13.5 extract and NRE probe, and complex "i", between IVT-RBP-Jk and NRE probe, as well as their parallel shifts with anti-RBP-Jk antibody, suggests that RBP-Jk is alone in the complex, and not part of a larger protein conglomerate. RBP-Jk protein may be modified directly in such a manner that its mobility in EMSA's does not change. RBP-Jk may also associate transiently with a developmentally regulated and tissue specific factor that does not remain with RBP-Jk as it binds DNA, but alters its affinity for different sequences selectively. A factor may in fact be associated with RBP-Jk in some extracts, but not alter the overall migration of the complex due to other changes in conformation. It is also possible that additional proteins bind the NRE and RBP-Jk probe sequences precluding or potentiating RBP-Jk complex formation in a differential manner. These would presumably not be present in the IVT-RBP-Jk preparation. The co-migration of complexes "C", "C-prime", "i" and "i-prime" indicates that such a scheme would have to occur via competitive binding of another factor or a transient interaction.

(b) Potential Relevance of the NRE/RBP-Jk Interaction

RBP-Jk is a sequence-specific DNA binding protein that possesses intrinsic transcriptionally repressive activity (Henkel et al 1994; Hsieh and Hayward 1995; Dou et al 1994). It has been evolutionarily conserved (Matsunami et al 1989; Furukawa et al 1991), as has the signal transduction cascade to which it belongs, namely the Notch signaling pathway (Jarriault et al 1995; Jarriault et al 1998). In Drosophila, the Notch pathway and *RBP-Jk/Su(H)* are involved in the segregation of neuroblasts from epidermoblasts in the initial stages of neural development, and are later involved in lateral specification events between at least some sibling neural cells that affects their acquisition of neuronal or non-neuronal fates (reviewed in Artavanis Tsakonas et al 1998; Greenwald 1998). During neuroblast segregation, RBP-Jk/Su(H) relays the Notch signal and negatively regulates the formation of neuroblasts in a cell intrinsic manner. Following neuroblast formation, RBP-Jk/Su(H) presumably relays the Notch signal,

again in cell intrinsic manner, to inhibit neuronal formation. Thus the Notch pathway and RBP-Jk/Su(H) seem to mediate binary decisions between alternative cell fates.

In mammals, the molecular components of the Notch pathway have been conserved, as has the integrity of the signaling cascade (Jarriault et al 1995; Jarrialut et al 1998). The mammalian Notch pathway appears to mediate binary decisions as it does in Drosophila, however the decisions seem to be different. In the mammalian nervous system, the Notch pathway appears to govern whether or not a neuronal precursor differentiates, rather than whether a cell becomes neural, which has already been decided by this stage (Green 1994). In particular, disruption of the *RBP-Jk/Su(H)* gene in transgenic mice led to precocious neuronal differentiation and gene expression (de la Pompa et al 1997), suggesting the transcription factor normally represses neuronal gene expression and differentiation in neural precursor cells.

The results presented in this report suggest RBP-Jk/Su(H) may normally repress neuronal differentiation through the direct repression of neuronal genes. The NRE may link RBP-Jk/Su(H) directly to these neuronal genes and place them under the control of the Notch pathway. In addition, the Notch pathway has been proposed to repress neurite outgrowth in mature neurons of the adult nervous system (Sestan et al 1999), as well as to repress neuronal differentiation in the developing nervous system (Nye et al 1994; Lardelli et al 1996). The NRE may provide a mechanism for repressing growth associated neuronal genes. Among the best characterized growth associated neuronal genes are *Tα1* (reviewed in Miller et al 1996) and *GAP-43* (Strittmatter et al 1992; 1995), both of which contain the NRE and are tightly coupled to neurite growth. The Notch pathway may negatively regulate neurite outgrowth through the direct repression of growth associated neuronal genes via an interaction between the NRE and RBP-Jk/Su(H).

However, not all neuronal genes containing the NRE are growth associated, as their patterns of expression differ. Though mature neurons diminish their growth, they presumably do not de-differentiate and stop expressing NRE-containing neuronal genes that are the hallmarks of the neuronal phenotype. Just as sequence differences between

these neuronal genes may distinguish their dynamic behaviours, so might these sequences distinguish their responsiveness to Notch signaling and RBP-Jk/Su(H) activity differentially in different contexts. This report describes RBP-Jk/Su(H) DNA binding sequences that may interact with RBP-Jk/Su(H) containing complexes differently in different tissues despite the ubiquitous expression of RBP-Jk/Su(H). Such a phenomenon has also been described previously (Shirakata et al 1996). Specific sequences may make the NRE's of growth associated genes responsive to an RBP-Jk/Su(H)-mediated Notch signal in adult neurons, and such regulation may underlie the inhibition of growth. Specific sequences may make NRE's of neuronal genes not associated with growth non-responsive to the signal in the context of adult neurons. Both groups of genes may be responsive to an RBP-Jk/Su(H)-mediated Notch signal in neural precursors and immature neurons that represses precocious expression.

The mechanism by which RBP-Jk/Su(H) may mediate NRE activity is unclear and may or may not involve the Notch pathway. The Notch intracellular domain is thought to activate transcription in association with RBP-Jk/Su(H) (Hsieh et al 1996). However RBP-Jk possesses intrinsic transcriptionally repressive activity (Hsieh et al 1996; Henkel et al 1994; Hsieh and Hayward 1995). In gel shift assays, it appeared that RBP-Jk bound the NRE alone, as a complex formed by *in vitro* translated RBP-Jk/Su(H) protein co-migrated with one formed by embryonic and adult brain nuclear extract. Whether a Notch- RBP-Jk/Su(H) complex bound the NRE is unknown. In addition, the ability of RBP-Jk/Su(H) to regulate *Tα1* promoter activity in an NRE-dependent manner has not been demonstrated.

If the NRE exerts transcriptionally repressive activity on other neuronal genes, as suggested by manipulation in the context of the *L1* (Kallunki et al 1997), it might suggest that neuronal differentiation, like neural induction (Green 1994), involves a derepressive mechanism. The loss of pre-existing neuronal gene repression in precursors could contribute to the timing of neuronal gene induction and neuronal differentiation. This mechanism might involve the Notch pathway and/or RBP-Jk/Su(H), though whether RBP-Jk/Su(H) can bind the NRE sequences of other neuronal genes is unknown.

According to this theory, both negative and positive factors would determine the timing of neuronal gene induction and neuronal differentiation. A number of studies have manipulated the timing of differentiation with gross morphological (and usually lethal) consequences. Development of the Drosophila eye involves both positive and negative regulators of neuronal differentiation (Brown et al 1992; Brown et al 1994). These factors work coordinately to control the timing of differentiation in a constellation of precursor cells. During Drosophila eye development, precursor cells proliferate, migrate and differentiate in a coordinated and stereotypical manner. The initial eye disc is a thin epithelial sheet of precursor cells. Cells differentiate successively from posterior to anterior, with all the cells at a given A/P position across the surface of the disc synchronized in their differentiation. The result is a wave front of differentiation called the morphogenetic furrow, which progresses across the eye leaving differentiated cells behind it while undifferentiated cells lie anteriorly and await its arrival. Neuronal differentiation in the developing eye is dependent on the positively acting bHLH transcription factor atonal. Loss of *atonal* function leads to a loss of neurons in the eye, while overexpression leads to the formation of supernumerary neurons (Jarman et al 1994). As previously described, the activity of *atonal* is antagonized by the bHLH factor encoded by *hairy* and the HLH factor encoded by *emc* (Brown et al 1994). Overexpression of each of these genes inhibits neuronal formation while loss of both genes leads to precocious neuronal differentiation coincident with precocious atonal expression ahead of the morphogenetic furrow (Brown et al 1994). Triggering such precocious neuronal differentiation results in gross morphological abnormalities in the eye, revealing that coordinated differentiation is critical for formation of the tissue.

A similar situation may exist in the developing mammalian telencephalon. The negative transcriptional regulator *Hes1*, the mammalian ortholog of *E(spl)*, is required for telencephalic development (Ishibashi et al 1995). Disruption of the *Hes1* gene in transgenic mice leads to precocious neuronal differentiation in the developing telencephalon and gross morphological abnormalities including failed closure of the anterior neural tube (Ishibashi et al 1995). These results suggest that *Hes1* may normally

repress neuronal differentiation and potentiate proliferation in neural precursor cells. In its absence, precursor cells appeared to differentiate prematurely, and such differentiation may deplete the pool of precursors to the point where not enough cells are available to complete the formation of the anterior nervous system. Several positively acting bHLH genes are expressed in the telencephalon, including *Mash1*, *ngn1*, and *ngn2* (Ma et al 1997; Sommer et al 1996; Lo et al 1991; Guillemot and Joyner 1993). *Mash1* mutant mice display morphological abnormalities in the telencephalon, possibly due to perturbations in the coordination and timing of neuronal differentiation (Tuttle et al 1999; Casarosa et al 1999; Hirsch et al 1998; Torii et al 1999; Horton et al 1999). In cultured hippocampal neurons, *Hes1* and *Mash1* have been found to exert antagonistic effects on differentiation (Castella et al 1999), and similar findings have been reported from studies focussing on the mammalian retina (Tomita et al 1996a, b). In addition, loss of function and gain of function studies *in vivo* have suggested opposing roles for *Hes1* (Ishibashi et al 1994; Ishibashi et al 1995), and the positively acting factors *ngn1* (Ma et al 1996; Ma et al 1998), *ngn2* (Fode et al 1998) and *Mash1* (Guillemot et al 1993; Tomita et al 1996a, b). Studies in Xenopus have also suggested that the forced early expression of the positively acting bHLH gene *NeuroD* can cause precocious neuronal differentiation (Lee et al 1995). These results suggest that positive and negative regulatory mechanisms control the timing of neuronal differentiation in mammals, a scheme to which the negative regulation of neuronal genes by RBP-Jk/Su(H) might contribute. As previously mentioned, disruption of the *RBP-Jk/Su(H)* gene in transgenic mice led to precocious neuronal differentiation and early embryonic lethality.

The winged helix transcription factor encoded by the *BF-1* gene may be similarly involved in the formation of the telencephalon. *BF-1* mutant mice exhibit severe morphological and gene expression defects in the telencephalon, particularly in the ventral domain (Xuan et al 1995). These defects are accompanied by the precocious expression of neuronal genes and a decrease in the number of proliferating precursors (Xuan et al 1995). An avian oncogene related to *BF-1* has been identified, suggesting that endogenous *BF-1* may regulate proliferation (Li and Vogt 1993). *BF-1* may therefore contribute to the enormous increase in cell number observed in the telencephalon, and

may influence directly or indirectly the timing of neuronal differentiation. The consequences of underproliferation and precocious differentiation in the telencephalon are dramatic as illustrated by *BF-1* mutant mice (Xuan et al 1995). *In vivo* studies by Caviness et al have suggested that the majority of the neurons of the cerebrum are formed in a relatively short period of time (approximately six days) (Caviness et al 1995). Based on *in vivo* measurements of proliferation, it has been hypothesized that the precursor pool expands up until E14 at which point there is a net contraction in the size of the pool (Caviness et al 1995). Minor changes in proliferation or the timing of differentiation are predicted to greatly alter the number of neurons produced and presumably the morphology of the tissue which is sculpted from these cells (Caviness et al 1995). Timing may be particularly important in the telencephalon where formation of the neocortex demands the production of a great number of neurons. This may require controls unique to the region, and may also make the region particularly sensitive to the manipulation of unique or widespread controls.

A comparison of *ΔNRE-nlacZ* mice and the previously characterized *nestin-lacZ* transgenic mice (in which all neural progenitor cells express β-gal) (Zimmerman et al 1994) revealed that the *ΔNRE-nlacZ* transgene was expressed in only a subset of precursor cells. This restriction may reflect redundant repressive mechanisms or an absence of stimulatory mechanisms operating on the remaining *Tα1* promoter sequence in non-expressing precursor cells. Notably, the remaining *Tα1* promoter sequence contains an additional putative RBP-Jk/Su(H) binding site, but does not contain any of the elements previously implicated in the repression of neuronal gene expression (Takebayashi et al 1995; Weber and Skeene 1997; Chong et al 1995; Schoenherr and Anderson 1995). Regardless of mechanism, the *ΔNRE-nlacZ* expression pattern revealed heterozygosity among neural precursor cells *in vivo*, as has been previously observed (Lillien 1998). These differences may have been due to molecular differences between precursor cells in different lineages at equivalent stages of development or alternatively may have reflected precursor cells of common lineages at different developmental stages (Lillien 1998).

Structurally and biochemically similar "determination" and "differentiation" factors belonging to the bHLH family of transcription factors are expressed successively at different stages of neuronal development (Lee 1997). In the absence of *RBP-Jk/Su(H)* function, determination factors may activate neuronal genes as differentiation factors are normally thought to do at later stages. Removing the NRE may have unmasked an interaction between such early inducers of neuronal specification and a neuronal gene, and revealed a requirement for early repression.

Neuronal "determination" that have been found in precursor cells of the mammalian CNS and PNS include *Mash1* (Johnson et al 1990), *ngn1* (Ma et al 1997) and *ngn2* (Sommer et al 1996). These transcription factors fall into the bHLH class, and are believed to heterodimerize with products of the *E2A* gene, the ubiquitously expressed homolog of *da*. Mash1/E2A heterodimers bind the E-box DNA sequence and can activate transcription in an E-box dependent manner *in vitro* (Johnson et al 1992). The NRE itself contains an E-box that overlaps with the RBP-Jk/Su(H) site, suggesting a direct mechanism by which RBP-Jk/Su(H) could compete with positive bHLH factors to repress *Tα1* promoter activity. Interestingly, several E-box sequences are found outside of the ΔNRE region of the *Tα1* promoter, providing a possible mechanism for *ΔNRE-nlacZ* gene activation by bHLH factors in neural progenitor cells. The remaining consensus RBP-Jk/Su(H) site lying outside of the NRE region may establish a threshold for gene activation that is not overcome by these positive factors at all stages of neural precursor cell development, and this may be partially responsible for the restriction of *ΔNRE-nlacZ* expression to a subset of neural precursor cells. Biochemical studies of RBP-Jk/Su(H) have revealed that the transcription factor need not compete for binding sites in order to repress transcriptional activation. RBP-Jk/Su(H) can interact with the basal transcription factors TFIIA and TFIID to repress transcriptional activation directly (Olave et al 1998), and cn interact with the histone deacetylase HDAC1 to repress transcription through histone modification (Kao et al 1998).

(v) The Interrelationship of Multiple Aspects of Neuronal Differentiation

The generation of a neuron from a neural precursor cell involves a number of changes. Three intrinsic scoreable aspects of neuronal differentiation are the cessation of cell division, induction of constituent neuronal genes, and morphological elaboration. We have focussed on the regulation of neuronal gene induction and tried to draw conclusions about the control of neuronal differentiation generally. To do this, we must know how these processes relate to one and other, and whether or not they (and any other aspects of neuronal differentiation) are coordinately controlled by common regulatory mechanisms. Are terminal mitosis and neuronal gene induction coordinately regulated? Does cessation of cell division drive neuronal gene induction or vice versa? Does constituent neuronal gene induction drive morphological differentiation?

It should first be noted that a subpopulation of neural precursor cells normally express neuronal genes and initiate morphological differentiation prior to cell cycle exit. Neural precursors of the sympathetic nervous system express constituent neuronal genes and begin to elaborate processes prior to terminal mitosis (Rohrer and Thoenen 1987; Dicicco-Bloom et al 1990). Notably, this population also expresses the *Tα1-nlacZ* transgene (Gloster et al 1999). These findings demonstrate that the processes of cell cycle exit and other aspects of neuronal differentiation are separable in mammals. However, the regulation of cell cycle exit and/or other aspects of neuronal differentiation may be distinct in the sympathetic precursor population, and may not reflect the independence of the processes in all neural precursor cells.

Studies in muscle and adipocyte differentiation have suggested that constituent gene expression and cell cycle exit are coordinately regulated. In both of these systems transcription factors that regulate constituent gene expression and stimulate differentiation interact directly with molecules that regulate the cell cycle (Gu et al 1993). Moreover, the ability of these transcription factors to stimulate differentiation depends upon these interactions (Gu et al 1993). Interestingly, the molecules regulating the cell

cycle are required not only for cell cycle exit, but also for the induction of constituent genes (Gu et al 1993; Scneider et al 1994). This suggests that transcription factors and components of the cell cycle machinery may form complexes that coordinately induce constituent genes and cell cycle exit.

The tumour suppressor retinoblastoma gene product (Rb), best known for its role in the inhibition of cell cycle progression (reviewed in Weinberg 1995), is required for myogenesis (Novitch et al 1996), adipogenesis (Chen et al 1996), and neurogenesis (Jacks et al 1992; Slack et al 1998). Viral oncoproteins that inactivate Rb also inhibit myogenesis (eg. Tedesco et al 1995), supporting a role for Rb in muscle differentiation. A number of transcription factors of the bHLH class are also required for myogenesis and are sufficient to initiate the process in a number of non-muscle cell types (reviewed in Megeney and Rudnicki 1995). The myogenic bHLH factors are able to induce muscle specific genes and to arrest cell growth (Megeney and Rudnicki 1995). These proteins interact molecularly with the retinoblastoma gene product and this interaction is required for muscle gene induction, cell cycle arrest, and muscle differentiation (Gu et al 1993). It is hypothesized that these interactions tie constituent gene expression to cell cycle arrest, as the retinoblastoma protein is thought to be liberated and free to interact with the myogenic bHLHs at the time of cell cycle arrest. What remains unclear is how this association then initiates irreversible changes precluding another cell division and why Rb is required for the activation of muscle specific genes by the myogenic bHLH factors. The mechanism may involve Rb's previously reported ability to interact with the histone deacetylase "HDAC1" (Brehm et al 1998; Magnaghi-Jaulin et al 1998), or its ability to prevent other transcription factors from contacting basal transcription factors and affecting transcription (Weintraub et al 1995). It is also interesting to note that activation of the Notch pathway can inhibit myogenesis (Nofzinger et al 1999; Shawber et al 1996), suggesting that whatever the mechanism, it may be opposed by molecular components of the Notch pathway at some point.

Similarly, adipocyte differentiation also depends upon particular transcription factors and Rb. The C/EBP gene encodes a transcription factor of the leucine zipper family (Lowherd

et al 1999). Members of this transcription factor family bind DNA as dimers through a basic stretch of amino acids preceeding the leucine zipper motif (Cowherd et al 1999). C/EBP is required for the differentiation of adipocytes, though unlike the myogenic bHLHs, C/EBP is not sufficient to convert other cell types to an adipocytic fate (Cowherd et al 1999). C/EBP does however interact molecularly with the retinoblastoma protein, like the myogenic bHLHs, and this interaction is required for the differentiation of adipocytes (Chen et al 1996). *In vitro* studies have shown that in the absence of Rb, adipocytes are unable differentiate, and that the introduction of exogenous Rb restores their ability to differentiate (Chen et al 1996).

Experiments utilizing mice that are homozygous null at the *Rb* locus have revealed that *Rb* is also required for neurogenesis (Jacks et al 1992; Slack et al 1998). Mice lacking a functional *Rb* gene have severe myeloid and neural defects, and die by embryonic day 15 with extensive cell death observed in the brain (Jacks et al 1992). In the developing frontal cortex of these embryos, neuron-specific gene expression is detected and ectopic mitoses are observed in the developing cortical plate (Slack et al 1998). The cell death and ectopic mitoses may be related, as inappropriate cell cycle progression in presumably postmitotic neurons has been hypothesized to trigger cell death (Ross 1996; Heintz 1993).

The results of *Rb* gene disruption suggest that *Rb* is required for cell cycle exit, but not for neuronal gene induction. They also suggest that the two processes might be separable, though whether cells undergoing mitosis in ectopic locations also expressed neuronal genes is unclear. These results have been supported by *in vitro* studies demonstrating that the Rb family of proteins is not required for neuronal gene induction nor the survival of differentiated neurons, but is required for an early event in neuronal differentiation (Slack et al 1998). Whether *Rb*-deficient neural precursor cells expressed neuronal genes without undergoing terminal mitosis *in vivo* is not known, and whether other members of the Rb family are required for neuronal gene expression and terminal mitosis in the absence of *Rb* is unknown. Other members of the Rb family are elevated in cells cultured from mice lacking *Rb*, suggesting they may compensate for a loss of *Rb* function

(Callaghan et al 1999). In addition, disruption of the Rb family member *p130* led to the loss of a variety of neuronal subtypes (LeCouter et al 1998). The number of motor neurons and sensory neurons was substantially decreased in *p130* mutants and correlated with an extensive and substantial increase in the amount of apoptosis observed during development. Interestingly, ectopic proliferation in regions normally occupied by postmitotic neurons was also evident, making the consequences of *Rb* disruption and *p130* disruption quite similar.

Conversely, in overexpression studies, forced expression of *Rb* induced growth arrest in several cell types *in vitro* and *in vivo* (Lipinski and Jacks 1999) and also induced neuronal differentiation in several neuroblastoma cell lines (Raschella et al 1998). Whether differentiation was directly or indirectly induced, and whether this induction can be observed *in vivo* is not known. However, the dwarfism observed in transgenic mice overexpressing *Rb* may have reflected a drop in the number of precursors through perturbations in both growth arrest and differentiation (Bignon et al 1993).

