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Etude génomique des programmes de différenciation neuronale régulés par les facteurs de transcription proneuraux Neurogénines et Mash1 au cours du développement du télencéphale de la souris

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ABBREV I ATIONS

amos	absent multidendritic neurons and olfactory sensillia
asc	achaete-scute complex
ato	atonal
bHLH	basic helix loop helix
cato	cousin of atonal
CNS	central nervous system
CP	cortical plate
DKO	double mutant
es	external sense organ
GABA	gamma amino butiric acid
GAD	glutamic acid decarboxylase
GOF	gain of function
Hes	hairy and enhancer of slit homolog
Id	inhibitor of differentiation
KO	single mutant
LacZ	beta-galactosidase
LGE	lateral ganglionic emminence
Mash1	mouse acheate-scute homolog 1
Math	mouse atonal homolog
MGE	medial ganglionic emminence
MZ	marginal zone
NB	neuroblast
NeuroD	neurogenic differentiation
Ngns	neurogenines
N^{icd}	Notch intracellular domain
PNS	preipheral nervous system
SOP	sensory organ precursor
SVZ	subventricular zone
v/v	volume/volume
VZ	ventricular zone
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

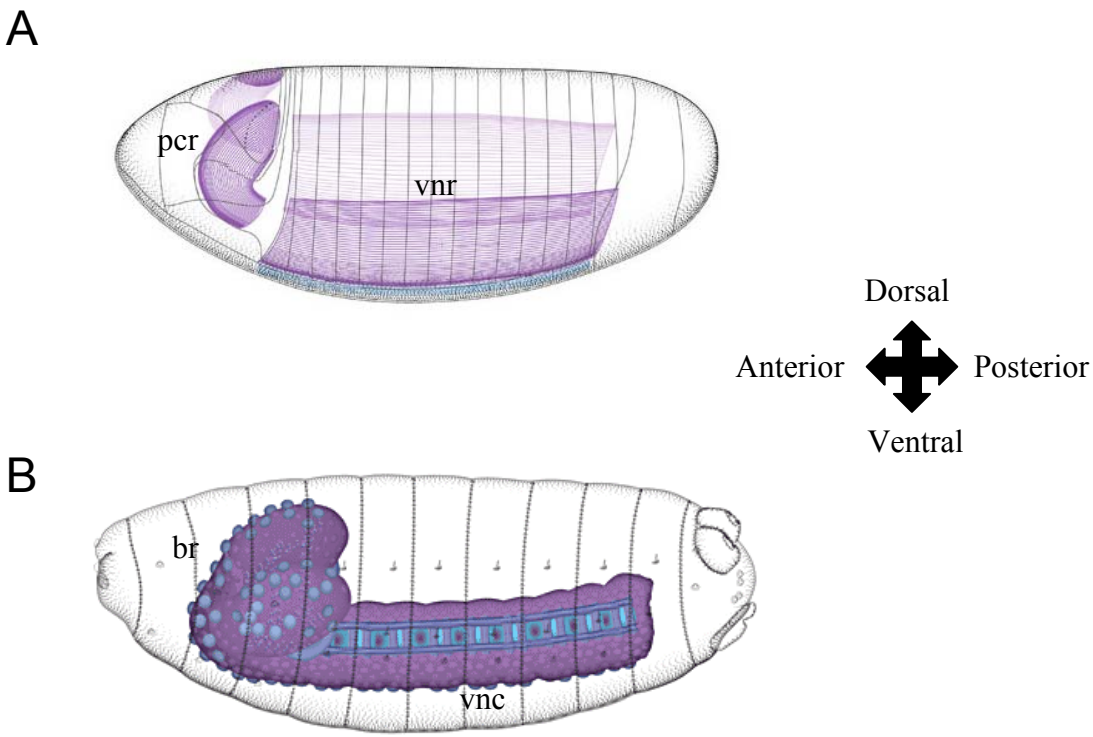


Figure 1: View of *Drosophila* CNS at larval stage 5 (A) and larval stage 17 (B). Neural precursors in the CNS arise from specialized region of the ectoderm (light violet lines). Neurogenic regions from the ventro-lateral part give rise to the ventral nerve cord, and anterior neurogenic regions give rise to the brain. pcr: procephalic region, vrn: ventral neurogenic region, br: brain, vnc: ventral nerve cord. (From *Atlas of Drosophila Development* by Volker Hartenstein, published by Cold Spring Harbor Laboratory Press, 1993).