The vertebrate *Id* genes are homologs of the Drosophila *emc* gene, and play widespread roles in the control of cell differentiation and proliferation (reviewed in Norton et al 1998). There are currently four identified *Id* genes, which are widely expressed in distinct and overlapping domains (Norton et al 1998). The genes are dynamically regulated in a tissue-specific manner and differentially regulated in different cell lines. *Id1*, *Id2*, and *Id3* are expressed in neural precursor cells in the developing nervous system. Both *Id1* and *Id3* are expressed in neural precursor cells of the developing CNS (Norton et al 1998). *Id1/Id3* homozygous double mutant mice die late in embryogenesis and are notably smaller by E11.5 (Lyden et al 1999). Large deficits are observed in the telencephalon of mutants, correlating with decreased numbers of proliferating precursor cells and accelerated expression of neuronal bHLH genes and postmitotic neuronal markers in the area (Lyden et al 1999). *Id2* is highly expressed in the embryonic ventricular zones and excluded from the mantle layer and cortical plate in the spinal cord and brain respectively (Neuman et al 1993). Similar expression is also observed in Xenopus, where two *Id* genes have been identified and are expressed in the developing nervous system (Zhang et al

1995; Wilson and Mohun 1995). Culture studies imply that downregulation of *Id2* expression is a pre-requisite for neuronal differentiation and that sustained *Id2* expression can inhibit differentiation (Iavarone et al 1994). Whether *Id* gene regulation, like *emc*, is independent of the Notch pathway (Artavanis-Tsakonas et al 1995) remains to be seen.

The *Id* gene products appear to interface directly with cell cycle machinery. Id2 interacts *in vitro* and *in vivo* with the Rb, and Id1 and Id3 interact with Rb *in vitro* (Norton et al 1998). The HLH domain of Id2 is required for this interaction, as is the pocket domain of Rb. The pocket domain is conserved and required for the inhibition of cell growth by Rb (Lipinski and Jacks 1999). It is found in the related proteins p107 and p130 which also regulate cell growth (Lipinski and Jacks 1999) and bind Id2 (Norton et al 1998). It has been proposed that the Id proteins function in neural progenitor cells within the ventricular zone by binding bHLH specification/differentiation factors and inhibiting their DNA-binding activity, as well as by binding Rb (and other family members) thereby potentiating proliferation (inhibiting cell cycle arrest). Studies of myogenesis have revealed that Id1 can inhibit muscle cell differentiation by antagonizing the activity of myogenic bHLH transcription factors (Jen et al 1992). Biochemical studies have also revealed that Id is required for the G1/S transition of the cell cycle (Norton et al 1998). Based on these findings it is tempting to suggest that *Id* gene products may regulate neuronal differentiation. Their downregulation may release previously bound proneural/differentiation bHLH factors and Rb simultaneously, thereby coordinating cell cycle exit and neuronal gene induction. The overt *in vivo* crisis point at E11.5 in *Id1/Id3* double mutants is a time of extensive proliferation and roughly marks the beginning of neurogenesis in the basal ganglia (Sussel et al 1998), an area severely affected in these mutant mice (Lyden et al 1999). It is conceivable that neural precursors from *Id1/Id3* mutants might experience difficulty re-entering the cell cycle after each division.

However certain findings conflict with this hypothesis. Recently it was found that forced expression of *Id2* in avian neural crest progenitor cells induced neuronal differentiation rather than inhibiting it (Martinsen and Bronner-Fraser 1998). Furthermore, disruption of the *Id2* gene in mice did not result in an overt neural phenotype (Yokota et al 1999). The

mechanism responsible for the differentiation promoting action of *Id2* is not clear, nor is it clear that it is a cell autonomous effect. The lack of an overt phenotype in transgenic mice may be complicated by gene redundancy and mechanisms of compensation. Interestingly, E-boxes are found in the *Id2* promoter (Neuman et al 1995), and Id2 is believed to negatively autoregulate the *Id2* gene by binding and inhibiting bHLH factors that stimulate its expression. If present in other *Id* genes, E-boxes may make cross regulation and compensation possible.

A role for other cell cycle molecules in the regulation of neurogenesis may also follow from studies of myogenesis. The cyclin-dependent kinase inhibitors (cdki's) are a family of proteins that inhibit one or more of the cyclin-dependent protein kinases that function at discrete stages of the cell cycle promoting progression through it (reviewed in Sherr and Roberts 1999). By inhibiting these kinases, cdki's antagonize cell cycle progression and are capable of arresting a cell at specific stages. It has been demonstrated that the cdki *p21* is induced coincident with MyoD induction and the initiation of myogenesis in culture (Skapek et al 1995; Halevy et al 1995). This induction may account in part for the growth arrest promoted by MyoD (Wientraub et al 1994). Similarly, several cdki's have been found to be elevated coincident with neuronal differentiation in culture models (Kranenburg et al 1995). Whether or not these factors fall under the control of the similar proneural/differentiation bHLH factors in this neural context and whether this connection is direct or indirect remains unknown.

An interesting connection between the transcription factor MEF2C and the tumour suppressor protein Rb has been made in the context of muscle development. The activity of MEF2C correlates with the presence of active Rb protein (Novitch et al 1999; Wilson-Rawls et al 1999). MEF2C is competent to bind DNA in the absence of Rb, but does not activate transcription nor stimulate muscle differentiation in cooperation with MyoD (Novitch et al 1999; Wilson-Rawls et al 1999). In the presence of Rb, MEF2C activates transcription and induces myogenesis in cooperation with MyoD (Novitch et al 1999; Wilson-Rawls et al 1999). Thus the ability of MEF2C to activate transcription may be regulated by Rb, which could tie cell cycle exit to muscle gene expression. The

mechanism could involve Rb's previously described abilities to interact with HDAC1 and to prevent other factors from contacting basal transcription factors (Brehm et al 1998; Magnaghi-Jaulin et al 1998; Weintraub et al 1995). Coupled with the fact that Notch opposes MEF2C DNA binding activity and myogenesis coincidentally (Kopan et al 1994), it appears that MEF2C may be a pivotal point of control in myogenesis. Whether a similar mechanism operates in neurogenesis, where *MEF2C* is known to be coexpressed with *Mash1* (Leifer et al 1993) and to cooperate with Mash1 to activate transcription (Black et al 1996), is unknown. Interestingly, activity-dependent neuronal survival in developing neurons has been shown to require *MEF2C* (Mao et al 1999). In addition, the expression of Mash1 in cultured neural crest stem cells drove them to differentiation by the criteria of morphology and gene expression (Lo et al 1999). This is similar to *MyoD*'s ability to direct myogenic differentiation (Weintraub et al 1994), but is the only reported context in which this aspect of *Mash1* activity can operate. Terminal mitosis was presumably achieved, but whether Mash1 interacts with molecules of the cell cycle analogous to MyoD is uncertain. An interaction between Rb and Mash1 has not been detected.

The necdin protein may also play a role in the induction and maintenance of growth arrest during neuronal differentiation, but through a distinct mechanism. *Necdin* encodes a nuclear protein and is expressed in nearly all postmitotic neurons (Aizawa et al 1992). Necdin promotes growth arrest and appears to do so through molecular interaction with the transcription factor E2F (Hayashi et al 1995; Taniura et al 1998). The association with E2F is believed to target the transcription factor for degradation via the ubiquitin pathway, inhibiting proliferation normally promoted by E2F. This mechanism may contribute to locking neurons out of the cell cycle. Interestingly, E2F also interacts with Rb and this interaction is modulated during G1 of the cell cycle (reviewed in Weinberg 1995). Inhibition of this interaction through the modification of Rb results in entry into S phase, while failure to inhibit this interaction results in growth arrest (Weinberg 1995). This is a key point of regulation in the cell cycle, and it appears that factors regulating and maintaining neuronal differentiation may target distinct components of the cell cycle apparatus at different times in order to perform their functions. The physiological

function of necdin has been called into question recently by the results of gene disruption in transgenic mice (Tsai et al, 1999). The chromosomal location of necdin had implicated it in Prader Willi syndrome, a condition that is associated with a specific chromosomal deletion and symptomatically presents with mental retardation (Nakada et al 1998). None of the effects characteristic of Prader Willi syndrome, nor those resulting from a corresponding chromosomal deletion in mice were produced by specific deletion of the necdin gene. Moreover, no gross anatomical abnormalities were identified in the nervous system of necdin null mice.

There is also evidence for the involvement of the Notch pathway in the regulation of proliferation, which potentially connects the pathway to all three aspects of neuronal differentiation considered. Whether the regulation of different aspects of neuronal differentiation by *Notch* occurs through common mechanisms is an interesting and unresolved issue. Notch pathway manipulation at the level of the receptor, the ligand, or downstream transcription factors affects cell differentiation and proliferation in the developing Drosophila eye (reviwed in Artavanis-Tsakonas et al 1999). In C. elegans, the glp-1 (Notch) protein is expressed in the developing germ cell line (reviewed in Greenwald 1998). Loss of glp-1 or ligand function leads to a loss of germ cells, which fail to proliferate in response to induction from an adjacent cell (Greenwald 1998). Conversely, a glp-1 gain of function mutation causes germ cell hyperproliferation (Greenwald 1998). In mice, a retrovirus expressing an activated mammalian *Notch* allele is sufficient to induce tumour formation in predisposed mammalian cells (Copabianco et al 1997). The expression of an activated *Notch* allele may also affect proliferation in progenitor cells of the rat retina (Bao and Cepko 1997). Overexpression of *Hes1*, a mammalian *E(spl)* gene, inhibits neuronal differentiation and possibly the proliferation of precursor cells in the mouse CNS (Ishibashi et al 1994). Epstein Barr virus normally utilizes the host *RBP-Jk/Su(H)* gene in the transformation of mammalian B cells (Henkel et al 1994), potentially implicating *RBP-Jk/Su(H)* in proliferation control. In addition, a naturally occurring translocation of the *Notch* locus in humans is associated with B cell lymphoma (Ellisen et al 1991), suggesting Notch pathway activation may be involved in B cell proliferation in humans.

The function of the constituent neuronal gene *GAP-43* has been addressed with respect to neuronal growth. Overexpression of *GAP-43* in postmitotic neurons was sufficient to cause neurite growth (Aigner et al 1995), while disruption of the *GAP-43* gene led to growth cone dysfunction and defective neurite outgrowth (Stritmatter et al 1995). This suggests *GAP-43* must be tightly regulated in mature neurons, possibly by contact mediated inhibitory mechanisms such as that of the Notch pathway. Overexpression of the *peripherin* gene, which encodes a neuronal intermediate filament protein and contains an NRE (Belecky-Adams et al 1993), promotes the death of mature motor neurons (Beaulieu et al 1999), suggesting its regulation must also be tightly controlled. The consequences of *Tα1* deregulation are unknown. However, *Tα1* mRNA accumulation has been uncoupled from neuronal growth in mature nervous system following injury (Tetzlaff et al 1991). Whether α-tubulin protein synthesis is also uncoupled in this situation, and whether this uncoupling is due to downstream mechanisms unique to the CNS is unknown.

Post-translational control of tubulin protein synthesis has been described previously, whereby excess free tubulin protein destabilizes its encoding mRNA by an as yet undetermined mechanism (Cleveland et al 1983; reviewed in Cleveland 1989). Posttranscriptional regulation of *Tα1 α-tubulin* expression has also been intimated by a comparison of *Tα1-nlacZ* and endogenous *Tα1* gene expression following injury (Wu et al 1997). In peripheral nerve, the loss of target contact without loss of axons led to an induction of *Tα1* promoter activity while causing only a minimal increase in *Tα1* mRNA expression (Wu et al 1997). This sugests that following injury, transcriptional mechanisms elevated *Tα1* expression while post-transcriptional mechanisms may have monitored the free α-tubulin protein and affected *Tα1* mRNA stability accordingly in order to fine-tune the amount of tubulin present. According to this hypothesis, *Tα1* and *Tα1-nlacZ* would both be sensitive to the loss of target contact and would be induced at the transcriptional level, but only the *Tα1-nlacZ* mRNA level would increase as it escaped posttranscriptional regulation. A neccessity for such fine tuning may be indicated by the dire consequences of structural gene overexpression in neurons (Beaulieu et al

1999). Whether forced expression of *Tα1* in the PNS would cause growth similar to that seen with *GAP-43* overexpression or death as with *peripherin* over expression is unknown. Finally, the consequences of ectopically expressing any of these genes in undifferentiated neural precursor cells is unknown.

In summary, it appears that constituent gene expression and cell cycle exit could potentially be coordinately regulated by a number of mechanisms based on evidence of such coordination in myogenesis. In some mechanisms, molecules involved in cell cycle regulation may interact directly with those controlling constituent gene expression and participate in the regulation of constituent genes in some manner. Thus cell cycle exit and constituent gene expression may not be independent and lie downstream of a master regulatory mechanism. However, there is much less evidence for such operative mechanisms in neurogenesis than there is in myogenesis. Finally, the morphological elaboration characteristic of neuronal differentiation appears to be a consequence of constituent gene expression, and control over structural constituent gene expression appears to be important for survival, neurite growth, and ultimately the function of neural networks. The NRE may function in the control of early neuronal gene induction and morphological elaboration, as well as growth associated gene regulation and morphological elaboration in mature neurons. This regulation may be mediated by RBP-Jk and its intrinsic transcriptionally repressive activity, and may involve the Notch pathway, which has been implicated in the regulation of all three aspects of neuronal differentiation discussed.

Materials and Methods

(a) Generation of Transgenic Mice

(i) Tα1-nlacZ Transgene Construction

Construction of the Tα1-nlacZ transgene and Tα1-nlacZ transgenic mice has been previously described (Gloster et al 1994). Standard screening and cloning methods were employed as detailed in Maniatis et al 1989. Briefly, a 1.9kb fragment of genomic DNA was isolated after probing a Wistar rat lambda-DASH genomic library with end-labeled oligonucleotides corresponding to the previously published 5' untranslated region and immediately 5' flanking region of the Tα1 α-tubulin gene (Lemischka and Sharp 1982). The 1.9kb fragment of genomic DNA contained 1.6kb of 5' flanking sequence, the 5' untranslated sequence (99 nucleotides), the translation start site (exon 1) and 200 nucleotides of intron 2. An Sst1/Nco1 partial digestion product that contained 1028 nucleotides of 5' flanking sequence, the 5' untranslated region (99 nucleotides), and the ATG translation start site was isolated. This fragment was fused to a modified E. coli lacZ gene in a puc19 vector that contained an N-terminal nuclear translocation signal sequence derived from SV40 T antigen (Kalderon et al 1984), and the murine protoamine 1 gene from +95 to +625 (Peschon et al 1987) at the C-terminus of lacZ. This protoamine 1 gene fragment provided an intron and a polyadenylation signal. The resulting vector was denoted Tα1-nlacZ:puc19.

(ii) ΔNRE-nlacZ Transgene Construction

Mutagenesis of the 1028-nucleotide Tα1 promoter fragment was performed using the Promega "Altered Sites" site directed mutagenesis kit and protocol. Standard cloning methods were employed as detailed in Maniatis et al 1989. A 5.8kb SalI/HindIII fragment from the Tα1-nlacZ-pUC19 vector which carried the full 1028-nucletode Tα1

promoter sequence, the 5' untranslated sequence and the modified lacZ gene, was inserted into SalI/HindIII digested "pALTER" (Promega). The resultant vector, "Tα1-nlacZ-pALTER" was used to transform E. coli strain JM109 , which were subsequently infected with bacteripohage R408 (Promega). Single stranded Tα1-nlacZ-pALTER sequence was purified from bacteriophage, and site directed mutagenesis was performed to delete a specific 66-nucleotide sequence from the 1028-nucleotide 5' flanking sequence of the Tα1 gene. The sequence from –674 to –609 inclusive (Gloster et al 1994) was deleted. A mutagenic single stranded oligonucleotide, (AACCACTAAGGGCGGGTGGTCTATTCATAC) and the single stranded selection oligonucleotide "amp-r" (Promega) were annealed to the single stranded Tα1-nlacZ-pALTER, synthesis was completed *in vitro* according to the protocol and using the DNA polymerase provided, and the resultant mutant vector was used to transform the E. coli repair mutant strain MutS. The amp-r oligonucleotide corrected a preexisting mutation in the gene conferring ampicillin resistance in pALTER, and transformants carrying the mutant vector were selected in ampicillin. Mutated Tα1-nlacZ-pALTER was purified and sequenced using the "Sequenase" kit and protocol (Promega), employing the di-deoxy method (Sanger et al 1977) and ^{32}-P dCTP (Amersham). The Tα1 sequencing oligonucleotide (CAAAATAACCGCAGT) was used to prime the sequencing reaction.

The 66-nucleotide deletion removed the NRE and its flanking sequences from the Tα1 promoter fragment, leaving the resultant "ΔNRE promoter". The deleted sequence included a number of consensus sequences representing potential binding sites for bHLH transcription factors (E-box), retinoic acid receptors (RARE), the estrogen receptor (ERE), the SP1 zinc finger transcription factor (SP1), the conserved transcription factor RBP-Jk/Su(H) and a gamma-interferon responsive factor (IRE) (Gloster et al 1994).

The ΔNRE promoter remained upstream of the 5' untranslated Tα1 gene sequence, the translation start site, and the modified lacZ gene carrying an SV40 nuclear localization sequence and the murine protoamine-1 gene fragment, creating the "ΔNRE-nlacZ" transgene. Figure 2 shows schematically how ΔNRE-nlacZ was constructed.

(iii) ΔFRE-nlacZ Transgene Construction

Site directed mutagenesis was performed similarly a second time using the "Altered Sites" kit and protocol (Promega) to delete a specific 184-nucleotide sequence from the 1028-nucleotide 5' flanking sequence of the Tα1 gene, generating the ΔFRE (forebrain response element) promoter. The sequence from –674 and –491 inclusive (Gloster et al 1994) was deleted. Single stranded Tα1-nlacZ-pALTER was purified from bacteriophage and a complimentary strand was synthesized in vitro using the selection "amp-r" oligonucleotide and the mutagenic oligonucleotide AACCACTAAGGGCGGTCATTCCCATAGCTC as primers for synthesis. Transformants carrying the mutant "ΔFRE-nlacZ-pALTER" plasmid were selected in ampicillin. ΔFRE-nlacZ-pALTER was purified and sequenced using the same method and sequencing primer as for ΔNRE-nlacZ.

The deleted sequence included the 66-nucleotide segment that was deleted to generate the ΔNRE promoter, as well as 3' flanking sequence. In addition to the consensus sequences common to the 66-nucleotide deletion, the 184-nucleotide deletion also removed a conserved 30-nucleotide sequence that constitutes a tandem repeat of a homeodomain consensus element. This putative homeodomain-binding element is located 41 nucleotides 5' of the 3' end of the deleted 66-nucleotide NRE-containing sequence. Figure 2 shows schematically how ΔFRE-nlacZ was constructed.

(iv) Generation of Transgenic Mice

Transgenic mice were generated by injecting purified linearized transgene DNA into the pronuclei of either CD1 (Tα1-nlacZ) or C3H (ΔNRE-nlacZ and ΔFRE-nlacZ) single cell embryos. Embryo purification, DNA injection, and implantation of injected embryos was performed according to standard methods as detailed in Hogan et al 1986.

The Tα1-nlacZ transgene fragment was excised from Tα1-nlacZ:puc19 using HindIII and SalI. Digestion products were electrophoresed and the 5.8kb transgene fragment was isolated using the "Qiaex Gel Extraction" kit (Qiagen). Transgenic mice were generated in the laboratory of John Roder (University of Toronto) by injecting a linearized and gel purified Tα1-nlacZ transgene fragment into the pronuclei of CD1 single cell embryos. Surviving embryos were implanted into pseudopregnant females at embryonic day 0.5 (the morning of plug detection). Between 20 and 30 embryos were implanted per female to avoid small litter sizes. Eight distinct founder Tα1-nlacZ transgenic lines were identified by genotype analysis (described below) , and X-gal staining (described below) of E14.5 embryos suggested that five of these lines expressed the transgene robustly in the nervous system. One of these lines is described in the present report, namely line "K6".

The 5.8kb ΔNRE-nlacZ transgene fragment was excised from ΔNRE-nlacZ-pALTER using SalI and HindIII, and was purified using the Qiaex gel extraction kit (Qiagen). ΔNRE-nlacZ-pALTER transgenic mice were generated in the laboratory of Dr. Alan Peterson (Royal Victoria Hospital, McGill University) by injecting the purified ΔNRE-nlacZ transgene fragment into the pronuclei of C3H. Embryos were implanted into false pregnant females and litters were analyzed for the presence of the transgene. Five distinct founder lines were identified by genotype analysis (described below) and X-gal staining (described below) indicated that three of these expressed the transgene at E14.5. Two expressing lines were used for subsequent analysis, namely lines 9 and 23.

The 5.6kb ΔFRE-nlacZ transgene was excised from ΔFRE-nlacZ-pALTER using SalI and HindIII, and the fragment was purified using the Qiaex gel extraction kit (Qiagen). Transgenic mice were generated similarly in the laboratory of Dr. Alan Peterson by injecting the purified ΔFRE-nlacZ transgene fragment into the pronuclei of C3H embryos. The transgene was detected in six founder mice (described below) and X-gal staining of these lines (described below) suggested that three of these expressed the transgene at E14.5. Two expressing lines were used for subsequent analysis, namely lines 1 and 17.

(v) Genotyping Transgenic Mice

Genomic DNA was isolated (Maniatis et al 1989) from tail clippings of founder mice born of pseudopregnant females receiving implants. Genomic DNA was analyzed for incorporation of the appropriate transgene by Southern blot analysis (Maniatis et al 1989) and by PCR analysis (Ausubel et al 1996). For Southern blot analysis, EcoRI was used to digest 5μg tail DNA overnight, and the digestion reaction was electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-labelled oligonucleotide corresponding to 35 nucleotides of the Tα1 promoter, from –332 to -298 inclusively (TTATCCTAACTACAGTTTAAGCTCCGTATAATCAC). The oligonucleotide was end-labelled using T4 polynucleotide kinase and ^{32}P gamma-ATP at 37°C for one hour according to standard methods (Maniatis et al 1989), and then separated from free nucleotide by spinning through "Nick" sephadex columns (Boehringer Manheim) at 2000rpm for 20min. To block non-specific binding of the probe, the nitrocellulose was preincubated in blocking solution that included DNA from herring sperm and salmon sperm (Maniatis et al 1989). The oligonucleotide probe was incubated with the nitrocellulose overnight at 50°C and then washed three times in phosphate-bufferred saline (Manitais et al 1989) before being exposed overnight to XAR film (Kodak) at -70°C.