INTROD U C TION

I Role of proneural genes

1 Characterization of proneural genes

A In Drosophila

The fruit fly *Drosophila melanogaster* is a major model system for the study of molecular mechanisms that control the development of the nervous system. In *Drosophila*, the entire nervous system develops from neuroectodermal cells which delaminate from the surface epithelium and move into the interior of the embryo to form neural precursor cells called neuroblasts. The central nervous system (CNS) arises from the ventral most regions of the neuroectoderm, while the peripheral nervous system (PNS) arises from more lateral areas (figure 1). The CNS of *Drosophila* is composed of the brain and the ventral nerve cord, while its PNS is formed by four different types of sensory organs: the external sensory organs (*es*), the chordotonal organs (*cho*), the multidendritic neurons (*md*) and the photoreceptor of the eyes.

The first step of neural determination in the fly is the singling-out of neural precursors from the neuroectodermal epithelium. Before this selection, all neuroectodermal cells are equivalent and have the potential to become either neuroblasts or epidermoblasts. The choice between those two fates relies on the expression of proneural genes, a group of transcription factors from the basic Helix-Loop-Helix family (bHLH) which instruct neuroectodermal cells to form neural precursors (Jan and Jan, 1994). Initially, proneural genes are expressed equivalently in groups of cells called proneural cluster. Every cell in the proneural cluster has the potential to become a neural progenitor, but progressively, cell-cell interactions mediated by the neurogenic proteins Delta and Notch restrict the expression of proneural genes in one single cell, the future neuroblast (Artavanis-Tsakonas et al., 1999). This process, called lateral inhibition, is dependent on negative and positive feed back loops between proneural genes and the neurogenic genes *Delta* and *Notch* (Hartenstein and Posakony, 1990; Heitzler et al., 1996; Van Doren et al., 1994) (see, lateral inhibition and neurogenic genes, p12). Briefly, proneural genes activate the expression of the ligand Delta which in turn activates its receptor Notch in neighbouring cells. Activation of Notch receptor by Delta leads to the inhibition of proneural genes expression in these cells. This process allows the accumulation of proneural protein in one cell within the proneural cluster (the future selected neural precursor) while the other cells downregulate the expression of proneural genes and become epidermoblasts. Therefore, the process of lateral inhibition allows the selection of neural progenitor