For PCR analysis, the 5' primer was a 15mer (ATCCCCATGGTGACC) corresponding to Tα1 promoter sequence from –51 to -37, and the 3' primer was a 15mer corresponding to the lacZ sequence +536 to +522 (ATCACCGCGAGGCGG). The PCR reaction produced an ~650bp product spanning the Tα1/lacZ junction in the transgene. Twenty-five cycles were used to amplify the 650bp sequence from ~500ng of genomic DNA (Ausubel et al 1996).

(b) Analysis of Whole Embryos and Adult Brain Sections

(i) Animals

For the studies described here, transgenic males that were homozygous for *Tα1-nlacZ* were mated to control CD1 females, and transgenic males homozygous for *ΔNRE-nlacZ* or *ΔFRE-nlacZ* were mated to control C3H females. Mice were paired in the evening, and the morning of vaginal plug detection was designated embryonic day 0.5. This convention has also been followed in the papers cited in this report. Pregnant females were anaesthetized with sodium pentobarbital (70mg/kg), and litters were removed by cesarian section.

For adult mouse brain sections, mice were anaesthetized with sodium pentobarbital (35mg/kg) and perfused transcardially with 25mL of phosphate-buffered saline followed immediately by 25mL of ice-cold 4% paraformaldehyde, 0.1 M NaH_2PO_4. Brains were removed and sliced coronally into 1-2mm thick sections using razor blades, rinsed in 2mM $MgCl_2$, 0.01% sodium, deoxycholate, 0.02M NaH_2PO_4, 0.15M Na_2HPO_4, and 0.02% NP-40 at pH7.4, and then stained in X-gal staining solution (described below) overnight at 37°C.

(ii) Histology

The reporter gene used in promoter assays encoded a modified isoform of the enzyme β-galactosidase that was designed to be translocated to the nucleus (described above). In whole embryos and adult brain slices and sections, promoter activity was indirectly measured by assaying reporter gene activity. Whole embryos and adult brain slices were incubated with the β-gal ligand "X-gal" (Sigma) 1mg/ml in staining solution (described below) to assay for β-gal activity. The presence of β-gal activity was inferred from the deposition of an insoluble blue reaction product in the nucleus. To detect *β-gal*

expression in cultured cortical precursor cells, a mouse monoclonal anti-β-gal primary antibody (5'-3', Boulder CO) was used for immunolabeling.

For β-galactosidase staining, embryos were fixed for 5 min at 4°C in 4% paraformaldehyde, 0.1M NaH_2PO_4. Embryos were subsequently rinsed three times in "rinse buffer" comprised of 2mM $MgCl_2$, 0.01% sodium, deoxycholate, 0.02M NaH_2PO_4, 0.15M Na_2HPO_4, and 0.02% NP-40 at pH7.4 for 15 minutes per rinse. Embryos were then stained in a "staining solution" containing all the components of the rinse buffer with 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ added. After staining for 6 hours at 37°C, embryos were fixed for 24 hours in 4% paraformaldehyde, 0.1M NaH_2PO_4 at 4°C.

Adult brain slices were then rinsed three times in phosphate-buffered saline, and subsequently fixed for 24h in 4% paraformaldehyde, 0.1M NaH_2PO_4 at 4°C. For histology, adult brain slices were cryoprotected in graded sucrose solutions (10%, 12%, 16% and 18%). Brain slices were left in each solution for 2 hours at 4°C, or until they sank in solution. The slices were then embedded in "tissue tek", frozen on dry ice, and sectioned on a cryostat at 14μm thickness. Sections were dried at 37°C for 3 hours, counterstained with eosin/70% ethanol 1:1 (eosin stock 1.0g eosin/ 1000mL 70% ethanol, 5mL glacial acetic acid), dehydrated in ascending concentrations of ethanol (95%, 100%) for 2 minutes per stage, and then cleared with xylene for 10 minutes, followed by coverslipping.

(c) Embryonal Mouse Cortical Precursor Cultures

(i) Culture

The preparation of cortical progenitors from mouse embryos was based on the method described by Ghosh et al. (1995) for rat cultures. The dorsal aspect of the telencephalic vesicle was collected from E10.5 Tα1-nlacZ line K6 or E10.0 ΔNRE-nlacZ line 23

mouse embryos in ice-cold Hank's balanced salt solution (HBSS) (Gibco BRL) and triturated with a fire polished Pasteur pipette in culture medium consisting of Neurobasal medium (Gibco BRL) 0.5 mM glutamine, penicillin-streptomycin, 1% N2 supplement (Gibco BRL) and bFGF (40 ng/ml; Collaborative Research Inc., Bedford, MA). Small clusters of cells were plated on chamber slides (Nunc Inc.) at densities ranging from 20-50,000 cells per chamber. Cultures were brought up to volume with supplemented neurobasal medium (as above) and incubated at 37°C in 5% CO_2. We have previously shown that cortical neurons generated from E12.5 mouse progenitor cells in this manner can be maintained for at least 3 weeks under these conditions (Gloster et al 1999).

(ii) Immunohistochemistry and Hoecsht Staining

After 24 hours, the neurobasal culture media was aspirated and cells were rinsed briefly with PBS and fixed by immersion in 4% paraformaldehyde, 0.1M NaH_2PO_4, for 15 minutes at room temperature. Cultures were then washed with PBS and blocked for 30 minutes with buffer containing 5% normal goat serum (Sigma), 0.2% Tween-20, 1% glycine, 1% BSA (Sigma), and 5% milk. Cultures were then incubated overnight with the two primary antibodies, anti-β-galactosidase; rabbit polyclonal (IgG), (5 Prime 3 Prime; Boulder, CO, 1:500), and mouse monoclonal anti β-III tubulin (TUJ1) (Dr. A. Frankfurter, 1:300), containing 5% goat serum and 5% rat serum. Cells were subsequently washed three times with PBS, and incubated for 1 hour with buffer containing both CY3-conjugated goat-anti-rabbit (1:200) (Jackson) and CY2-conjugated goat-anti-mouse (IgG) (1:200) (Jackson) secondary antibodies. Cultures were then washed with PBS twice and stained with Hoecsht dye to visualize nuclei.

To visualize cell nuclei in cortical cultures, Hoechst stain (Sigma) was applied. Hoecsht stain was diluted 1:1000 in phosphate-buffered saline and 500μL of the dilution was added to chambers of fixed cells for 2 minutes at room temperature. The stain was then aspirated and chambers were washed three times with phosphate-buffered saline for 5

minutes. Hoechst staining was performed after immunostaining for β-III tubulin and β-gal was completed (ie. following washes that followed secondary antibody incubation).

(d) Nuclear Protein Preparation, *in vitro* Protein Synthesis and Electrophoretic Mobility Shift Assays

(i) Nuclear Protein Preparation

Whole E10.0 and E13.5 embryos, adult brain and liver, and postnatal day two brain samples were obtained and immersed in ice-cold 0.32M sucrose. Tissue was homogenized in 4 volumes of homogenization solution (0.5M sucrose, 10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 10% glycerol, 1mM EDTA, 1mM DTT, 1mM PMSF, 1μg/μl aprotinin/leupeptin/PEP A, 100μM vanadate) using a potter homogenizer. Approximately 10 strokes were used to homogenize tissues. Homogenate was collected, aliquotted into 1.5ml microfuge tubes and spun at 4000xg in a microfuge for 5 minutes at 4°C, the supernatant was removed, and the nuclear pellet was resuspended in 1 volume of lysis buffer (20mM HEPES pH7.9, 20% glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.3M NaCl, 0.5mM DTT, 0.5mM PMSF). Nuclei were incubated in lysis buffer for 30 minutes on ice, homogenized with a pipetteman, spun at 12000g for 20 minutes, and the supernatant was collected in 50μl aliquots, quick frozen in a dry ice ethanol bath, and stored at –80°C.

E12.5 cultured cortical precursor cells at one day in vitro and four days in vitro were harvested with 1 ml of TEN buffer (40 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl), and extracts were prepared according to the method of Dignam et al 1983, with some modifications. Briefly, cell pellets were resuspended in 30 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2 ,10 mMKCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mM dithiothreitol). After three cycles of freeze-thaw, cytoplasmic extracts were recovered by centrifugation at 12,000 rpm for 1 min and the supernatant was retrieved. Nuclear pellets were resuspended in 20 ml of buffer B (20 mM

Hepes, pH 7.9, 1.5 mM MgCl2 , 420 mM KCl, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM dithiothreitol). Following a 30-min incubation at 4 °C, nuclear extracts were spun down at 12,000 rpm for 5 min, and the supernatants were recovered. Extracts were either used immediately or quick frozen in a dry ice ethanol bath and stored at -80°C. The Biorad "Protein Standardization" kit and protocol, based on the Bradford colorimetric protein quantitiation assay and utilizing a bovine serum albumin protein standard, was used to determine the concentration of protein in each sample.

(ii) *In vitro* Translation

In vitro translated protein was synthesized using the "TNT T7 Coupled Reticulocyte Lysate System" kit and protocol (Promega). cDNAs encoding RBP-Jk and REST and under the direction of a T7 sequence were kindly provided by Dr. Susan Hayward (Bethesda) and Dr. Gail Mandel (New York) (Chong et al 1996) respectively. Amplification of the cDNA vectors was carried out according to standard methods as detailed in Maniatis et al 1989. For the in vitro translation, briefly, 100ng cDNA was incubated with 10µl of canine reticulocyte cytoplasmic lysate, 1µl of T7 RNA polymerase, and a premade mixture of tRNA and 22 amino acids. The transcription and translation reactions took place in the same tube, according to the Promega protocol, and proceeded at 37°C for one hour. To analyze protein production, the above procedure was used with the exception that the premade amino acid micture lacked methionine and ^{35}-S labelled methionine (Amersham) was added to the synthesis reaction according to the Promega protocol. One fifth of this reaction product was run on an TBE-buffered SDS-containing 8.5% polyacrylamide gel alongside mid range molecular weight markers (Biorad), the gel was dried at 60°C for 4 hours and exposed to XAR film (Kodak) overnight at room temperature.

(iii) Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts and *in vitro* translated proteins prepared as described above were incubated in 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl2 ,5 mMEDTA, pH 8, 5% glycerol, and 1 mM dithiothreitol.with 1.5 mg of poly(dI-dC). In the labelling reactions, 1.25ng of single complementary strands comprising each probe (Tα1, RBP-Jk or RE1) were indivdually radioactively labelled with ^{32}P-ATP (Amersham) using the T4 polynucleotide kinase according to standard procedures as detailed in Maniatis et al 1989. Following the one hour labelling reaction at 37°C , the labelled single stranded oligonucleotides were purified by spinning through a sephadex column ("Nick" column, Boehringer Manheim) at 2000rpm for 20 minutes. Isolated complementray oligonucleotides were then annealed by co-incubation in a 50µl total volume, followed by heating to 100°C and a slow one hour cooling to room temperature using an ice bath. Following the annealing reaction, the mixture was diluted to 250µl and stored at -20°C.

Ten picograms (1µl) of the probe (approximately 20,000 cpm) was added to 1-5µg of nuclear protein extract or 1µl of in vitro translated protein reaction product for 15 min at room temperature. Samples were then loaded on a tris/glycine buffered 5% polyacrylamide gel (30:1) and separated by electrophoresis at 8 V/cm for 2 h in 50 mMTris, 0.38 M glycine, and 1 mM EDTA, pH 8.5. Gels were then dried for four hours at 60°C and exposed to XAR film (Kodak) overnight at -70°C.

In competition experiments, unlabeled oligonucleotide was incubated with nuclear extract or *in vitro* translated protein for 15 mintues at room temperature prior to addition of probe. The entire sample was subsequently loaded and run as described. All probe incubations were done at room temperature.

The oligonucleotides used in gel shift assays were as follows :

Tα1 : CTGCCTCTGCC TCCCA GGTGCTGTTGAGGG

ΔNRE: CTGCCTCTGCC **GTTAC** GGTGCTGTTGAGGG

RBP-Jk: GGAAACACGCCGTGGGAAAAAATTTGGC

RE1: GGGTTTCAGAACCACGGACAGCACCAG

(iv) Supershifts

Anti-RBP-Jk poly IgG antiserum was kindly provided by Dr. John Coligan (Bethesda, MD) for use in gel shift assays. The antibody was raised against a GST-RBP-Jk fusion protein containing RBP-Jk sequence from amino acids 1 to 242. Anti-TrkB poly IgG antiserum, raised against an extracellular peptide of TrkB, was used as control and was kindly provided by Dr. David Kaplan (Montreal, PQ). In supershift experiments, 1μL of serum was pre-incubated with nuclear protein in 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl2 ,5 mMEDTA, pH 8, 5% glycerol, and 1 mM dithiothreitol with 1.5 mg of poly(dI-dC) for 15 minutes at room temperature, prior to inclusion of the probe. Following incubation with probe for 15 minutes, the entire reaction was used for EMSA as described.

References

Abu-Issa R, Cavicchi S. 1996. Genetic interactions among vestigial, hairy, and Notch suggest a role of vestigial in the differentiation of epidermal and neural cells of the wing and halter of Drosophila melanogaster. Journal of Neurogenetics **10**(4):239-46.

Acampora D, Mazan S, Avantaggiato V, Barone P, Tuorto F, Lallemand Y, Brûlet P, Simeone A. 1996. Epilepsy and brain abnormalities in mice lacking the Otx1 gene. Nature Genetics **14**(2):218-22.

Acampora D, Mazan S, Tuorto F, Avantaggiato V, Tremblay JJ, Lazzaro D, di Carlo A, Mariano A, Macchia PE, Corte G, Macchia V, Drouin J, Brûlet P, Simeone A. 1998. Transient dwarfism and hypogonadism in mice lacking Otx1 reveal prepubescent stage-specific control of pituitary levels of GH, FSH and LH. Development **125**(7):1229-39.

Acampora D, Simeone A. 1999. The TINS Lecture. Understanding the roles of Otx1 and Otx2 in the control of brain morphogenesis. Trends Neurosci. **22**(3):116-22.

Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G. 1999. Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development **126**(17):3795-809.

Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, Brenner HR, Caroni P. 1995. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell **83**(2):269-78.

Aizawa T, Maruyama K, Kondo H, Yoshikawa K. 1992. Expression of necdin, an embryonal carcinoma-derived nuclear protein, in developing mouse brain. Brain Research. Developmental Brain Research **68**(2):265-74.

Akazawa C, Ishibashi M, Shimizu C, Nakanishi S, Kageyama R. 1995. A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. JBC **270**: 8730-8738.

Akazawa C, Sasai Y, Nakanishi S, Kageyma R.1992. Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. JBC **267**: 21879-21885.

Alder J, Cho NK, Hatten ME. 1996. Embryonic precursor cells from the rhombic lip are specified to a cerebellar granule neuron identity. Neuron **17**(3):389-99.

Allen T, Lobe CG. 1999. A comparison of Notch, Hes and Grg expression during murine embryonic and post-natal development. Cellular and Molecular Biology **45**(5):687-708.

Allende ML, Weinberg ES. 1994. The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant. Developmental Biology **166**(2):509-30.

Alexandre D, Clarke JD, Oxtoby E, Yan YL, Jowett T, Holder N. 1996. Ectopic expression of Hoxa-1 in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. Development **122**(3):735-46.

Alonso MC, Cabrera CV. 1988. The achaete-scute gene complex of Drosophila melanogaster comprises four homologous genes. Embo Journal **7**(8):2585-91.

Al-Shawi RJ, Kinnaird J, Burke J, Bishop JO. 1990. Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. Mol. Cell. Biol. **10**: 1192-1198.

Altaba AR, Cox C, Jessell TM, Klar A. 1993. Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. PNAS **90**(17):8268-72.

Altman J. 1969. Autoradiographic and hstological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol **137**: 433-458.

Anderson DJ. 1995. A molecular switch for the neuron-glia developmental decision. Neuron **15**(6):1219-22.

Anderson DJ. 1997. Cellular and molecular biology of neural crest cell lineage determination. Trends in Genetics **13**(7):276-80.

Anderson SA, Qiu M, Bulfone A, Eisenstat DD, Meneses J, Pedersen R, Rubenstein JL. 1997. Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron **19**(1):27-37.

Ang SL, Jin O, Rhinn M, Daigle N, Stevenson L, Rossant J. 1996. A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. Development **122**(1):243-52.

Appel B, Eisen JS. 1998. Regulation of neuronal specification in the zebrafish spinal cord by Delta function. Development **125**(3):371-80.

Arndt M, Bank U, Frank K, Sabel BA, Ansorge S, Lendeckel UR. 1999. espl, a rat homologue of drosophila groucho, is differentially expressed after optic nerve crush and mediates NGF-induced survival of PC12 cells. Febs Letters **457**(2):246-50.

Artavanis-Tsakonas S, Matsuno K, Fortini M E 1995. Notch signaling. Science **268**: 225-232.

Artavanis-Tsakonas S, Rand MD, Lake RJ. 1999. Notch signaling: cell fate control and signal integration in development. Science **284**(5415):770-6.

Aruga J, Yokota N, Hashimoto M, Furuichi T, Fukuda M, Mikoshiba K. 1994. A novel zinc finger protein, Zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. J Neurochem **63**: 1880-1890.

Austin CP, Feldman DE, Ida JA Jr, Cepko CL. 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development **121**(11):3637-50.

Austin J, Kimble J. 1989. Transcript analysis of glp-1 and lin-12, homologous genes required for cell interactions during development of C. elegans. Cell **58**(3):565-71

Ausubel FM, Brent R, Kingstin RE, Moore DD, Seidman JG, Smith JA, Struhl K. Current Protocols in Molecular Biology; John Wiley and Sons Inc., New York, 1998.

Avisar N, Shiftan L, Ben-Dror I, Havazelet N, Vardimon L. 1999. A silencer element in the regulatory region of glutamine synthetase controls cell type-specific repression of gene induction by glucocorticoids. JBC **274**(16):11399-407.

Bailey AM, Posakony JW. 1995. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes and Development **9**(21):2609-22.

Baker NE, Mlodzik M, Rubin GM. 1990. Spacing differentiation in the developing Drosophila eye: a fibrinogen-related lateral inhibitor encoded by scabrous. Science **250**(4986):1370-7.

Balcells L, Modolell J, Ruiz-Gómez M. 1988. A unitary basis for different Hairy-wing mutations of Drosophila melanogaster. Embo Journal 7(12):3899-906.

Balling R, Mutter G, Gruss P, Kessel M. 1989. Craniofacial abnormalities induced by ectopic expression of the homeobox gene Hox-1.1 in transgenic mice. Cell **58**(2):337-47.

Bally-Cuif L, Dubois L, Vincent A. 1998. Molecular cloning of Zcoe2, the zebrafish homolog of Xenopus Xcoe2 and mouse EBF-2, and its expression during primary neurogenesis. Mechanisms of Development **77**(1):85-90.

Bamji SX, Miller FD. 1996. Comparison of the expression of a T alpha 1:nlacZ transgene and T alpha 1 alpha-tubulin mRNA in the mature central nervous system. Journal of Comparative Neurology **374**(1):52-69.

Bao ZZ, Cepko CL. 1997. The expression and function of Notch pathway genes in the developing rat eye. Journal of Neuroscience **17**(4):1425-34.

Barbaric S, Münsterkötter M, Svaren J, Hörz W. 1996. The homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast PHO5 promoter. Nucleic Acids Research **24**(22):4479-86.

Barth KA, Wilson SW. 1995. Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. Development **121**: 1755-1768.

Bayer SA, Altman J. *Neocortical Development*. New York: Raven Press, 1991.

Beato M. 1989. Gene regulation by steroid hormones. Cell **56**(3):335-44.

Beaudet L, Charron G, Houle D, Tretjakoff I, Peterson A, Julien JP. 1992. Intragenic regulatory elements contribute to transcriptional control of the neurofilament light gene. Gene **116**(2):205-14.

Begley CG, Lipkowitz S, Göbel V, Mahon KA, Bertness V, Green AR, Gough NM, Kirsch IR. 1992. Molecular characterization of NSCL, a gene encoding a helix-loop-helix protein expressed in the developing nervous system. PNAS **89**(1):38-42.

Belecky-Adams T, Wight DC, Kopchick JJ, Parysek LM. 1993. Intragenic sequences are required for cell type-specific and injury-induced expression of the rat peripherin gene. Journal of Neuroscience **13**(12):5056-65.

Bellefroid EJ, Bourguignon C, Hollemann T, Ma Q, Anderson DJ, Kintner C, Pieler T. 1996. X-MyT1, a Xenopus C2HC-type zinc finger protein with a regulatory function in neuronal differentiation. Cell **87**(7): 1191-1202.

Ben-Arie N, Bellen HJ, Armstrong DL, McCall AE, Gordadze PR, Quo Q, Matzuk MM, Zoghbi HY. 1997. Math1 is essential for genesis of cerebellar granule neurons. Nature **390**: 169-172.

Bender LB, Kooh PJ, Muskavitch MA. 1993. Complex function and expression of Delta during Drosophila oogenesis. Genetics **133**(4):967-78.

Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY. 1999. Math1: an essential gene for the generation of inner ear hair cells. Science **284**: 1837-1841.

Bernardoni R, Miller AA, Giangrande A. 1998. Glial differentiation does not require a neural ground state. Development **125**(16):3189-200.

Berry LW, Westlund B, Schedl T. 1997. Germ-line tumor formation caused by activation of glp-1, a Caenorhabditis elegans member of the Notch family of receptors. Development **124**(4):925-36.

Bessis A, Champtiaux N, Chatelin L, Changeux JP. 1997. The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain. PNAS **94**(11):5906-11.

Bettenhausen B, Hrabe de Angelis M, Simon D, Guénet JL, Gossler A. 1995. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. Development **121**(8):2407-18.

Bhat KM. 1996. The patched signaling pathway mediates repression of gooseberry allowing neuroblast specification by wingless during Drosophila neurogenesis. Development **122**(9): 2921-2932.

Bier E, Vaessin H, Younger-Shepherd S, Jan LY, Jan YN. 1992. deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. Genes and Development **6**(11):2137-51.

Bierkamp C, Campos-Ortega JA. 1993. A zebrafish homologue of the Drosophila neurogenic gene Notch and its pattern of transcription during early embryogenesis. Mechanisms of Development **43**(2-3):87-100.

Bignon YJ, Chen Y, Chang CY, Riley DJ, Windle JJ, Mellon PL, Lee WH. 1993. Expression of a retinoblastoma transgene results in dwarf mice. Genes and Development **7**(9):1654-62.

Black BL, Ligon KL, Zhang Y, Olson EN. 1996. Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. JBC **271**(43):26659-63.

Black BL, Olson EN. 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annual Review of Cell and Developmental Biology **14**:167-96.

Blader P, Fischer N, Gradwohl G, Guillemont F, Strähle U. 1997. The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. Development **124**(22):4557-69.

Blaschke AJ, Staley K, Chun J. 1996. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development **122**(4):1165-74.

Blumberg B, Bolado J Jr, Moreno TA, Kintner C, Evans RM, Papalopulu N. 1997. An essential role for retinoid signaling in anteroposterior neural patterning. Development **124**(2):373-9.

Bodmer R, Carretto R, Jan YN. 1989. Neurogenesis of the peripheral nervous system in Drosophila embryos: DNA replication patterns and cell lineages. Neuron **3**(1):21-32.

Bodner M, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. 1998. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell **55**(3):505-18.

Boncinelli E, Gulisano M, Broccoli V. 1993. Emx and Otx homeobox genes in the developing mouse brain. Journal of Neurobiology **24**(10):1356-66.

Boudjelal M, Taneja R, Matsubara S, Bouillet P, Dolle P, Chambon P. 1997. Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes and Development **11**(16):2052-65.

Bourguignon C, Li J, Papalopulu N. 1998. XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. Development **125**(24):4889-900.

Brand M, Campos-Ortega J A. 1989. Second site modifiers of the split mutation of Notch define genes involved in neurogenesis in Drosophila melanogaster. Roux's Arch. Dev. Biol. **198**: 275-285.

Brand M, Jarman AP, Jan LY, Jan YN. 1993. asense is a Drosophila neural precursor gene and is capable of initiating sense organ formation. Development **119**(1):1-17.

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature, **391**(6667):597-601.

Brewster R, Lee J, Ruiz i Altaba A. 1998. Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. Nature **393**(6685): 579-583.

Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell TM, Rubenstein JL, Ericson J. 1999. Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature **398**(6728):622-7.

Brou C, Logeat F, Lecourtois M, Vandekerckhove J, Kourilsky P, Schweisguth F, Israël A. 1994. Inhibition of the DNA-binding activity of Drosophila suppressor of hairless and of its human homolog, KBF2/RBP-J kappa, by direct protein-protein interaction with Drosophila hairless. Genes and Development **8**(20):2491-503.

Brown NL, Kanekar S, Vetter ML, Tucker PK, Gemza DL, Glaser T. 1998. Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development **125**(23):4821-33.

Brown NL, Sattler CA, Paddock SW, Carroll SB 1995. Hairy and emc negatively regulate morphogenetic furrow progression in the Drosophila eye. Cell **80**: 879-887.

Brown NL, Sattler CA, Markey DR, Carroll SB. 1991. hairy gene function in the Drosophila eye: normal expression is dispensable but ectopic expression alters cell fates. Development **113**(4):1245-56.

Brunner D, Dücker K, Oellers N, Hafen E, Scholz H, Klämbt C. 1994. The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature **370**(6488):386-9.

Bulfone A, Puelles L, Porteus MH, Frohman MA, Martin GR, Rubenstein JL. 1993. Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. Journal of Neuroscience **13**(7):3155-72.

Bulfone A, Kim HJ, Puelles L, Porteus MH, Grippo JF, Rubenstein JL. 1993. The mouse Dlx-2 (Tes-1) gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. Mechanisms of Development **40**(3):129-40.

Bürglin TR. 1994. A Caenorhabditis elegans prospero homologue defines a novel domain. Trends Biochem Sci **19**(2):70-1.

Burrill JD, Moran L, Goulding MD, Saueressig H. 1997. PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. Development **124**(22):4493-503.

Burrows RC, Wancio D, Levitt P, Lillien L. 1997. Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron **19**(2):251-67.

Cabrera CV. 1990. Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta. Development **110**(1):733-42.

Cabrera CV, Alonso,\ MC. 1991. Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila. Embo Journal **10**(10):2965-73.

Cabrera CV, Alonso MC, Huikeshoven H. 1994. Regulation of scute function by extramacrochaete in vitro and in vivo. Development **120**(12):3595-603

Caggiano M, Kauer JS, Hunter DD. (1994) Globose basal cells are neuronal progenitors in thenolfactory epithelium: a lineage analysis using a replication incompetent retrovirus. Neuron **13**: 339-352.

Callaghan DA, Dong L, Callaghan SM, Hou YX, Dagnino L, Slack RS. 1999. Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. Developmental Biology **207**(2):257-70.

Campos AR, Grossman D, White K. 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. Journal of Neurogenetics **2**(3):197-218.

Campos-Ortega JA, Jiménez F. 1980. The effect of X-chromosome deficiencies on neurogenesis in Drosophila. Basic Life Sciences **16**:201-22.

Campos-Ortega JA, Lehmann R, Jimenez F, Dietrich U. 1984. A genetic analysis of early neurogenesis in Drosophila. In *Organizing Principles of Neural Development*, ed. Sharma S C. pp. 129-144. New York/ London: Plenum.

Campos-Ortega J A, Hartenstein V. 1985. *The embryonic development of Drsophila melanogaster*. Berlin/New York/Heidelberg: Springer-Verlag.

Campos-Ortega JA. 1988. Cellular interactions during early neurogenesis of Drosophila melanogaster. Trends in Neurosciences **11**(9):400-5.

Campos-Ortega J A, Jan YN 1991. Genetic and molecular bases of neurogenesis in Drosophila Melanogaster. Ann. Rev. Neurosci. **14**: 399-420.

Campos-Ortega JA. 1994. Cellular interactions in the developing nervous system of Drosophila. Cell **77**(7):969-75.

Campos-Ortega JA. 1996. Numb diverts notch pathway off the tramtrack. Neuron **17**(1):1-4.

Campuzano S, Carramolino L, Cabrera CV, Ruíz-Gómez M, Villares R, Boronat A, Modolell J. 1985. Molecular genetics of the achaete-scute gene complex of D. melanogaster. Cell **40**(2):327-38.

Capobianco AJ, Zagouras P, Balumueller CM, Artavanis-Tsakonas S, Bishop JM. 1997. Neoplastic transformation by truncated alleles of human Notch1/TAN1 and Notch2. MCB **17**: 6265-6273.

Caric D, Gooday D, Hill RE, McConnell SK, Price DJ. 1997. Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax-6. Development **124**(24): 5087-5096.

Carpenter EM, Goddard JM, Chisaka O, Manley NR, Capecchi MR. 1993. Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development **118**(4):1063-75.

Carrol SB, Laughon A, Thalley BS. 1988. Expression, function, and regulation of the hairy segmentation protein in the Drosophila embryo. Genes Dev **2**: 883-890.

Casarosa S, Fode C, Guillemot F. 1999. Mash1 regulates neurogenesis in the ventral telencephalon. Development **126**(3):525-34.

Castella P, Wagner JA, Caudy M. 1999. Regulation of hippocampal neuronal differentiation by the basic helix-loop-helix transcription factors HES-1 and MASH-1. Journal of Neuroscience Research **56**(3):229-40.

Catron KM, Wang H, Hu G, Shen MM, Abate-Shen C. Comparison of MSX-1 and MSX-2 suggests a molecular basis for functional redundancy. Mechanisms of Development **55**(2):185-99.

Cau E, Gradwohl G, Fode C, Guillemot F. 1997. Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development **124**(8):1611-2.

Caudy M, Vässin H, Brand M, Tuma R, Jan LY, Jan YN. 1988. daughterless, a Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to myc and the achaete-scute complex. Cell **55**(6):1061-7.

Caudy M, Grell EH, Dambly-Chaudière C, Ghysen A, Jan LY, Jan YN. 1988. The maternal sex determination gene daughterless has zygotic activity necessary for the formation of peripheral neurons in Drosophila. Genes and Development **2**(7):843-52.

Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M, Bejsovec A. 1998. Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature **395**(6702):604-8.

Caviness VS Jr. 1982. Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. Brain Research **256**(3):293-302.

Caviness VS Jr, Takahashi T, Nowakowski RS. 1995. Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci. **18**(9):379-83.

Caviness VS Jr, Takahashi T, Nowakowski RS. 1999. The G1 restriction point as critical regulator of neocortical neuronogenesis. Neurochemical Research, **24**(4):497-506.

Cepko CL, Austin CP, Yang X, Alexiades M, Ezzeddine D. 1996. Cell fate determination in the vertebrate retina. PNAS **93**: 589-595.

Chalepakis G, Goulding M, Read A, Strachan T, Gruss P. 1994. Molecular basis of splotch and Waardenburg Pax-3 mutations. PNAS **91**(9):3685-9.

Chen G, Nguyen PH, Courey AJ. 1998. A role for Groucho tetramerization in transcriptional repression. Molecular and Cellular Biology **18**(12):7259-68.

Chen PL, Riley DJ, Chen Y, Lee WH. 1996. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes and Development **10**(21):2794-804.

Chen Z, Paquette AJ, Anderson DJ. 1998. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. Nature Genetics **20**(2):136-42.

Chenn A, McConnell SK. 1995. Celavage oreintation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell **82**(4): 631-641.

Chen Y, Solursh M. 1995. Mirror-image duplication of the primary axis and heart in Xenopus embryos by the overexpression of Msx-1 gene. Journal of Experimental Zoology **273**(2):170-4.

Chisaka O, Musci TS, Capecchi MR. 1992. Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene Hox-1.6. Nature **355**(6360):516-20.

Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C. 1995. Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature **375**(6534):761-6.

Chitnis A, Kintner C. 1995. Neural induction and neurogenesis in amphibian embryos. Perspectives on Developmental Neurobiology **3**(1):3-15.

Chitnis A, Kintner C. 1996. Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. Development **122**(7): 2295-2301.

Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altschuller YM, Frohman MA, Krasner SD, Mandel GM. 1995. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell **80**(6): 949-957.

Choudhury BK, Kim J, Kung HF, Li SS. 1997. Cloning and developmental expression of Xenopus cDNAs encoding the Enhancer of Split groucho and related proteins. Gene **195**: 41-48.

Chu-Lagraff Q, Wright DM, McNeil LK, Doe CQ. 1991. The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila. Development **2**:79-85.

Clerc RG, Corcoran LM, LeBowitz JH, Baltimore D, Sharp PA. 1988. The B-cell-specific Oct-2 protein contains POU box- and homeo box-type domains. Genes and Development **2**(12):1570-81.

Cleveland DW. 1989. Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. Trends Biochem Sci **13**(9):339-43.

Cleveland DW. 1989. Gene regulation through messenger RNA stability. Current Opinion in Cell Biology **1**(6):1148-53.

Cleveland D, Pittenger MF, Feramisco JR. 1983. Elevation of tubuin level by microinjection supresses new tubulin synthesis. Nature **305**: 738-740.

Coffman CR, Skoglund P, Harris WA, Kintner CR. 1993. Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos. Cell **73**(4):659-71.

Cohen SM, Brönner G, Küttner F, Jürgens G, Jäckle H. 1989. Distal-less encodes a homoeodomain protein required for limb development in Drosophila. Nature **338**(6214):432-4.

Cohen SM, Jürgens G. 1990. Mediation of Drosophila head development by gap-like segmentation genes. Nature **346**(6283):482-5.

Conlon RA, Reaume AG, Rossant J. 1995. Notch1 is required for the coordinate segmentation of somites. Development **121**(5):1533-45.

Corbin V, Michelson AM, Abmayr SM, Neel V, Alcamo E, Maniatis T, Young MW. 1991. A role for the Drosophila neurogenic genes in mesoderm differentiation. Cell **67**(2):311-23.

Cordes SP, Barsh GS. 1994. The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. Cell **79**: 1025-1034.

Couso JP, Martinez Arias A. 1994. Notch is required for wingless signaling in the epidermis of Drosophila. Cell **79**(2):259-72.

Cowherd RM, Lyle RE, McGehee RE Jr. 1999. Molecular regulation of adipocyte differentiation. Seminars in Cell and Developmental Biology **10**(1):3-10.

Cowing DW, Kenyon C. 1992. Expression of the homeotic gene mab-5 during Caenorhabditis elegans embryogenesis. Development **116**(2):481-90.

Coyle-Thompson CA, Banerjee U. 1993. The strawberry notch gene functions with Notch in common developmental pathways. Development, **119**(2):377-95.

Craig CG, Tropepe V, Morshead CM, Reynolds BA, Weis S, van der Kooy D. 1996. In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci **16**: 2649-2658.

Cronmiller C, Schedl P, Cline TW. 1988. Molecular characterization of daughterless, a Drosophila sex determination gene with multiple roles in development. Genes and Development **2**(12):1666-76.

Cubas P, de Celis JF, Campuzano S, Modolell J. 1991. Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. Genes and Development **5**(6):996-1008.

Cubas P, Modolell J, Ruiz-Gómez M. 1994. The helix-loop-helix extramacrochaetae protein is required for proper specification of many cell types in the Drosophila embryo. Development **120**(9):2555-66.

Cubas P, Modolell J. 1992. The extramacrochaetae gene provides information for sensory organ patterning. Embo Journal **11**(9):3385-93.

Culí J, Modolell J. 1998. Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. Genes and Development **12**(13):2036-47.

Dambly-Chaudiere C, Ghysen A. 1987. Independent subpatterns of sense organs require independent genes of the achaete-scute complex in Drosophila larvae. Genes Deve. **1**: 297-306.

Danciger E, Mettling C, Vidal M, Morris R, Margolis F. 1989. Olfactory marker protein gene: its structure and olfactory neuron-specific expression in transgenic mice. PNAS **86**(21):8565-9.

Danielian PS, McMahon AP. 1996. Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. Nature **383**(6598):332-4.

Daubas P, Salmon AM, Zoli M, Geoffroy B, Devillers-Thiéry A, Bessis A, Médevielle F, Changeux JP. 1993. Chicken neuronal acetylcholine receptor alpha 2-subunit gene exhibits neuron-specific expression in the brain and spinal cord of transgenic mice. PNAS **90**(6):2237-41.

Davis A, Temple S. 1994. A self renewing multipotential stem cell in embryonic rat cerebral cortex. Nature **372**: 263-266.

Dawson SR, Turner DL, Weintraub H, Parkhurst SM. 1995. Specificity for hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. Mol Cell Biol **15**: 6923-6931.

DeCamillis M, Cheng NS, Pierre D, Brock HW. 1992. The polyhomeotic gene of Drosophila encodes a chromatin protein that shares polytene chromosome-binding sites with Polycomb. Genes and Development **6**(2):223-32.

de la Pompa JL, Wakeham A, Correia KM, Samper E, Brown S, Aguilera RJ, Nakano T, Honjo T, Mak TW, Rossant J, Conlon RA. 1997. Conservation of the Notch signaling pathway in mammalian neurogenesis. Development, **124**(6):1139-48.

Dehni G, Liu Y, Husain J, Stifani S. 1995. TLE expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. Mechanisms of Development **53**(3):369-81.

DiCicco-Bloom E, Townes-Anderson E, Black IB. 1990. Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. Journal of Cell Biology **110**(6):2073-86.

Dickman ED, Thaller C, Smith SM. 1997. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. Development **124**(16):3111-21.

Diederich RJ, Matsuno K, Hing H, Artavanis-Tsakonas S. 1994. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development **120**(3):473-81.

Dietrich U, Campos-Ortega JA. 1984. The expression of neurogenic loci in imaginal epidermal cells of Drosophila melanogaster. Journal of Neurogenetics **1**(4):315-32.

Dittrich R, Bossing T, Gould AP, Technau GM, Urban J. 1997. The differentiation of the serotonergic neurons in the Drosophila ventral nerve cord depends on the combined

functionof the zinc finger proteins Eagle and Huckebein. Development **124**(13): 2515-2525.

Doe CQ, Chu-LaGraff Q, Wright DM, Scott MP. 1991. The prospero gene specifies cell fates in the Drosophila central nervous system. Cell **65**(3):451-64.

Doe CQ, Goodman CS. 1985. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. Developmental Biology **111**(1):206-19.

Dornseifer P, Takke C, Campos-Ortega JA. 1997. Overexpression of a zebrafish homologue of the Drosophila neurogenic gene Delta perturbs differentiation of primary neurons and somite development. Mechanisms of Development **63**(2):159-71.

Dou C, Ye X, Stewart C, Lai E, Li SC. 1997. TWH regulates the development of subsets of spinal cord neurons. Neuron **18**(4):539-51.

Dou S, Zeng X, Cortes P, Erdjument-Bromage H, Tempst P, Honjo T,Vales LD. 1994. The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. Molecular and Cellular Biology **14**(5):3310-9.

Dubois L, Bally-Cuif L, Crozatier M, Moreau J, Paquereau L, Vincent A. 1998. XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in Xenopus. Current Biology **8**(4):199-209.

Dupé V, Davenne M, Brocard J, Dollé P, Mark M, Dierich A, Chambon P, Rijli FM. 1997. In vivo functional analysis of the Hoxa-1 3' retinoic acid response element (3'RARE). Development **124**(2):399-410.

Easter SS Jr, Ross LS, Frankfurter A. 1993. Initial tract formation in the mouse brain. Journal of Neuroscience **13**(1):285-99.

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mophler J, McMahon JA, McMahon AP. 1993. Sonic hedgehog, a member of a family of of putative signaling molecules, is implicated I the regulation of CNS polarity. Cell **75**: 1417-1430.

Edelman GM, Jones FS. 1995. Developmental control of N-CAM expression by Hox and Pax gene products. Philos Trans Royal Soc Lond **349**: 305-312.

Eichmann A, Grapin-Botton A, Kelly L, Graf T, Le Douarin NM, Sieweke M. 1997. The expression pattern of the mafB/kr gene in birds and mice reveals that the kresiler phenotype does not represent a null mutant. Mech Develop **65**: 111-122.

Eisenstat DD, Liu JK, Mione M, Zhong W, Yu G, Anderson SA, Ghattas I, Puelles L, Rubenstein JL. 1999. DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. Journal of Comparative Neurology **414**(2):217-37.

Ellis HM, Spann DR, Posakony JW. 1990. extramacrochaetae, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. Cell **61**(1):27-38.

Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J. 1991. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal trasnlocations in T lymphoblastic neoplasms. Cell **66**: 649-661.

Emery JF, Bier E. 1995. Specificity of CNS and PNS regulatory subelements comprising pan-neural enhancers of the deadpan and scratch genes is achieved by repression. Development **121**(11):3549-60.

Epstein DJ, Vekemans M, Gros P. 1991. Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. Cell **67**(4):767-74.

Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J. 1997. Pax6 control progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell **90**(1): 169-180.

Ericson J, Thor S, Edlund T, Jessell TM, Yamada T. 1992. Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. Science **256**(5063):1555-60.

Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM, Keithley EM, Rapaport DH, Ryan AF, Rosenfeld MG. 1996. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature **381**: 603-606.

Faissner A, Kruse J, Nieke J, Schachner M. 1984. Expression of neural cell adhesion molecule L1 during development, in neurological mutants and in the peripheral nervous system. Brain Research **317**(1):69-82.

Fanarraga ML, Charitae J, Hage WJ, De Graaff W, Deschamps J. 1997. Hoxb-8 gin-of-function transgenic mice exhibit alterations in the peripheral nervous system. J Neurosci. Methods **71**: 11-18.

Fehon RG, Kooh PJ, Rebay I, Regan CL, Xu T, Muskavitch MA, Artavanis-Tsakonas S. 1990. Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell **61**(3):523-34.

Feng L, Heintz N. 1995. Differentiating neurons activate transcription of the brain lipid-binding protein gene in radial glia through a novel regulatory element. Development **121**(6): 1719-1730.

Fernandez AS, Pieau C, Repérant J, Boncinelli E, Wassef M. 1998. Expression of the Emx-1 and Dlx-1 homeobox genes define three molecularly distinct domains in the

telencephalon of mouse, chick, turtle and frog embryos: implications for the evolution of telencephalic subdivisions in amniotes. Development **125**(11):2099-111.

Filosa S, Rivera-Pérez JA, Gómez AP, Gansmuller A, Sasaki H, Behringer RR, Ang SL. 1997. Goosecoid and HNF-3beta genetically interact to regulate neural tube patterning during mouse embryogenesis. Development **124**(14):2843-54.

Finney M, Ruvkun G. 1990. The unc-86 gene product couples cell lineage and cell identity in C. elegans. Cell **63**(5):895-905.

Finney M, Ruvkun G, Horvitz HR. 1988. The C. elegans cell lineage and differentiation gene unc-86 encodes a protein with a homeodomain and extended similarity to transcription factors. Cell **55**(5):757-69.

Finkelstein R, Perrimon N. 1990. The orthodenticle gene is regulated by bicoid and torso and specifies Drosophila head development. Nature **346**(6283):485-8.

Finkelstein R, Boncinelli E. 1994. From fly head to mammalian forebrain: the story of otd and Otx. Trends in Genetics **10**(9):310-5.

Fishell G, Rossant J, van der Kooy D. 1990. Neuronal lineages in chimeric mouse forebrain are segregated between compartments and in the rostrocaudal and radial planes. Developmental Biology **141**(1):70-83.

Fishell G, Mason CA, Hatten ME. 1993. Dispersion of neural progenitors within the germinal zones of the forebrain. Nature **362**(6421):636-8.

Fishell G. 1997. Regionalization in the mammalian telencephalon. Current Opinion in Neurobiology **7**(1):62-9.

Fisher AL, Caudy M. 1998. Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. Genes and Development **12**(13):1931-40.

Fisher AL, Ohsako S, Caudy M. 1996. The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Molecular and Cellular Biology **16**(6):2670-7.

Fitzgerald K, Wilkinson HA, Greenwald I. 1993. glp-1 can substitute for lin-12 in specifying cell fate decisions in Caenorhabditis elegans. Development **119**(4):1019-27.

Fleming RJ, Scottgale TN, Diederich RJ, Artavanis-Tsakonas S. 1990. The gene Serrate encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in Drosophila melanogaster. Genes and Development **4**(12A):2188-201.

Flores GV, Daga A, Kalhor HR, Banerjee U. 1998. Lozenge is expressed in pluripotent precursor cells and patterns multiple cell types in the Drosophila eye through the control of cell-specific transcription factors. Development **125**(18): 3681-3687.

Fode C, Gradwohl G, Morin X, Dierichh A, LeMeur M, Goridis C, Guillemeot F. 1998. The bHLH protein neuroenin2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron **20**: 483-494.

Foerst-Potts L, Sadler TW. 1997. Disruption of Msx-1 and Msx-2 reveals roles for these genes in craniofacial, eye, and axial development. Developmental Dynamics **209**(1):70-84.

Folberg A, Nagy Kovács E, Luo J, Giguère V, Featherstone MS. 1999. RARbeta mediates the response of Hoxd4 and Hoxb4 to exogenous retinoic acid. Developmental Dynamics **215**(2):96-107.

Fortini ME, Artavanis-Tsakonas S. 1994. The suppressor of hairless protein participates in notch receptor signaling. Cell **79**(2):273-82.

Frantz G, McConnell SK. 1996. Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron **17**(1):55-61.

Franz T, Kothary R. 1993. Characterization of the neural crest defect in Splotch (Sp1H) mutant mice using a lacZ transgene. Brain Research. Developmental Brain Research **72**(1):99-105.

Frasch M, Chen X, Lufkin T. 1995. Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and Drosophila. Development **121**(4):957-74.

Freund CL, Gregory-Evans CY, Furukawa T, Papaioannou M, Looser J, Ploder L, Bellingham J, Ng D, Herbrick JA, Duncan A, Scherer SW, Tsui LC, Loutradis-Anagnostou A, Jacobson SG, Cepko CL, Bhattacharya SS, McInnes RR. 1997. Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. Cell **91**(4):543-53.

Frohman MA, Martin GR, Cordes SP, Halamek LP, Barsh GS. 1993. Altered rhombomere-specific gene expression and hyoid bone differentiation in the mouse segmentation mutant, kreisler (kr). Development **117**: 925-936.

Fujii H, Hamada H. 1993. A CNS-specific POU transcription factor, Brn-2, is required for establishing mammalian neural cell lineages. Neuron **11**(6):1197-206.

Furukawa T, Kawaichi M, Matsunami N, Ryo H, Nishida Y, Honjo T. 1991. The Drosophila RBP-J kappa gene encodes the binding protein for the immunoglobulin J kappa recombination signal sequence. JBC **266**(34):23334-40.

Furukawa T, Morrow EM, Cepko CL. 1997. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell **91**(4):531-41.

Furukawa T, Kozak CA, Cepko CL. 1997. rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. PNAS **94**(7):3088-93.

Furukawa T, Maruyama S, Kawaichi M, Honjo T. 1992. The Drosophila homolog of the immunoglobulin recombination signal-binding protein regulates peripheral nervous system development. Cell **69**(7):1191-7.

Gale E, Prince V, Lumsden A, Clarke J, Holder N, Maden M. 1996. Late effects of retinoic acid on neural crest and aspects of rhombomere. Development **122**(3):783-93.

Gan L, Wang SW, Huang Z, Klein WH. 1999. POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification. Developmental Biology **210**(2):469-80.

Gan L, Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J. 1996. POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. PNAS **93**(9):3920-5.

Garcia-Bellido A. 1979. Genetic analysis of the achaete-scute system of Drosophila melanogaster. Genetics **91**: 491-520.

Garcia-Bellido A, Santamaria P. 1978. Developmental analysis of the achaete-scute system of Drsophila melanogaster. Genetics **88**: 469-486.

Garel S, Marain F, Mattaei MG, Vesque C, Vincent A, Charnay P. 1997. Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. Developmental Dynamics **210**: 191-205.

Garrell J, Modolell J. 1990. The Drosophila extramacrochaetae locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. Cell **61**(1):39-48.

Ghosh A, Greenberg ME. 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron **15**(1): 89-103.

Ghyselinck NB, Dupé V, Dierich A, Messaddeq N, Garnier JM, Rochette-Egly C, Chambon P, Mark M. 1997. Role of the retinoic acid receptor beta (RARbeta) during mouse development. International Journal of Developmental Biology **41**(3):425-47.

Ghysen A, Dambly-Chaudière C. 1988. From DNA to form: the achaete-scute complex. Genes and Development **2**(5):495-501.

Ghysen A, Dambly-Chaudiere C. 1989. Genesis of the Drosophila peripheral nervous system. Trends in Genetics **5**(8):251-5.

Gloster A, El-Bizri H, Bamji SX, Rogers D, Miller FD. 1999. Early induction of Tα1 α-tubulin transcription in neurons of the developing nervous system. Journal of Comp. Neurol. **405**(1): 45-60.

Göbel V, Lipkowitz S, Kozak CA, Kirsch IR. 1992. NSCL-2: a basic domain helix-loop-helix gene expressed in early neurogenesis. Cell Growth and Differentiation **3**(3):143-8.

Gómez-Skarmeta JL, Rodríguez I, Martínez C, Culí J, Ferrés-Marcó D, Beamonte D, Modolell J. 1995. Cis-regulation of achaete and scute: shared enhancer-like elements

drive their coexpression in proneural clusters of the imaginal discs. Genes and Development 9(15):1869-82.

Gonzales F, Romani S, Cubas P, Modolell J, Campuzano S. 1989. Molecular analusis of asense, a member of the achaete-scute complex of Drosophila melanogaster, and its novel role in optic lobe development. EMBO J. **8**: 3553-3562.

Good DJ, Porter FD, Mahon KA, Parlow AF, Westphal H, Kirsch IR. 1997. Hypogonadism and obesity in mice with a targeted deletion of the Nhlh2 gene. Nature Genetics **15**(4):397-401.

Gorman MJ, Girton JR. 1992. A genetic analysis of deltex and its interaction with the Notch locus in Drosophila melanogaster. Genetics **131**(1):99-112.

Gould A, Itasaki N, Krumlauf R. 1998. Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. Neuron **21**(1):39-51.

Goulding MD, Chalepakis G, Deutsch U, Erslius JR, Gruss P. 1991. Pax-3, a novel murine DNA-binding protein expressed during early neurogenesis. EMBO J **10**: 1135-1147.

Graba Y, Aragnol D, Pradel J. 1997. Drosophila Hox complex downstream targets and the function of homeotic genes,. Bioessays **19**: 379-388.

Grbavec D, Lo R, Liu Y, Greenfield A, Stifani S. 1999. Groucho/transducin-like enhancer of split (TLE) family members interact with the yeast transcriptional co-repressor SSN6 and mammalian SSN6-related proteins: implications for evolutionary conservation of transcription repression mechanisms. Biochemical Journal **337** (1):13-7.

Grbavec D, Lo R, Liu Y, Stifani S. 1998. Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor,

interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. European Journal of Biochemistry **258**(2):339-49.

Grbavec D, Stifani S. 1996. Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. Biochemical and Biophysical Research Communications **223**(3):701-5.

Green JB. 1994. Roads to neuralness: embryonic neural induction as derepression of a default state. Cell **77**(3):317-20.

Greenwald I, Cole M, Paterson B, Fire A. 1997. A C. elegans E/Daughterless bHLH protein marks neuronal but not striated muscle development. Development **124**(11):2179-2189.

Greenwald I. 1998. LIN-12/Notch signaling: lessons from worms and flies. Genes and Development **12**(12):1751-62.

Grindley JC, Davidson DR, Hill RE. 1995. The role of Pax-6 in eye and nasal development. Development **121**(5):1433-42.

Groves AK, George KM, Tissier-Seta JP, Engel JD, Brunet JF, Anderson DJ. 1995. Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. Development **121**(3): 887-901.

Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V, Nadal-Ginard B. 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell **72**(3):309-24.

Gu Y, Hukriede NA, Fleming RJ. 1995. Serrate expression can functionally replace Delta activity during neuroblast segregation in the Drosophila embryo. Development **121**(3):855-65.

Guillemot F, Joyner AL. 1993. Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mechanisms of Development **42**(3):171-85.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL. 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell **75**(3):463-76.

Guillemot F, Nagy A, Auerbach A, Rossant J, Joyner AL. 1994. Essential role of Mash-2 in extraembryonic development. Nature **371**(6495):333-6.

Guo M, Bier E, Jan LY, Jan YN. 1995. tramtrack acts downstream of numb to specify distinct daughter cell fates during asymmetric cell divisions in the Drosophila PNS. Neuron **14**(5):913-25.

Guo M, Jan LY, Jan YN. 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron **17**(1):27-41.

Hackett BP, Bingle CD, Gitlin JD. 1996. Mechanisms of gene expression and cell fate determination in the developing pulmonary epithelium. Annual Review of Physiology **58**:51-71.

Haddon C, Smithers L, Schneider-Maunoury S, Coche T, Henrique D, Lewis J. 1998. Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. Development **125**(3):359-70.

Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar AB. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science **267**(5200):1018-21.

Hamaguchi Y, Matsunami N, Yamamoto Y, Honjo T. 1989. Purification and characterization of a protein that binds to the recombination signal sequence of the immunoglobulin J kappa segment. Nucleic Acids Research **17**(22):9015-26.

Hamaguchi Y, Yamamoto Y, Iwanari H, Maruyama S, Furukawa T, Matsunami N, Honjo T. 1992. Biochemical and immunological characterization of the DNA binding protein (RBP-J kappa) to mouse J kappa recombination signal sequence. Journal of Biochemistry **112**(3):314-20.

Harbers K, Jahner D, Jaenisch R. 1981. Microinjection of cloned retroviral genomes into mouse zygotes: Integration and expression in the animal. Nature **293**: 540-541.

Hatenstein V. 1988. Development of Drosophila larval sensory organs: Spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation. Development **102**: 869-886.

Hartenstein V, Posakony JW. 1990. A dual function of the Notch gene in Drosophila sensillum development. Developmental Biology **142**(1):13-30.

Hartenstein AY, Rugendorff A, Tepass U, Hartenstein V. 1992. The function of the neurogenic genes during epithelial development in the Drosophila embryo. Development **116**(4):1203-20.

Hassan B, Vaessin H. 1997. Daughterless is required for the expression of cell cycle genes in peripheral nervous system precursors of Drosophila embryos. Developmental Genetics **21**: 117-122.

Hatini V, Tao W, Lai E. 1994. Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. Journal of Neurobiology **25**(10):1293-309.

Hatta K, Kimmel CB, Ho RK, Walker C. 1991. The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. Nature **350**: 339-341.

Hayashi Y, Matsuyama K, Takagi K, Sugiura H, Yoshikawa K. 1995. Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons. Biochemical and Biophysical Research Communications **213**(1):317-24.

Heberlein U, Hariharan IK, Rubin GM. 1993. Star is required for neuronal differentiation in the Drosophila retina and displays dosage-sensitive interactions with Ras1. Developmental Biology, **160**(1):51-63.

Heintz N. 1993. Cell death and the cell cycle: a relationship between transformation and neurodegeneration? Trends in Biochemical Sciences **18**(5):157-9.

Heitzler P, Bourouis M, Ruel L, Carteret C, Simpson P. 1996. Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development **122**(1):161-71.

Helms AW, Johnson JE. 1998. Progenitors of dorsalcommisural interneurons are defined by MATH1 expression. Development **125**(5): 919-928.

Henkel T, Ling PD, Hayward SD, Peterson MG. 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. Science **265**(5168):92-5.

Henrique D, Adam J, Myat A, Chitnis A, Lewis J, Ish-Horowicz D. 1995. Expression of a Delta homologue in prospective neurons in the chick. Nature **375**(6534):787-90.

Henkel T, Ling PD, Hayward SD, Peterson MG. 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. Science **265**(5168):92-5.

Hing HK, Sun X, Artavanis-Tsakonas S. 1994. Modulation of wingless signaling by Notch in Drosophila. Mechanisms of Development **47**(3):261-8.

Hinz U, Giebel B, Campos-Ortega JA. 1994. The basic-helix-loop-helix domain of Drosophila lethal of scute protein is sufficient for proneural function and activates neurogenic genes. Cell **76**(1):77-87.

Hirsch MR, Tiveron MC, Guillemot F, Brunet JF, Goridis C. 1998. Control of noradrenergic differentiation and Phox2a expression by MASH-1 in the central and peripheral nervous system. Development **125**: 599-608.

Hirth F, Reichert H. 1999. Conserved genetic programs in insect and mammalian brain development. Bioessays **21**(8):677-84.

Homyk T Jr, Isono K, Pak WL. 1985. Developmental and physiological analysis of a conditional mutation affecting photoreceptor and optic lobe development in Drosophila melanogaster. Journal of Neurogenetics **2**(5):309-24.

Hobert O, Tessmar K, Ruvkun G. 1999. The Caenorhabditis elegans lim-6 LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons. Development **126**(7):1547-62.

Hobert O, Moerman DG, Clark KA, Beckerle MC, Ruvkun GA. 1999. conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in Caenorhabditis elegans. Journal of Cell Biology **144**(1):45-57.

Hobert O, D'Alberti T, Liu Y, Ruvkun G. 1998. Control of neural development and function in a thermoregulatory network by the LIM homeobox gene lin-11. Journal of Neuroscience **18**(6):2084-96.

Hobert O, Mori I, Yamashita Y, Honda H, Ohshima Y, Liu Y, Ruvkun G. 1997. Regulation of interneuron function in the C. elegans thermoregulatory pathway by the ttx-3 LIM homeobox gene. Neuron **19**(2):345-57.

Hogan B, Costantini F, Lacy E. Manipulating the Mouse Embryo, A Laboratory Manual. Cold Spring Harbor Press, 1986.

Holst BD, Wang Y, Jones FS, Edelman GM. 1997. A binding site for Pax proteins regulates expression of the gene for the neural cell adhesion molecule in the embryonic spinal cord. PNAS **94**(4):1465-70.

Horton S, Meredith A, Richardson JA, Johnson JE. 1999. Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Molecular and Cellular Neurosciences **14**(4-5):355-69.

Hosoya T, Takizawa K, Nitta K, Hotta Y. 1995. glial cells missing: a binary switch between neuronal and glial cell determination in Drosophila. Cell **82**(6): 1025-1036.

Hromas R, Costa R. 1995. The hepatocyte nuclear factor-3/forkhead transcription regulatory family in development, inflammation, and neoplasia. Critical Reviews in Oncology/Hematology **20**(1-2):129-40.

Hsieh JJ, Hayward SD. 1995. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. Science **268**(5210):560-3.

Hsieh JJ, Henkel T, Salmon P, Robey E, Peterson MG, Hayward SD. 1996. Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Molecular and Cellular Biology **16**(3):952-9.

Husain J, Lo R, Grbavec D, Stifani S. 1996. Affinity for the nuclear compartment and expression during cell differentiation implicate phosphorylated Groucho/TLE1 forms of higher molecular mass in nuclear functions. Biochemical Journal **317** (2):523-31.

Hynes M, Stone DM, Dowd M, Pitts-Meek S, Goddard A, Gurney A, Rosenthal A. 1997. Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. Neuron **19**(1):15-26.

i Altaba AR. 1998. Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. Development **125**(12): 2203-2212.

Iavarone A, Garg P, Lasorella A, Hsu J, Israel MA. 1994. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes and Development **8**(11):1270-84.

Ingraham HA, Chen RP, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG. 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell **55**(3):519-29.

Ishibashi M, Sasai Y, Nakanishi S, Kageyama R. 1993. Molecular characterization of HES-2, a mammalian helix-loop-helix factor structurally related to Drosophila hairy and Enhancer of split. European Journal of Biochemistry **215**(3):645-52.

Ishibashi M, Ang SL, Shiota K, Nakanishi S, Kageyama R, Guillemot F. 1995. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes and Development **9**(24):3136-48.

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R. 1994. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. Embo Journal, **13**(8):1799-805.

Ishiguro H, Kim KS, Joh TH. 1995. Identification of a negative regulatory element in the 5'-flanking region of the human dopamine beta-hydroxylase gene. Brain Research. Molecular Brain Research **34**(2):251-61.

Itoh F, Nakane T, Chiba S. 1997. Gene expression of MASH-1, MATH-1, neuroD and NSCL-2, basic helix-loop-helix proteins, during neural differentiation in P19 embryonal carcinoma cells. Tohoku Journal of Experimental Medicine **182**(4):327-36.

Iulianella A, Lohnes D. 1997. Contribution of retinoic acid receptor gamma to retinoid-induced craniofacial and axial defects. Developmental Dynamics **209**(1):92-104.

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. 1992. Effects of an Rb mutation in the mouse. Nature **359**(6393):295-300.

Jaenisch R, Jahner P, Nobois P, Simon I, Lohler J, Harbers K, Grotkopp D. 1981. Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. Cell **24**: 519-529.

Jarman AP, Grau Y, Jan LY, Jan YN. 1993. atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell **73**(7):1307-21.

Jarman AP, Brand M, Jan LY, Jan YN. 1993. The regulation and function of the helix-loop-helix gene, asense, in Drosophila neural precursors. Development **119**(1):19-29.

Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN. 1994. Atonal is the proneural gene for Drosophila photoreceptors. Nature, 1994 **369**(6479):398-400.

Jarman AP, Ahmed I. 1998. The specificity of proneural genes in determining Drosophila sense organ identity. Mechanisms of Development **76**(1-2):117-25.

Jarman AP, Sun Y, Jan LY, Jan YN. 1995. Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. Development **121**(7):2019-30.

Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A. 1995. Signalling downstream of activated mammalian Notch. Nature **377**(6547):355-8.

Jarriault S, Le Bail O, Hirsinger E, Pourquié O, Logeat F, Strong CF, Brou C, Seidah NG, Israel A. 1998. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Molecular and Cellular Biology **18**(12):7423-31.

Jen Y, Weintraub H, Benezra R. 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes and Development **6**(8):1466-79.

Jiang YJ, Brand M, Heisenberg CP, Beuchle D, Furutani-Seiki M, Kelsh RN, Warga RM, Granato M, Haffter P, Hammerschmidt M, Kane DA, Mullins MC, Odenthal J, van Eeden FJ, Nüsslein-Volhard C. 1996. Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. Development **123**:205-16.

Jiménez F, Campos-Ortega JA. 1979. A region of the Drosophila genome necessary for CNS development. Nature **282**(5736):310-2.

Jiménez F, Campos-Ortega JA. 1987. Genes in subdivision 1B of the Drosophila melanogaster X-chromosome and their influence on neural development. Journal of Neurogenetics **4**(4):179-200.

Jiménez G, Paroush Z, Ish-Horowicz D. 1997. Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. Genes and Development **11**(22):3072-82.

Jiralerspong S, Patel PI. 1996. Regulation of the hypoxanthine phosphoribosyltransferase gene: in vitro and in vivo approaches. Proceedings of the Society for Experimental Biology and Medicine **212**(2):116-27.

Johansen KM, Fehon RG, Artavanis-Tsakonas S. 1989. The notch gene product is a glycoprotein expressed on the cell surface of both epidermal and neuronal precursor cells during Drosophila development. Journal of Cell Biology **109**(5):2427-40.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisén J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. Cell **96**(1):25-34.

Johe K, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RDG. 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. **10**(24): 3129-40.

Johnson JD, Zhang W, Rudnick A, Rutter WJ, German MS. 1997. Transcriptional synergy between LIM-homeodomain proteins and basic helix-loop-helix proteins: the LIM2 domain determines specificity. Molecular and Cellular Biology **17**(7):3488-96.

Johnson JE, Birren SJ, Anderson DJ. 1990. Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. Nature **346**(6287):858-61.

Johnson JE, Birren SJ, Saito T, Anderson DJ. 1992. DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer. PNAS **89**(8):3596-600.

Johnston LA, Edgar BA. 1998. Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. Nature **394**(6688):82-4.

Jones BW, Fetter RD, Tear G, Goodman CS. 1995. glial cells missing: a genetic switch that controls glial cell versus neuronal cell fate. Cell **82**(6): 1013-1023.

Joutel A, Tournier-Lasserve E. 1998. Notch signalling pathway and human diseases. Seminars in Cell and Developmental Biology, 1998 Dec, 9(6):619-25.

Joyner AL. 1996. Engrailed, Wnt and Pax genes regulate midbrain--hindbrain development. Trends in Genetics **12**(1):15-20.

Kallunki P, Edelman GM, Jones FS. 1997. Tissue-specific expression of the L1 cell adhesion molecule is modulated by the neural restrictive silencer element. Journal of Cell Biology **138**(6):1343-54.

Kallunki P, Edelman GM, Jones FS. 1998. The neural restrictive silencer element can act as both a repressor and enhancer of L1 cell adhesion molecule gene expression during postnatal development. PNAS **95**(6):3233-8.

Kao HY, Ordentlich P, Koyano-Nakagawa N, Tang Z, Downes M, Kintner CR, Evans RM, Kadesch T. 1998. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes and Development **12**(15):2269-77.

Karns LR, Ng SC, Freeman JA, Fishman MC. 1987. Cloning of complementary DNA for GAP-43, a neuronal growth-related protein. Science **236**(4801):597-600.

Kastner P, Mark M, Ghyselinck N, Krezel W, Dupé V, Grondona JM, Chambon P. 1997. Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development **124**(2):313-26.

Kastner P, Messaddeq N, Mark M, Wendling O, Grondona JM, Ward S, Ghyselinck N, Chambon P. 1997. Vitamin A deficiency and mutations of RXRalpha, RXRbeta and RARalpha lead to early differentiation of embryonic ventricular cardiomyocytes. Development **124**(23):4749-58.

Kauffmann RC, Li S, Gallagher PA, Zhang J, Carthew RW. 1996. Ras1 signaling and transcriptional competence in the R7 cell of Drosophila. Genes and Development **10**(17):2167-78.

Kaur S, Singh G, Stock JL, Schreiner CM, Kier AB, Yager KL, Mucenski ML, Scott WJ Jr, Potter SS. 1992. Dominant mutation of the murine Hox-2.2 gene results in developmental abnormalities. Journal of Experimental Zoology **264**(3):323-36.

Kaushal S, Schneider JW, Nadal-Ginard B, Mahdavi V. 1994. Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. Science **266**(5188):1236-40.

Kawano H, Fukuda T, Kubo K, Horie M, Uyemura K, Takeuchi K, Osumi N, Eto K, Kawamura K. 1999. Pax-6 is required for thalamocortical pathway formation in fetal rats. Journal of Comparative Neurology **408**(2):147-60.

Kidd S, Baylies MK, Gasic GP, Young MW. 1989. Structure and distribution of the Notch protein in developing Drosophila. Genes and Development **3**(8):1113-29.

Kidd S, Kelley MR, Young MW. 1986. Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors. Molecular and Cellular Biology **6**(9):3094-108.

Kidd S, Lieber T, Young MW. 1998. Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes and Development **12**(23):3728-40.

Kilpatrick T, Bartlett PF. 1995. Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. Journal of Neuroscience **15**(5):3653-61.

Kim CH, Bae YK, Yamanaka Y, Yamashita S, Shimizu T, Fujii R, Park HC, Yeo SY, Huh TL, Hibi M, Hirano T. 1997. Overexpression of neurogenin induces ectopic expression of HuC in zebrafish. Neurosci Letters **239**: 113-116.

King PH, Levine TD, Fremeau RT Jr, Keene JD. 1994. Mammalian homologs of Drosophila ELAV localized to a neuronal subset can bind in vitro to the 3' UTR of mRNA encoding the Id transcriptional repressor. Journal of Neuroscience **14**(4):1943-52.

Klein T, Arias AM. 1998. Interactions among Delta, Serrate and Fringe modulate Notch activity during Drosophila wing development. Development **125**(15):2951-62.

Klueg KM, Muskavitch MA. 1999. Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in Drosophila. Journal of Cell Science **112** :3289-97.

Knoblich JA, Jan LY, Jan YN. 1995. Asymmetric segregation of Numb and Prospero during cell division. Nature **377**(6550):624-7.

Knust E, Dietrich U, Tepass U, Bremer KA, Weigel D, Vässin H, Campos-Ortega JA. 1987. EGF homologous sequences encoded in the genome of Drosophila melanogaster, and their relation to neurogenic genes. Embo Journal **6**(3):761-6.

Ko HS, Fast P, McBride W, Staudt LM. 1988. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. Cell **55**(1):135-44.

Köhler J, Schäfer-Preuss S, Buttgereit D. 1996. Related enhancers in the intron of the beta1 tubulin gene of Drosophila melanogaster are essential for maternal and CNS-specific expression during embryogenesis. Nucleic Acids Research **24**(13):2543-50.

Kooh PJ, Fehon RG, Muskavitch MA. 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. Development **117**(2):493-507.

Koop KE, MacDonald LM, Lobe CG. 1996. Transcripts of Grg4, a murine groucho-related gene, are detected in adjacent tissues to other murine neurogenic gene homologues during embryonic development. Mechanisms of Development **59**(1):73-87.

Kopan R, Nye J.S., Weintraub H. 1994. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development **120**: 2385-2396.

Kopczynski CC, Alton AK, Fechtel K, Kooh PJ, Muskavitch MA. 1988. Delta, a Drosophila neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. Genes and Development **2**(12B):1723-35.

Korzh V, Sleptsova I, iao J, He J, Gong Z. 1998. Expression of zebrafish bHLH genes ngn1 and nrd defines distinct stages of neural differentiation. Developmental Dynamics **213**: 92-104.

Koushika SP, Lisbin MJ, White K. 1996. ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Current Biology **6**(12):1634-41.

Kranenburg O, Scharnhorst V, Van der Eb AJ, Zantema A. 1995. Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. Journal of Cell Biology **131**(1):227-34.

Kraner SD, Chong JA, Tsay HJ, Mandel G. 1992. Silencing the type II sodium channel gene: a model for neural-specific gene regulation. Neuron **9**(1):37-44.

Krezel W, Dupé V, Mark M, Dierich A, Kastner P, Chambon P. 1996. RXR gamma null mice are apparently normal and compound RXR alpha +/-/RXR beta -/-/RXR gamma -/- mutant mice are viable. PNAS **93**(17):9010-4.

Kroll KL, Salic AN, Evans LM, Kirschner MW. 1998. Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. Development **125**: 3247-3258.

Krumlauf R. 1994. Hox genes in vertebrate development. Cell **78**(2):191-201.

Krushel LA, Johnston JG, Fishell G, Tibshirani R, van der Kooy D. 1993. Spatially localized neuronal cell lineages in the developing mammalian forebrain. Neuroscience **53**(4):1035-47.

Krushel LA, Fishell G, van der Kooy D. 1995. Pattern formation in the mammalian forebrain: striatal patch and matrix neurons intermix prior to compartment formation. European Journal of Neuroscience **7**(6):1210-9.

Kudrycki KE, Buiakova O, Tarozzo G, Grillo M, Walters E, Margolis FL. 1998. Effects of mutation of the Olf-1 motif on transgene expression in olfactory receptor neurons. Journal of Neuroscience Research **52**(2):159-72.

Kuhn HG, Svendsen CN. 1999. Origins, functions, and potential of adult neural stem cells. Bioessays **21**(8):625-30.

LaBonne C, Bronner-Fraser M. 1999. Molecular mechanisms of neural crest formation. Annual Review of Cell and Developmental Biology **15**:81-112.

LaBonne SG, Mahowald AP. 1985. Partial rescue of embryos from two maternal-effect neurogenic mutants by transplantation of wild-type ooplasm. Developmental Biology **110**(1):264-7.

LaBonne SG, Sunitha I, Mahowald AP. 1989. Molecular genetics of pecanex, a maternal-effect neurogenic locus of Drosophila melanogaster that potentially encodes a large transmembrane protein. Developmental Biology **136**(1):1-16.

Labosky PA, Winnier GE, Jetton TL, Hargett L, Ryan AK, Rosenfeld MG, Parlow AF, Hogan BL. 1997. The winged helix gene, Mf3, is required for normal development of the diencephalon and midbrain, postnatal growth and the milk-ejection reflex. Development **124**(7):1263-74.

Labouesse M, Hartwieg E, Horvitz HR. 1996. The Caenorhabditis elegans LIN-26 protein is required to specify and/or maintain all non-neuronal ectodermal cell fates. Development **122**(9):2579-88.

Lai ZC, Rubin GM. 1992. Negative control of photoreceptor development in Drosophila by the product of the yan gene, an ETS domain protein. Cell **70**(4):609-20.

Lakin ND, Morris PJ, Theil T, Sato TN, Möröy T, Wilson MC, Latchman DS. 1995. Regulation of neurite outgrowth and SNAP-25 gene expression by the Brn-3a transcription factor. Journal of Biological Chemistry **270**(26):15858-63.

Lambie EJ, Kimble J. 1991. Two homologous regulatory genes, lin-12 and glp-1, have overlapping functions. Development **112**(1):231-40.

Lanford PJ, Lan Y, Jiang R, Lindsell C, Weinmaster G, Gridley T, Kelley MW. 1999. Notch signalling pathway mediates hair cell development in mammalian cochlea. Nature Genetics **21**(3):289-92.

Lardelli M, Williams R, Mitsiadis T, Lendahl U. 1996. Expressionof the Notch-3 intracellular domain in mouse central nervous system progenitor cells is lethal and leads to disturbed neural tube development. Mech Develop **59**: 177-190.

Lazarova DL, Spengler BA, Biedler JL, Ross RA. 1999. HuD, a neuronal-specific RNA-binding protein, is a putative regulator of N-myc pre-mRNA processing/stability in malignant human neuroblasts. Oncogene **18**(17):2703-10.

Leconte L, Semonin O, Zvara A, Boisseau S, Poujeol C, Julien JP, Simonneau M. 1994. Both upstream and intragenic sequences of the human neurofilament light gene direct expression of lacZ in neurons of transgenic mouse embryos. Journal of Molecular Neuroscience **5**(4): 273-95.

Leconte L, Santha M, Fort C, Poujeol C, Portier MM, Simonneau M. 1996. Cell type-specific expression of the mouse peripherin gene requires both upstream and intragenic sequences in transgenic mouse embryos. Brain Research. Developmental Brain Research **92**(1):1-9.

Lecourtois M, Schweisguth F. 1995. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes and Development 9(21):2598-608.

Lecourtois M, Schweisguth F. 1998. Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. Current Biology 8(13):771-4.

Le Douarin NM, Dupin E. 1993. Cell lineage analysis in neural crest ontogeny. Journal of Neurobiology 24(2):146-61.

Le Douarin NM, Dupin E, Ziller C. 1994. Genetic and epigenetic control in neural crest development. Current Opinion in Genetics and Development 4(5):685-95.

Lee JE. 1997. Basic helix-loop-helix genes in neural development. Current Opinion in Neurobiology 7(1):13-20.

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268(5212):836-44.

Lee YM, Osumi-Yamashita N, Ninomiya Y, Moon CK, Eriksson U, Eto K. 1995. Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. Development 121(3):825-37.

Lehmann R, Dietrich U, Jimenez F, Campos-Ortega J A. 1981. Mutations of early neurogenesis in Drosophila. Roux's Arch. Dev. Biol. 190: 226-229.

Lehmann R, Jimenez F, Dietrich U, Campos-Ortega JA. 1983. On the phenotype and development of mutants of early neurogenesis in Drosophila melanogaster. Roux's Arch. Dev. Biol. 192: 62-74.

Leifer D, Krainc D, Yu YT, McDermott J, Breitbart RE, Heng J, Neve RL, Kosofsky B, Nadal-Ginard B, Lipton SA. 1993. MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. PNAS **90**(4):1546-50.

Leon C, Lobe CG. 1997. Grg3, a murine Groucho-related gene, is expressed in the developing nervous system and in mesenchyme-induced epithelial structures. Developmental Dynamics **208**(1):11-24.

Li CM, Yan RT, Wang SZ. 1999. Misexpression of cNSCL1 disrupts retinal development. Molecular and Cellular Neurosciences **14**(1):17-27.

Li H, Zeitler PS, Valerius MT, Small K, Potter SS. 1996. Gsh-1, an orphan Hox gene, is required for normal pituitary development. Embo Journal **15**(4):714-24.

Li J, Vogt PK. 1993. The retroviral oncogene qin belongs to the transcription factor family that includes the homeotic gene fork head. PNAS **90**(10):4490-4.

Liao J, He J, Yan T, Korzh V, Gong Z. 1999. A class of neuroD-related basic helix-loop-helix transcription factors expressed in developing central nervous system in zebrafish. Dna and Cell Biology **18**(4):333-44.

Lieber T, Kidd S, Alcamo E, Corbin V, Young MW. 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes and Development **7**(10):1949-65.

Lillien L. 1998. Neural progenitors and stem cells: mechanisms of progenitor heterogeneity. Curr Opin Neurobiol **8**(1): 37-44.

Lin JH, Saito T, Anderson DJ, Lance-Jones C, Jessell TM, Arber S. 1998. Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell **95**(3):393-407.

Lindsell CE, Boulter J, diSibio G, Gossler A, Weinmaster G. 1996. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Molecular and Cellular Neurosciences **8**(1):14-27.

Ling PD, Hsieh JJ, Ruf IK, Rawlins DR, Hayward SD. 1994. EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1. Journal of Virology **68**(9):5375-83.

Liu JK, Ghattas I, Liu S, Chen S, Rubenstein JL. 1997. Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. Developmental Dynamics **210**(4):498-512.

Lipinski MM, Jacks T. 1999. The retinoblastoma gene family in differentiation and development. Oncogene **18**(55):7873-82.

Lo LC, Johnson JE, Wuenschell CW, Saito T, Anderson DJ. 1991. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes and Development **5**(9):1524-37.

Lo LC, Anderson DJ. 1995. Postmigratory neural crest cells expressing c-ret display restricted developmental and proliferative capacities. Neuron **15**: 527-539.

Lo L, Morin X, Brunet JF, Anderson DJ. 1999. Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. Neuron **22**: 693-705.

Lo L, Tiveron MC, Anderson DJ. 1998. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development **125**(4): 609-620.

Lo LC, Johnson JE, Wuenschell CW, Saito T, Anderson DJ. 1991. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes and Development **5**(9):1524-37.

Lobe CG. 1997. Expression of the helix-loop-helix factor, HES-3, during embryo development suggests a role in early midbrain-hindbrain patterning. Mech Dev **62**: 227-237.

Lohnes D, Mark M, Mendelsohn C, Dollé P, Decimo D, LeMeur M, Dierich A, Gorry P, Chambon P. 1995. Developmental roles of the retinoic acid receptors. Journal of Steroid Biochemistry and Molecular Biology **53**(1-6):475-86.

Lönnerberg P, Lendahl U, Funakoshi H, Arhlund-Richter L, Persson H, Ibáñez CF. 1995. Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice. PNAS **92**(9):4046-50.

Lothian C, Lendahl U. 1997. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. European Journal of Neuroscience **9**(3):452-62.

Lothian C, Prakash N, Lendahl U, Wahlström GM. 1999. Identification of both general and region-specific embryonic CNS enhancer elements in the nestin promoter. Experimental Cell Research **248**(2):509-19.

Lumsden A, Krumlauf R. 1996. Patterning the vertebrate neuraxis. Science **274**(5290):1109-15.

Luo J, Pasceri P, Conlon RA, Rossant J, Giguère V. 1995. Mice lacking all isoforms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. Mechanisms of Development **53**(1):61-71.

Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R. 1999. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature **401**(6754):670-7.

Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ. 1998. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron **20**(3):469-82.

Ma Q, Kintner C, Anderson DJ. 1996. Identification of neurogenin, a vertebrate neruonal determination gene. Cell **87**(1): 43-52.

Ma Q, Sommer L, Cserjesi P, Anderson DJ. 1997. Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. J Neurosci **17**: 3644-3652.

Maden M, Holder N. 1992. Retinoic acid and development of the central nervous system. Bioessays **14**(7):431-8.

Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature, **391**:601-5.

Mallo M, Gendron-Maguire M, Harbison ML, Gridley T. 1995. Protein characterization and targeted disruption of Grg, a mouse gene related to the groucho transcript of the Drosophila Enhancer of split complex. Developmental Dynamics **204**(3):338-47.

Maniatis T, Sambrook J, Fritsch EF. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, 1989.

Manley NR, Capecchi MR. 1995. The role of Hoxa-3 in mouse thymus and thyroid development. Development **121**(7):1989-2003.

Manley NR, Capecchi MR. 1997. Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. Developmental Biology **192**(2):274-88.

Mansouri A, Hallonet M, Gruss P. 1996. Pax genes and their roles in cell differentiation and development. Current Opinion in Cell Biology **8**(6):851-7.

Mansouri A. 1998. The role of Pax3 and Pax7 in development and cancer. Critical Reviews in Oncogenesis **9**(2):141-9.

Manzanares M, Cordes S, Ariza-McNaughton L, Sadl V, Maruthainar K, Barsh G, Krumlauf R. 1999. Conserved and distinct roles of kreisler in regulation of the paralogous Hoxa3 and Hoxb3 genes. Development **126**: 759-769.

Manzanares M, Cordes S, Kwan CT, Sham MH, Barsh GS, Krumlauf R. 1997. Segmental regulation of Hoxb-3 by kreisler. Nature **387**: 191-195.

Mao Z, Nadal-Ginard B. 1996. Functional and physical interactions between mammalian achaete-scute homolog 1 and myocyte enhancer factor 2A. Journal of Biological Chemistry **271**(24):14371-5.

Mariani FV, Harland RM. 1998. XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue. Development **125**(24):5019-31.

Mark M, Lufkin T, Vonesch JL, Ruberte E, Olivo JC, Dollae P, Gorry P, Lumsden A, Chambon P. 1993. Two rhombomeres are altered in Hoxa-1 mutant mice. Development **119**: 319-338.

Marshall H, Nonchev S, Sham MH, Muchamore I, Lumsden A, Krumlauf R. 1992. Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature **360**: 737-741.

Martín-Bermudo MD, Carmena A, Jiménez F. 1995. Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. Development **121**(1):219-24.

Martínez C, Modolell J, Garrell J. 1993. Regulation of the proneural gene achaete by helix-loop-helix proteins. Molecular and Cellular Biology **13**(6):3514-21.

Martinsen BJ, Bronner-Fraser M. 1998. Neural crest specification regulated by the helix-loop-helix repressor Id2. Science **281**(5379):988-91.

Mastick GS, Davis NM, Andrew GL, Easter SS Jr. 1997. Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. Development **124**(10):1985-97.

Mathew TC, Miller FD. 1990. Increased expression of Tα1 α-tubulin mRNA during collateral and NGF-induced sprouting of sympathetic neurons. Dev. Biol. **141**: 84-92.

Mathew TC, Miller FD. 1993. Induction of Tα1 α-tubulin mRNA during neuronal regeneration is a function of the amount of axon lost. Dev. Biol. **158**: 467-474.

Matsunami N, Hamaguchi Y, Yamamoto Y, Kuze K, Kangawa K, Matsuo H, Kawaichi M, Honjo T. 1989. A protein binding to the J kappa recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif. Nature **342**(6252):934-7.

Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S. 1995. Mouse Otx2 functions in the formation and patterning of rostral head. Genes and Development **9**(21):2646-58.

Mayer-Proschel M, Kalyani AJ, Mujtaba T, Rao MS. 1997. Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. Neuron **19**(4):773-85.

McConnell SK, Kaznowski CE. 1991. Cell cycle dependence of laminar determination in developing neocortex. Science **254**(5029):282-5.

McCormick MB, Tamimi RM, Snider L, Asakura A, Bergstrom D, Tapscott SJ. 1996. NeuroD2 and neuroD3: distinct expression patterns and transcriptional activation potentials within the neuroD gene family. Molecular and Cellular Biology **16**(10):5792-800.

McDonald JA, Doe CQ. 1997. Establishing neuroblast-specific gene expression in the Drosophila CNS: huckebain is activated by Wingless and Hedgehog and repressed by Engrailed and Gooseberry. Development **124**: 1079-1087.

McEvilly RJ, Erkman L, Luo L, Sawchenko PE, Ryan AF, Rosenfeld MG. 1996. Requirements for Brn-3.0 in differentiation and survival of sensory and motor neurons. Nature **384**: 574-577.

McKay IJ, Lewis J, Lumsden A. 1997. Organization and development of facial motor neurons in the kresiler mutant mouse. European J Neurosci **9**: 1499-1506.

McKay IJ, Muchamore I, Krumlauf R, Maden M, Lumsden A, Lewis J. 1994. The kreisler mouse: a hindbrain segmentation mutant that lacks two rhombomeres. Development **120**: 2199-2211.

McLain K, Schreiner C, Yager KL, Stock JL, Potter SS. 1992. Ectopic expression of Hox-2.3 induces craniofacial and skeletal malformations in transgenic mice. Mechanisms of Development **39**(1-2):3-16.

McMahon AP, Bradley A. 1990. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell **62**(6):1073-85.

Meech R, Kallunki P, Edelman GM, Jones FS. 1999. A binding site for homeodomain and Pax proteins is necessary for L1 cell adhesion molecule gene expression by Pax-6 and bone morphogenetic proteins. PNAS **96**(5):2420-5.

Megeney LA, Rudnicki MA. 1995. Determination versus differentiation and the MyoD family of transcription factors. Biochemistry and Cell Biology **73**(9-10):723-32.

Mello CC, Schubert C, Draper B, Zhang W, Lobel R, Preiss JR. 1996. The PIE-1 protein and germline specification in C. elegans embryos. Nature **382**: 710-712.

Mercer EH, Hoyle GW, Kapur RP, Brinster RL, Palmiter RD. 1991. The dopamine β-hydroxylase gene promoter directs expression of E. coli lacZ to sympathetic and other neurons in adult transgenic mice. Neuron **7**: 703-716.

Millen KJ, Wurst W, Herrup K, Joyner AL. 1994. Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. Development **120**(3):695-706.

Miller FD, Mathew TC, Toma JG. 1991. Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. Journal of Cell Biology **112**(2):303-12.

Miller FD, Naus CCG, Durand M, Bloom FE, Milner RJ. 1987. Isotypes of α-tubulin are differentially regulated during neuronal maturation. J. Cell. Biol. **105**: 3065-3073.

Miller FD, Tetzlaff W, Bisby MA, Fawcett JW, Milner RJ. 1989. Rapid induction of the major embryonic α-tubulin mRNA, Tα1, during nerve regeneration in adult rats. J. Neurosci. **9**: 1452-1463.

Miller FD, Rogers D, Bamji SX, Slack RS, Gloster A. 1996. Analysis and manipulation of neuronal gene expression using the Tα1 α-tubulin promoter. Seminars in the Neurosciences **8**: 117-124.

Minoguchi S, Taniguchi Y, Kato H, Okazaki T, Strobl LJ, Zimber-Strobl U, Bornkamm GW, Honjo T. 1997. RBP-L, a transcription factor related to RBP-Jkappa. Molecular and Cellular Biology **17**(5):2679-87.

Minoo P, Su G, Drum H, Bringas P, Kimura S. 1999. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. Developmental Biology **209**(1):60-71.

Mirsky R, Jessen KR, Schachner M, Goridis C. 1986. Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats. Journal of Neurocytology **15**(6):799-815.

Miyama S, Takahashi T, Nowakowski RS, Caviness VS Jr. 1997. A gradient in the duration of the G1 phase in the murine neocortical proliferative epithelium. Cerebral Cortex, **7**(7):678-89.

Miyata T, Maeda T, Lee JE. 1999. NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes and Development **13**(13):1647-52.

Mlodzik M, Baker NE, Rubin GM. 1990. Isolation and expression of scabrous, a gene regulating neurogenesis in Drosophila. Genes and Development **4**(11):1848-61.

Modolell J. 1997. Patterning of the adult peripheral nervous system of Drosophila. Perspectives on Developmental Neurobiology **4**(4):285-96.

Moens CB, Cordes SP, Giorgianni MW, Barsh GS, Kimmel CB. 1998. Equivalence in the genetic control of hindbrain segmentation in fish and mouse. Development **125**: 381-391.

Moens CB, Yan YL, Appel B, Force AG, Kimmel CB. 1996. Development **122**: 3981-3990.

Monaghan AP, Kaestner KH, Grau E, Schütz G. 1993. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. Development **119**(3):567-78.

Moos M, Tacke R, Scherer H, Teplow D, Früh K, Schachner M. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. Nature **334**(6184):701-3.

Morin X, Cremer H, Hirsch MR, Kapur RP, Goridis C, Brunet JF. 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus I mice deficient for the homeobox gene Phox2a. Neuron **18**: 411-423.

Morrison A, Ariza-McNaughton L, Gould A, Featherstone M, Krumlauf R. 1997. HOXD4 and regulation of the group 4 paralog genes. Development **124**(16):3135-46.

Morrison A, Moroni MC, Ariza-McNaughton L, Krumlauf R, Mavilio F. 1996. In vitro and transgenic analysis of a human HOXD4 retinoid-responsive enhancer. Development **122**(6):1895-907.

Morrison SJ, Shah NM, Anderson DJ. 1997. Regulatory mechanisms in stem cell biology. Cell **88**: 287-298.

Morrow EM, Furukawa T, Lee JE, Cepko CL. 1999. NeuroD regulates multiple functions in the developing neural retina in rodent. Development **126**(1):23-36.

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron **13**: 1071-1082.

Muccielli ML, Martinez S, Pattyn A, Goridis C, Brunet JF. 1996. Otlx2, an Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. Molecular and Cellular Neurosciences **8**(4):258-71.

Murphy P, Topilko P, Schneider-Maunoury S, Seitanidou T, Baron-Van Evercooren A, Charnay P. 1996. The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. Development **122**(9): 2847-2857.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, et al. 1989a. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**(3):537-44.

Murre C, McCaw PS, Baltimore D. 1989b. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56**(5):777-83.

Na E, McCarthy M, Neyt C, Lai E, Fishell G. 1998. Telencephalic progenitors maintain anteroposterior identities cell autonomously. Current Biology **8**(17):987-90.

Nakada Y, Taniura H, Uetsuki T, Inazawa J, Yoshikawa K. 1998. The human chromosomal gene for necdin, a neuronal growth suppressor, in the Prader-Willi syndrome deletion region. Gene **213**(1-2):65-72.

Nakai S, Kawano H, Yudate T, Nishi M, Kuno J, Nagata A, Jishage K, Hamada H, Fujii H, Kawamura K, et al. 1995. The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. Genes and Development 9(24):3109-21.

Nakao K, Campos-Ortega JA. 1996. Persistent expression of genes of the enhancer of split complex suppresses neural development in Drosophila. Neuron **16**(2):275-86.

Nardelli J, Thiesson D, Fujiwara Y, Tsai FY, Orkin SH. 1999. Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. Developmental Biology **210**(2):305-21.

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo F, Leiter AB, Tsai MJ. 1997. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes and Development **11**(18):2323-34.

Naya FJ, Wu C, Richardson JA, Overbeek P, Olson EN. 1999. Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. Development **126**(10):2045-52.

Neuman T, Keen A, Zuber MX, Kristjansson GI, Gruss P, Nornes HO. 1993. Neuronal expression of regulatory helix-loop-helix factor Id2 gene in mouse. Developmental Biology **160**(1):186-95.

Neyt C,Welch M, Langston A, Kohtz J, Fishell G. 1997. A short-range signal restricts cell movement between telencephalic proliferative zones. Journal of Neuroscience **17**(23):9194-203.

Nofziger D, Miyamoto A, Lyons KM, Weinmaster G. 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development **126**(8):1689-702.

Nomura M, Takihara Y, Shimada K. 1994. Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: one of the early inducible clones encodes a novel protein sharing several highly homologous regions with a Drosophila polyhomeotic protein. Differentiation **57**(1):39-50.

Nonchev S, Maconochie M, Vesque C, Aparicio S, Ariza-McNaughton L, Manzanares M, Maruthainar K, Kuroiwa A, Brenner S, Charnay P, Krumlauf R. 1996. The conserved role of Krox-20 in directing Hox gene expression during vertebrate hindbrain segmentation. PNAS **93**(18):9339-45.

Nonchev S, Vesque C, Maconochie M, Seitanidou T, Ariza-McNaughton L, Frain M, Marshall H, Sham MH, Krumlauf R, Charnay P. 1996. Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. Development **122**(2):543-54.

Norton JD, Deed RW, Craggs G, Sablitzky F. 1998. Id helix-loop-helix proteins in cell growth and differentiation. Trends in Cell Biology **8**(2):58-65.

Novitch BG, Mulligan GJ, Jacks T, Lassar AB. 1996. Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. Journal of Cell Biology **135**(2):441-56.

Nye JS, Kopan R, Axel R. 1994. An activated Notch supresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. Development **120**: 2421-2430.

Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC. 1997. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nature Genetics **16**(3):235-42.

Ohsako S, Hyer J, Panganiban G, Oliver I Caudy M. 1994. Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. Genes Dev **8**: 2743-2755.

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R. 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. Embo Journal **18**(8):2196-207.

Okano HJ, Darnell RB. 1997. A hierarchy of Hu RNA binding proteins in developing and adult neurons. Journal of Neuroscience **17**(9):3024-37.

Olave I, Reinberg D, Vales LD. 1998. The mammalian transcriptional repressor RBP (CBF1) targets TFIID and TFIIA to prevent activated transcription. Genes and Development **12**(11):1621-37.

Oliver G, Sosa-Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P. 1993. Prox 1, a prospero-related homeobox gene expressed during mouse development. Mechanisms of Development **44**(1):3-16.

O'Neill EM, Rebay I, Tjian R, Rubin GM. 1994. The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell **78**(1):137-47.

Osumi N, Hirota A, Ohuchi H, Nakafuku M, Iimura T, Kuratani S, Fujiwara M, Noji S, Eto K. 1997. Pax-6 is involved in the specification of hindbrain motor neuron subtype. Development **124**(15):2961-72.

Pabst O, Herbrand H, Arnold HH. 1998. Nkx2-9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS. Mechanisms of Development **73**(1):85-93.

Packer AI, Crotty DA, Elwell V, Wolgemuth DJ. 1998. Expression of the murine Hoxa4 gene requires both autoregulation and a conserved retinoic acid response element. Development **125**(11):1991-8.

Papalopulu N, Kintner C. 1996. A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. Development **122**(11):3409-18.

Parody TR, Muskavitch MA. 1993. The pleiotropic function of Delta during postembryonic development of Drosophila melanogaster. Genetics **135**(2):527-39.

Parks AL, Muskavitch MA. 1993. Delta function is required for bristle organ determination and morphogenesis in Drosophila. Developmental Biology **157**(2):484-96.

Paroush Z, Finley RL Jr, Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D. 1994. Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell **79**(5):805-15.

Parr BA, Shea M, Vassileva G, McMahon AP. 1993. Wnt genes exhibit discrete domains of expression in the early embryionic CNS and limb buds. Development **119**: 247-261.

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF. 1997. Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development **124**(20): 4065-4075.

Pellegrini M, Mansouri A, Simeone A, Boncinelli E, Gruss P. 1996. Dentate gyrus formation requires Emx2. Development **122**(12):3893-8.

Perez SE, Rebelo S, Anderson DJ. 1999. Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development **126**: 1715-1728.

Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM. 1996. Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell **84**(2):309-320.

Pflugrad A, Meir JY, Barnes TM, Miller DM 3rd. 1997. The Groucho-like transcription factor UNC-37 functions with the neural specificity gene unc-4 to govern motor neuron identity in C. elegans. Development **124**(9):1699-709.

Phelan SA, Ito M, Loeken MR. 1997. Neural tube defects in embryos of diabetic mice: role of the Pax-3 gene and apoptosis. Diabetes **46**(7):1189-97.

Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL. 1997. The eye-specification proteins So and Eya form a complex and regulate multiple steps in Drosophila eye development. Cell **91**(7):881-891.

Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt F W, Orkin S H. 1996. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell **86**: 47-57.

Porter FD, Drago J, Xu Y, Cheema SS, Wassif C, Huang SP, Lee E, Grinberg A, Massalas JS, Bodine D, Alt F, Westphal H. 1997. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development **124**(15):2935-44.

Porteus MH, Bulfone A, Liu JK, Puelles L, Lo LC, Rubenstein, JL. 1994. DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. Journal of Neuroscience **14**(11):6370-83.

Portier MM, de Néchaud B, Gros F. 1983. Peripherin, a new member of the intermediate filament protein family. Developmental Neuroscience **6**(6):335-44.

Poulson DF. 1937. Chromosomal deficienceis and embryonic development of Drosophila melanogaster. PNAS **23**: 133-137.

Prasad BC, Ye B, Zackhary R, Schrader K, Seydoux G, Reed RR. 1998. unc-3, a gene required for axonal guidance in Caenorhabditis elegans, encodes a member of the O/E family of transcription factors. Development **125**(8): 1561-1568.

Pravtcheva D, Wise TL, Ensor NJ, Ruddle FH. 1994. Mosaic expression of an Hprt transgene integrated in a region of Y heterochromatin. J. Exp. Zool **268**: 452-468.

Price M, Lazarro D, Pohl T, Mattei MG, Ruther U, Olivo JC, Duboule D. 1992. Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain. Neuron 8: 241-255.

Price M, Lazzaro D, Pohl T, Mattei MG, Rüther U, Olivo JC, Duboule D, Di Lauro R. 1992. Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain. Neuron **8**(2):241-55.

Price M. 1993. Members of the Dlx- and Nkx2-gene families are regionally expressed in the developing forebrain. Journal of Neurobiology **24**(10):1385-99.

Prince VE, Moens CB, Kimmel CB, Ho RK. 1998. Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. Development **125**(3): 393-406.

Puelles L, Rubenstein JLR. 1993. Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. Trends Neurosci. **16**: 472-479.

Puigserver P, Ribot J, Serra F, Gianotti M, Bonet ML, Nadal-Ginard B, Palou A. 1998. Involvement of the retinoblastoma protein in brown and white adipocyte cell differentiation: functional and physical association with the adipogenic transcription factor C/EBPalpha. European Journal of Cell Biology **77**(2):117-23.

Qi H, Rand MD, Wu X, Sestan N, Wang W, Rakic P, Xu T, Artavanis-Tsakonas S. 1999. Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science **283**(5398):91-4.

Qian X, Davis AA, Goderie SK, Temple S. 1997. FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron **18**(1):81-93.

Qiu M, Bulfone A, Martinez S, Meneses J, Shimamura K, Pedersen RA, Rubenstein JL. 1995. Null mutation of Dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. Genes and Development **9**(20):2523-38.

Qiu M, Anderson S, Chen S, Meneses JJ, Hevner R, Kuwana E, Pedersen RA, Rubenstein JL. 1996. Mutation of the Emx-1 homeobox gene disrupts the corpus callosum. Developmental Biology **178**(1):174-8.

Qiu M, Shimamura K, Sussel L, Chen S, Rubenstein JL. 1998. Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. Mechanisms of Development **72**(1-2):77-88.

Rabinow L, Birchler JA. 1990. Interactions of vestigial and scabrous with the Notch locus of Drosophila melanogaster. Genetics **125**(1):41-50.

Rao Y, Bodmer R, Jan LY, Jan YN. 1992. The big brain gene of Drosophila functions to control the number of neuronal precursors in the peripheral nervous system. Development **116**(1):31-40.

Raschellà G, Tanno B, Bonetto F, Negroni A, Claudio PP, Baldi A, Amendola R, Calabretta B, Giordano A, Paggi MG. 1998. The RB-related gene Rb2/p130 in neuroblastoma differentiation and in B-myb promoter down-regulation. Cell Death and Differentiation **5**(5):401-7.

Ravassard P, Vallin J, Mallet J, Icard-Liepkalns C. 1997. Relax promotes ectopic neuronal differentiation in Xenopus embryos. PNAS **94**: 8602-8605.

Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S. 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell **67**(4):687-99.

Rebay I, Fehon RG, Artavanis-Tsakonas S. 1993. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell **74**(2):319-29.

Rebay I, Rubin GM. (1995) Yan function as a general inhibtor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell **81**(6): 857-866.

Reichert H, Boyan G. 1997. Building a brain: developmental insights in insects. Trends in Neurosciences **20**(6):258-64.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science **255**: 1707-1710.

Rhinn M, Dierich A, Shawlot W, Behringer RR, Le Meur M, Ang SL. 1998. Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. Development **125**(5):845-56.

Rhyu MS, Jan LY, Jan YN. 1994. Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell **76**: 477-491.

Roark M, Sturtevant MA, Emery J, Vaessin H, Grell E, Bier E. 1995. scratch, a pan-neural gene encoding a zinc finger protein related to snail, promotes neuronal development. Genes and Development **9**(19):2384-98.

Robinow S, White K. 1988. The locus elav of Drosophila melanogaster is expressed in neurons at all developmental stages. Developmental Biology **126**(2):294-303.

Robinow S, Campos AR, Yao KM, White K. 1988. The elav gene product of Drosophila, required in neurons, has three RNP consensus motifs. Science **242**(4885):1570-2.

Robinson GW, Wray S, Mahon KA. 1991. Spatially restricted expression of a member of a new family of murine Distal-less homeobox genes in the developing forebrain. New Biologist, 1991 Dec, 3(12):1183-94.

Robinson GW, Mahon KA. 1994. Differential and overlapping expression domains of Dlx-2 and Dlx-3 suggest distinct roles for Distal-less homeobox genes in craniofacial development. Mechanisms of Development **48**(3):199-215.

Rohrer H, Thoenen H. 1987. Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. J Neurosci **7**: 3739-3748.

Romani S, Campuzano S, Modolell J. 1987. The achaete-scute complex is expressed in neurogenic resions of Drosophila embryos. EMBO J. **6**: 2085-2092.

Roose J, Molenar, M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destraee O, Clevers H. 1998. The Xenopus Wnt effector XTcf-3 interacts with groucho-related trascriptional repressors. Nature **395**: 608-612.

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destrée O, Clevers H. 1998. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature **395**(6702):608-12.

Ross ME. 1996. Cell division and the nervous system: regulating the cycle from neural differentiation to death. Trends in Neurosciences **19**(2):62-8.

Ross RA, Lazarova DL, Manley GT, Smitt PS, Spengler BA, Posner JB, Biedler JL. 1997. HuD, a neuronal-specific RNA-binding protein, is a potential regulator of MYCN expression in human neuroblastoma cells. European Journal of Cancer **33**(12):2071-4.

Roztocil T, Matter-Sadzinski L, Alliod C, Ballivet M, Matter JM. 1997. NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. Development **124**(17):3263-72.

Rubenstein JLR, Martinez S, Shimamura K, Puelles L. (1994) The embryonic vertebrate forebrain: the Prosomeric model. Science **266**:578-580.

Rubenstein JL, Shimamura K, Martinez S, Puelles L. 1998. Regionalization of the prosencephalic neural plate. Annual Review of Neuroscience **21**:445-77.

Ruchoux MM, Maurage CA. 1997. CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Journal of Neuropathology and Experimental Neurology **56**(9):947-64.

Ruel L, Bourouis M, Heitzler P, Pantesco V, Simpson P. 1993. Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. Nature, **362**(6420):557-60.

Ruiz-Gómez M, Modolell J. 1987. Deletion analysis of the achaete-scute locus of Drosophila melanogaster. Genes and Development **1**(10):1238-46.

Rushlow CA, Hogan A, Pinchin SM, Howe KM, Lardelli M, Ish-Horowicz D. 1989. The Drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc. Embo Journal **8**(10):3095-103.

Saito T, Sawamoto K, Okano H, Anderson DJ, Mikoshiba K. 1998. Mammalian BarH homologue is a potential regulator of neural bHLH genes. Dev Biol **199**: 216-225.

Salser SJ, Loer CM, Kenyon C. 1993. Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system. Genes and Development **7**(9):1714-24.

Salser SJ, Kenyon C. 1996. A C. elegans Hox gene switches on, off, on and off again to regulate proliferation, differentiation and morphogenesis. Development **122**(5):1651-61

Salzberg A, Bellen HJ. 1996. Invertebrate versus vertebrate neurogenesis: variations on the same theme? Developmental Genetics **18**(1):1-10.

Sanlioglu S, Zhang X, Baader SL, Oberdick J. 1998. Regulation of a Purkinje cell-specific promoter by homeodomain proteins: repression by engrailed-2 vs. synergistic activation by Hoxa5 and Hoxb7. J Neurobiol **36**: 559-571.

Sasai Y. 1998. Identifying the missing links: genes that connect neural induction and primary neurogenesis in vertebrate embryos. Neuron **21**(3):455-8.

Sasai Y, Kageyama R, Tagawa Y, Shigemoto R, Nakanishi S. 1992. Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of Split. Genes Dev **6**: 2620-2634.

Sasaki H, Hogan BLM. 1993. Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development **118**: 47-59.

Sasaki H, Hogan BL. 1996. Enhancer analysis of the mouse HNF-3 beta gene: regulatory elements for node/notochord and floor plate are independent and consist of multiple sub-elements. Genes To Cells **1**(1):59-72.

Sasaki H, Hui C, Nakafuku M, Kondoh H. 1997. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development *124*(7):1313-22.

Sasner M, Buonanno A. 1996. Distinct N-methyl-D-aspartate receptor 2B subunit gene sequences confer neural and developmental specific expression. Journal of Biological Chemistry **271**(35):21316-22.

Scheidereit C, Cromlish JA, Gerster T, Kawakami K, Balmaceda CG, Currie RA, Roeder RG. 1988. A human lymphoid-specific transcription factor that activates immunoglobulin genes is a homoeobox protein. Nature *336*(6199):551-7.

Schier AF, Neuhauss SC, Harvey M, Malicki J, Solnica-Krezel L, Stainier DY, Zwartkruis F, Abdelilah S, Stemple DL, Rangini Z, Yang H, Driever W. 1996. Mutations affecting the development of the embryonic zebrafish brain. Development **123**:165-78.

Schneider-Maunoury S, Seitanidou T, Charnay P, Lumsden A. 1997. Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. Development **124**(6): 1215-1226.

Schneider-Maunoury S, Topilko P, Seitandou T, Levi G, Cohen-Tannoudji M, Pounin S, Babinet C, Charnay P. 1993. Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. Cell **75**: 1199-1214.

Schonemann MD, Ryan AK, McEvilly RJ, O'Connell SM, Arias CA, Kalla KA, Li P, Sawchenko PE, Rosenfeld MG. 1995. Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2. Genes and Development **9**(24):3122-35.

Schoenherr CJ, Anderson DJ. 1995. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science **267**(5202):1360-3.

Schoenherr CJ, Paquette AJ, Anderson DJ. 1996. Identification of potential target genes for the neuron-restrictive silencer factor. PNAS **93**(18):9881-6.

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature **381**(6579):235-8.

Schroeter EH, Kisslinger J, Kopan R. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature **393**(6683):382-6.

Schwarz M, Alvarez-Bolado G, Urbánek P, Busslinger M, Gruss P. 1997. Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. PNAS **94**(26):14518-23

Schweisguth F. 1995. Suppressor of Hairless is required for signal reception during lateral inhibition in the Drosophila pupal notum. Development **121**(6):1875-84.

Schweisguth F, Posakony JW. 1992. Suppressor of Hairless, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. Cell **69**(7):1199-212.

Sengupta P, Bargmann CI. 1996. Cell fate specification and differentiation in the nervous system of Caenorhabditis elegans. Developmental Genetics : **18**: 73-80.

Sestan N, Artavanis-Tsakonas S, Rakic P. 1999. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science **286**(5440):741-6.

Seydoux G, Mello CC, Pettitt J, Wood WB, Preiss JR, Fire A. 1996. Repression of gene expression in the embryonic germ lineage of C. elegans. Nature **382**: 713-716.

Shah NM, Marchionni MA, Isaacs I, Stroobant PW, Anderson DJ. 1994. Glial growth factor restricts mammalian neural crest stem cells ti a glial fate. Cell **77**: 349-360.

Shah N, Groves A, Anderson D J. 1996. Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. Cell **85**: 331-343.

Sharma K, Sheng HZ, Lettieri K, Li H, Karavanov A, Potter S, Westphal H, Pfaff SL. 1998. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell **95**(6):817-28.

Sharpe CR, Goldstone K. 1997. Retinoid receptors promote primary neurogenesis in Xenopus. Development **124**(2):515-23.

Shawber C, Boulter J, Lindsell CE, Weinmaster G. 1996. Jagged2: a serrate-like gene expressed during rat embryogenesis. Developmental Biology **180**(1):370-6.

Shawber C, Nofziger D, Hsieh JJ, Lindsell C, Bögler O, Hayward D, Weinmaster G. 1996. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development **122**(12):3765-73.

Shawlot W, Behringer RR. 1995. Requirement for Lim1 in head-organizer function. Nature **374**: 425-430.

Shepard SB, Broverman SA, Muskavitch MA. 1989. A tripartite interaction among alleles of Notch, Delta, and Enhancer of split during imaginal development of Drosophila melanogaster. Genetics **122**(2):429-38.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes and Development **13**(12):1501-12.

Shimamura K, Hartigan D, Martinez S, Puelles L, Rubenstein JLR. 1995. Longitudinal organization of the nterior neural plate and neural tube. Development **121**: 3923-3933.

Shimazaki T, Arsenijevic Y, Ryan AK, Rosenfeld MG, Weiss S. 1999. A role for the POU-III transcription factor Brn-4 in the regulation of striatal neuron precursor differentiation. Embo Journal **18**(2):444-56.

Shimeld SM, McKay IJ, Sharpe PT. 1996. The murine homeobox gene Msx-3 shows highly restricted expression in the developing neural tube. Mechanisms of Development **55**(2):201-10.

Shimizu C, Akazawa C, Nakanishi S, Kageyama R. 1995. MATH-2, a mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal, is specifically expressed in the nervous system. European Journal of Biochemistry **229**: 239-248.

Shirakata Y, Shuman JD, Coligan JE. 1996. Purification of a novel MHC class I element binding activity from thymus nuclear extracts reveals that thymic RBP-Jkappa/CBF1 binds to NF-kappaB-like elements. Journal of Immunology **156**(12):4672-9.

Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E. 1992. Nested expression domains of four homeobox genes in developing rostral brain. Nature **358**: 687-690.

Simeone A, Acampora D, Mallamaci A, Stornaiuolo A, D'Aprice M R, Nigro V, Boncinelli E. 1993. A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. EMBO J **12**: 2735-2747.

Simeone A, Gulisano M, Acampora D, Stornaiuolo A, Rambaldi M, Boncinelli E. 1992. Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. EMBO J **11**: 2541-2550.

Simpson P. 1995. Positive and negative regulators of neural fate. Cell **15**: 739-742.

Simpson P. 1990. Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of Drosophila. Development **109**(3):509-19.

Simpson P, Carteret C. 1989. A study of shaggy reveals spatial domains of expression of achaete-scute alleles on the thorax of Drosophila. Development **106**(1):57-66.

Skapek SX, Rhee J, Spicer DB, Lassar AB. 1995. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science **267**(5200):1022-4.

Skeath JB, Carroll SB. 1991. Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. Genes and Development **5**(6):984-95.

Smith J. 1999. T-box genes: what they do and how they do it. Trends Genetics **15**(4) 154-158.

Sommer L, Ma Q, Anderson DJ. 1996. neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. Molecular and Cellular Neurosciences **8**(4):221-41.

Spana EP, Doe CQ. 1995. The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. Development **121**(10):3187-95.

Spana EP, Doe CQ. 1996. Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron **17**(1):21-6.

Spana EP, Kopcyznski C, Goodman CS, Doe CQ. 1995. Asymmetric localization of numb autonomously determines sibbling neuron identity in the Drosophila CNS. Development **121**: 3489-3494.

Stemple DL, Anderson DJ. 1992. Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell **71**: 973-985.

Stifani S, Blaumueller CM, Redhead NJ, Hill RE, Artavanis-Tsakonas S. 1992. Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nature Genetics **2**(4):343.

Stout JT, Caskey CT. 1985. HPRT: gene structure, expression, and mutation. Annual Review of Genetics **19**:127-48.

Stoykova A, Fritsch R, Walther C, Gruss P. 1996. Forebrain patterning defects in Small eye mutant mice. Development **122**(11):3453-65.

Strittmatter SM, Vartanian T, Fishman MC. 1992. GAP-43 as a plasticity protein in neuronal form and repair. Journal of Neurobiology **23**(5):507-20.

Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC. 1995. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. Cell **80**(3):445-52.

Struhl G, Fitzgerald K, Greenwald I. 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell, 1993 **74**(2):331-45.

Studer M, Lumsden A, Ariz-McNaughton L, Bradley A, Krumlauf R. 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. Nature **384**: 630-634.

Studer M, Gavalas A, Marshall H, Ariza-McNaughton L, Rijli FM, Chambon P, Krumlauf R. 1998. Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. Development **125**(6):1025-36.

Suchanek B, Seeburg PH, Sprengel R. 1997. Tissue specific control regions of the N-methyl-D-aspartate receptor subunit NR2C promoter. Biological Chemistry **378**(8):929-34.

Sucov HM, Izpisúa-Belmonte JC, Gañan Y, Evans RM. 1995. Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. Development **121**(12):3997-4003.

Suda Y, Matsuo I, Kuratani S, Aizawa S. 1996. Otx1 function overlaps with Otx2 in development of mouse forebrain and midbrain. Genes To Cells **1**(11):1031-44.

Sun X, Artavanis-Tsakonas S. 1997. Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. Development **124**(17):3439-48.

Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. 1998. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development **125**(12):2213-21.

Swiatek PJ, Lindsell CE, del Amo FF, Weinmaster G, Gridley T. 1994. Notch1 is essential for postimplantation development in mice. Genes and Development **8**(6):707-19.

Szucsik JC, Witte DP, Li H, Pixley SK, Small KM, Potter SS. 1997. Altered forebrain and hindbrain development in mice mutant for the Gsh-2 homeobox gene. Developmental Biology **191**(2):230-42.

Taghert PH, Doe CQ, Goodman CS. 1984. Cell determination and regulation during development of neuroblasts and neurones in grasshopper embryo. Nature **307**(5947):163-5.

Tajbakhsh S, Buckingham ME. 1995. Lineage restriction of the myogenic conversion factor myf-5 in the brain. Development **121**(12): 4077-4083.

Takahashi T, Goto T, Miyama S, Nowakowski RS, Caviness VS Jr. 1999. Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. Journal of Neuroscience **19**(23):10357-71.

Takebayashi K, Akazawa C, Nakanishi S, Kageyama R. 1995. Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. JBC **270**(3):1342-9.

Takihara Y, Tomotsune D, Shirai M, Katoh-Fukui Y, Nishii K , Motaleb MA, Nomura M, Tsuchiya R ,Fujita Y, Shibata Y, Higashinakagawa T, Shimada K. 1997. Targeted

disruption of the mouse homologue of the Drosophila polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. Development **124**(19):3673-82.

Takuma N, Sheng HZ, Furuta Y, Ward JM, Sharma K, Hogan BL, Pfaff SL, Westphal H, Kimura S, Mahon KA. 1998. Formation of Rathke's pouch requires dual induction from the diencephalon. Development **125**(23):4835-40.

Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T, Honjo T. 1995. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Current Biology **5**(12):1416-23.

Tanabe Y, William C, Jessell TM. (1998) Specification of motor neuron identity by the MNR2 homeodomain protein. Cell **95**(1): 67-80.

Taniura H, Taniguchi N, Hara M, Yoshikawa K. 1998. Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. JBC **273**(2):720-8.

Tao W, Lai E. 1992. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron **8**(5):957-66.

Technau GM, Becker T, Campos-Ortega JA. 1988. Reversible commitment of neural and epidermal progenitor cells during embryogenesis of Drosophila melanogaster. Roux's Arch. Dev. Biol. **197**: 413-418.

Technau GM, Campos-Ortega JA. 1985. Fate mapping in wildtype Drosophila melanogaster. II. Injections of horseradish peroxidase in cells of the early gastrula stage. Roux's Arch. Dev. Biol. **194**: 196-212.

Tedesco D, Caruso M, Fischer-Fantuzzi L, Vesco C. 1995. The inhibition of cultured myoblast differentiation by the simian virus 40 large T antigen occurs after myogenin

expression and Rb up-regulation and is not exerted by transformation-competent cytoplasmic mutants. Journal of Virology **69**(11):6947-57.

Temple S, Qian X (1996) Vertebrate neural progenitor cells: subtypes and regulation. Curr Opin Neurobiol **6**: 11-17.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA. 1991. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. Journal of Neuroscience **11**(8):2528-44.

Thompson MA, Lee E, Lawe D, Gizang-Ginsberg E, Ziff EB. 1992. Nerve growth factor-induced derepression of peripherin gene expression is associated with alterations in proteins binding toa negative regulatory element. MCB **12**: 2501-2513.

Thomas U, Speicher SA, Knust E. 1991. The Drosophila gene Serrate encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. Development **111**(3):749-61.

Thor S, Andersson SG, Tomlinson A, Thomas JB. 1999. A LIM-homeodomain combinatorial code for motor-neuron pathway selection. Nature **397**(6714):76-80.

Thor S, Thomas JB. 1997. The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. Neuron **18**(3):397-409.

Timmusk T, Palm K, Lendahl U, Metsis M. 1999. Brain-derived neurotrophic factor expression in vivo is under the control of neuron-restrictive silencer element. JBC **274**(2):1078-84.

Tiret L, Le Mouellic H, Maury M, Brûlet P. 1998. Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development **125**(2):279-91.

Tiveron MC, Hirsch MR, Brunet JF. 1996. The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. J Neurosci. **16**: 7649-7660.

Tomita K, Nakanishi S, Guillemot F, Kageyama R. 1996. Mash1 promotes neuronal differentiation in the retina. Genes To Cells **1**(8):765-74.

Tomita K, Ishibashi M, Nakahara K, Ang SL, Nakanishi S, Guillemot F, Kageyama R. 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron **16**(4):723-34.

Torii Ma, Matsuzaki F, Osumi N, Kaibuchi K, Nakamura S, Casarosa S, Guillemot F, Nakafuku M. 1999. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development **126**(3):443-56.

Treacy MN, He X, Rosenfeld MG. 1991. I-POU: a POU-domain protein that inhibits neuron-specific gene activation. Nature **350**(6319):577-84.

Treier M, Bohman D, Mlodzik M. 1995. JUN cooperates with the ETS domain protein pointed to induce photoreceptor R7 fate in the Drosophila eye. Cell **83**(5):753-760.

Tsai TF, Armstrong D, Beaudet AL. 1999. Necdin-deficient mice do not show lethality or the obesity and infertility of Prader-Willi syndrome. Nature Genetics **22**(1):15-6.

Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL. 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell **79**(6):957-70.

Tun T, Hamaguchi Y, Matsunami N, Furukawa T, Honjo T, Kawaichi M. 1994. Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. Nucleic Acids Research **22**(6):965-71.

Turner DL, Cepko CL. 1987. Cell lineage in the rat retina: a common progenitor for neurons and glia persists late in development. Nature **328**: 131-136.

Tuttle R, Nakagawa Y, Johnson JE, O'Leary DD. 1999. Defects in thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-deficient mice. Development **126**(9):1903-16.

Uemura T, Shepherd S, Ackerman L, Jan LY, Jan YN. 1989. numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell **58**(2):349-60.

Uyttendaele H, Marazzi G, Wu G, Yan Q, Sassoon D, Kitajewski J. 1996. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development **122**(7):2251-9.

Vässin H, Vielmetter J, Campos-Ortega JA. 1985. Genetic interactions in early neurogenesis of Drosophila melanogaster. Journal of Neurogenetics **2**(5):291-308.

Vaessin H, Grell E, Wolff E, Bier E, Jan LY, Jan YN. 1991. prospero is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in Drosophila. Cell, 1991 **67**(5):941-53.

Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, Lee MK, Skvorak AB, Morton CC, Blumenfeld A, Frydman M, Friedman TB, King MC, Avraham KB. 1998. Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science **279**(5358):1950-4.

Valarchae I, Tissier-Seta JP, Hirsch MR, Martinez S, Goridis C, Brunet JF. 1993. The mouse homeodomain protein Phox2 regulates NCAM promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. Development **199**: 881-896.

Valsecchi C, Ghezzi C. Ballabio A, Rugarli EI. 1997. JAGGED2: a putative Notch ligand expressed in the apical ectodermal ridge and in sites of epithelial-mesenchymal interactions. Mechanisms of Development **69**(1-2):203-7.

Vandaele S, Nordquist DT, Feddersen RM, Tretjakoff I, Peterson AC, Orr HT. 1991. Purkinje cell protein-2 regulatory regions and transgene expression in cerebellar compartments. Genes and Development **5**(7):1136-48.

van der Wees J, Schilthuis JG, Koster CH, Diesveld-Schipper H, Folkers GE, van der Saag PT, Dawson MI, Shudo K, van der Burg B, Durston AJ. 1998. Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. Development **125**(3):545-56.

Van Doren M, Bailey AM, Esnayra J, Ede K, Posakony JW. 1994. Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. Genes and Development **8**(22):2729-42.

Van Doren M, Ellis HM, Posakony JW. 1991. The Drosophila extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. Development **113**(1):245-55.

Van Doren M, Powell PA, Pasternak D, Singson A, Posakony JW. 1992. Spatial regulation of proneural gene activity: auto- and cross-activation of achaete is antagonized by extramacrochaetae. Genes and Development **6**(12B):2592-605.

Verdi JM, Schmandt R, Bashirullah A, Jacob S, Salvino R, Craig CG, Lipshitz HD, McGlade CJ. 1996. Mammalian numb is an evolutionarily conserved signaling adapter protein that specifies cell fate. Curr Biol **6**: 1134-1145.

Verma-Kurvari S, Savage T, Gowan K, Johnson JE. 1996. Lineage-specific regulation of the neural differentiation gene MASH1. Developmental Biology **180**(2):605-17.

Vescovi AL, Reynolds BA, Fraser DD, Weiss S. 1993. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron **11**(5):951-66.

Villares R, Cabrera CV. 1987. The achaete-scute gene complex of D. melanogaster: conserved domains in a subset of genes required for neurogenesis and their homology to myc. Cell **50**(3):415-24.

Voyvodic JT. 1996. Cell death in cortical development: How much? Why? So what? Neuron **16**(4):693-6.

Wainwright SM, Ish-Horowicz D. 1992. Point mutations in the Drosophila hairy gene demonstrate in vivo requirements for basic, helix-loop-helix, and WRPW domains. Molecular and Cellular Biology **12**(6):2475-83.

Wakamatsu Y, Watanabe Y, Nakamura H, Kondoh H. 1997. Regulation of the neural crest cell fate by N-myc: promotion of ventral migration and neuronal differentiation. Development **124**(10): 1953-1962.

Wakamatsu Y, Weston JA. 1997. Sequential expression and role of Hu RNA-binding proteins during neurogenesis. Development **124**(17):3449-60.

Walldorf U, Gehring WJ. 1992. Empty spiracles, a gap gene containing a homeobox involved in Drosophila head development. Embo Journal **11**(6):2247-59.

Warren N, Price DJ. 1997. Roles of Pax-6 in murine diencephalic development. Development **124**(8):1573-82.

Wasylyk B, Hagman J, Gutierrez-Hartmenn A. 1998. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem **23**: 213-216.

Weber JRM, Skene JHP. 1997. Identification of a novel repressive element that contributes to neuron-specific gene expression. J Neurosci. **17**: 7583-7593.

Weinberg RA. 1995. The retinoblastoma protein and cell cycle control. Cell **81**(3):323-30.

Weiner JA, Chun J. 1997. Png-1, a nervous system-specific zinc finger gene, identifies regions containing postmitotic neurons during mammalian embryonic development. J Comp Neurol. **381**: 130-142.

Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science **251**(4995):761-6.

Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S, Dean DC. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature, **375**(6534):812-5.

Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, van der Kooy D. 1996. Is there a neural stem cell in the mammalian forebrain? Trends Neurosci **19**(9):387-93.

Wettstein DA, Turner DL, Kintner C. 1997. The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. Develop **124**: 693-702.

Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S. 1985. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell **43**:567-81.

White K. 1980. Defective neural development in Drosophila melanogaster embryos deficient for the tip of the X-chromosome. Dev. Biol. **80**: 322-344.

Williams BP, Price J. 1995. Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. Neuron **14**(6):1181-8.

Williams R, Lendahl U, Lardelli M. 1995. Complementary and combinatorial patterns of Notch gene family expression during early mouse development. Mech Develop **53**: 357-368.

Wilson R, Mohun T. 1995. XIdx, a dominant negative regulator of bHLH function in early Xenopus embryos. Mechanisms of Development **49**(3):211-22.

Wilson-Rawls J, Molkentin JD, Black BL, Olson EN. 1999. Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. Molecular and Cellular Biology **19**(4):2853-62.

Wrischnik LA, Kenyon CJ. 1997. The role of lin-22, a hairy/enhancer of split homolog, in patterning the peripheral nervous system of C. elegans. Development **124**(15):2875-88.

Wu W, Mathew TC, Miller FD (1993) Evidence that the loss of homeostatic signals induces regeneration-associated alterations in neuronal gene expression. Dev. Biol. **158**: 456-466.

Wu W, Gloster A, Miller FD. 1997. Transcriptional repression of the growth-associated T alpha1 alpha-tubulin gene by target contact. Journal of Neuroscience Research **48**(5):477-87.

Wurst W, Auerbach AB, Joyner AL. 1994. Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. Development **120**(7):2065-75.

Xiang M, Gao WQ, Hasson T, Shin JJ. 1998. Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. Development **125**(20):3935-46.

Xu PX, Woo I, Her H, Beier DR, Maas RL. 1997. Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. Development **124**(1):219-31.

Xu T, Artavanis-Tsakonas S. 1990. deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. Development **126**(3):665-77.

Xu T, Rebay I, Fleming RJ, Scottgale TN, Artavanis-Tsakonas S. 1990. The Notch locus and the genetic circuitry involved in early Drosophila neurogenesis. Genes and Development **4**(3):464-75.

Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E. 1995. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron **14**(6):1141-52.

Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C, Gendron-Maguire M, Rand EB, Weinmaster G, Gridley T. 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Human Molecular Genetics **8**(5):723-30.

Yan RT, Wang SZ. 1998. neuroD induces photoreceptor cell overproduction in vivo and de novo generation in vitro. Journal of Neurobiology **36**(4):485-96.

Yang X, Bahri S, Klein T, Chia W. 1997. Klumpfuss, a putative Drosophila zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage. Genes Dev **11**: 1396-1408.

Yang XW, Zhong R, Heintz N. 1996. Granule cell specification in the developing mouse brain as defined by expression of the zinc finger transcription factor RU49. Development **122**(2): 555-566.

Yao KM, White K. 1991. Organizational analysis of elav gene and functional analysis of ELAV protein of Drosophila melanogaster and Drosophila virilis. Molecular and Cellular Biology **11**(6):2994-3000.

Yao KM, White K. 1994. Neural specificity of elav expression: defining a Drosophila promoter for directing expression to the nervous system. Journal of Neurochemistry **63**(1):41-51.

Yaworsky P, Gardner DP, Kappen C. 1997. Transgenic analyses reveal developmentally regulated neuron- and muscle-specific elements in the murine neurofilament light chain gene promoter. JBC **272**(40):25112-20.

Yazdanbakhsh K, Fraser P, Kioussis D, Vidal M, Grosveld F, Lindenbaum M. 1993. Functional analysis of the human neurofilament light chain gene promoter. Nucleic Acids Research **21**(3):455-61.

Yedvobnick B, Smoller D, Young P, Mills D. 1998. Molecular analysis of the neurogenic locus mastermind of Drosophila melanogaster. Genetics **118**(3):483-97.

Yochem J, Greenwald I. 1989. glp-1 and lin-12, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell **58**(3):553-63.

Yochem J, Weston K, Greenwald I. 1988. The Caenorhabditis elegans lin-12 gene encodes a transmembrane protein with overall similarity to Drosophila Notch. Nature **335**(6190):547-50.

Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature **397**(6721):702-6.

Zeng C, Younger-Shepherd S, Jan LY, Jan YN. 1998. Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the Drosophila sensory organ lineage. Genes and Development **12**(8):1086-91.

Zhang F, Pöpperl H, Morrison A, Kovàcs EN, Prideaux V, Schwarz L, Krumlauf R, Rossant J, Featherstone MS. 1997. Elements both 5' and 3' to the murine Hoxd4 gene establish anterior borders of expression in mesoderm and neurectoderm. Mechanisms of Development **67**(1):49-58.

Zhang H, Reynaud S, Kloc M, Etkin LD, Spohr G. 1995. Id gene activity during Xenopus embryogenesis. Mechanisms of Development **50**(2-3):119-30.

Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature **381**(6579):238-41.

Zhang M, Kim HJ, Marshall H, Gendron-Maguire M, Lucas DA, Baron A, Gudas LJ, Gridley T, Krumlauf R, Grippo JF. 1994. Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. Development **120**: 2431-2442.

Zhao C,Emmons SW. 1995. A transcription factor controlling development of peripheral sense organs in C. elegans. Nature **373**: 74-78.

Zhao Y, Sheng HZ, Amini R, Grinberg A, Lee E, Huang S, Taira M, Westphal H. 1999. Control of hippocampal morphogenesis and neuronal differentiation by the LIM homeobox gene Lhx5. Science **284**(5417):1155-8.

Zhong WM, Feder JN, Jiang MM, Jan LY, Jan YN. 1996. Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron **17**: 43-53.

Zimmerman K, Shih J, Bars J, Collazo A, Anderson DJ. 1993. XASH-3, a novel Xenopus achaete-scute homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. Development **119**(1):221-32.

Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J, Vassileva G, McMahon A. 1994. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron **12**(1):11-24.