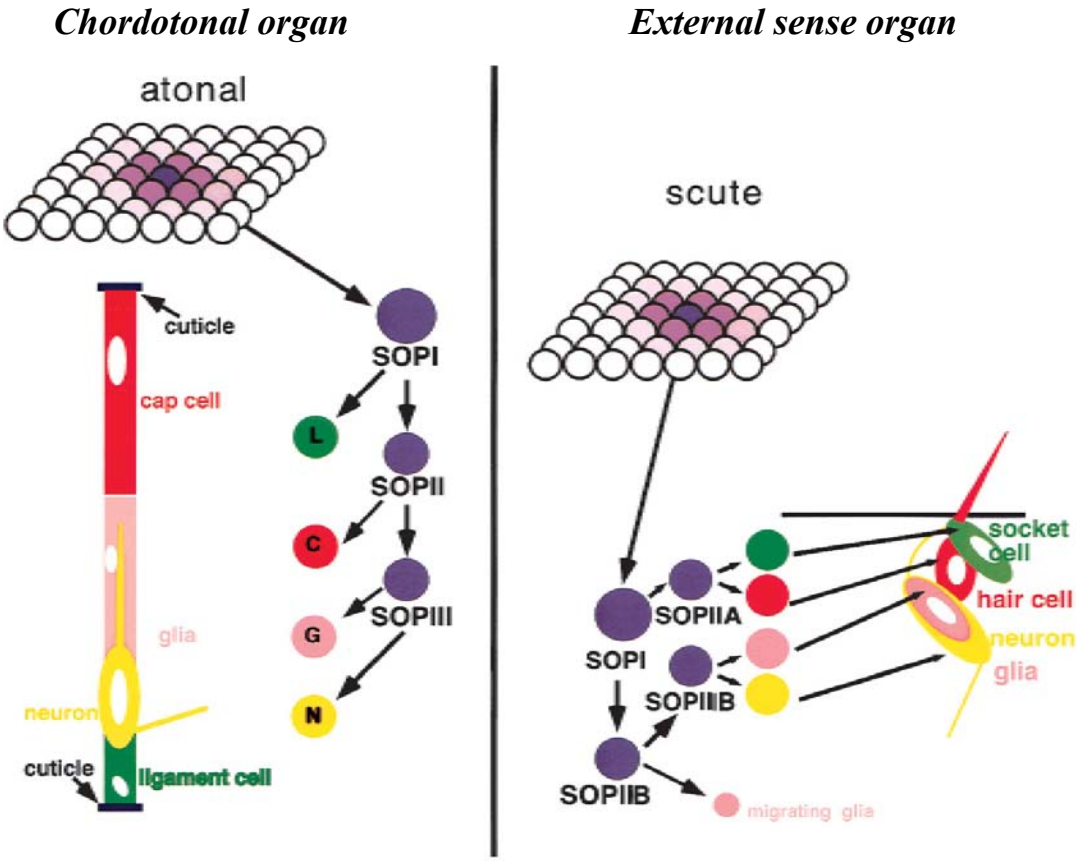


Figure 2: Assymetric division and generation of *Drosophila* sense organs. *atonal* is required for the selection of neuroblasts at the origin of chordotonal organs and *scute* for neuroblasts of external sense organs. After singling-out of neural progenitors, sensory organ precursors (SOP) undergo a stereotyped pattern of division producing a fixed number of progeny cells (neurons, glia and two types of "accessory" cells, hair and socket cells for external sense organs) at the origin of the sense organ. L: ligament cell, C: cap cell, G: glia, N: neuron (from Hassan and Bellen, 2000).

from an originally equivalent population of cells. In the CNS, the newly selected neural progenitor divides asymmetrically to produce another neuroblast and a ganglion mother cell (GMC) which will produce a limited number of neurons. Progenitors specified in the PNS divide also asymmetrically to produce sensory organ progenitor (SOP), which endow a stereotyped pattern of division to produce all cells composing sensory organs (neurons, glial cells, hair and socket cells in the case of *es* organs) (see figure 2) (Jimenez and Modolell, 1993).

a The achaete-scute complex

The role of *asc* in the determination of neural fates was established by genetic analysis of mutant flies lacking *es* organs (or bristles) (Villares and Cabrera, 1987; Ghysen and Dambly-Chaudiere, 1988). Four genes were isolated in this complex, all transcription factors belonging to the bHLH family: *ac* (*achaete*), *sc* (*scute*), *lethal of scute* (*l'sc*) and *asense* (*ase*). The gene *l'sc* is the main proneural determinant in the CNS, whereas *ac* and *sc* have a redundant function in the generation of SOP in *es* organs and a subsets of neuroblast in the CNS (Jimenez and Campos-Ortega, 1990). The last member of the *asc* complex, *ase*, is induced by proneural genes in the PNS during neural differentiation and has been shown to have a proneural role in the selection of neural progenitors in the wing margin (Dominguez and Campuzano, 1993; Brand et al., 1993).

b The *atonal* group

The gene *ato* was identified in a PCR screen for genes sharing similarity with the bHLH domain of *asc*. Sequence comparison in the bHLH domain showed that, while genes in the *asc* complex share 70% of identity, *ato* exhibits only 46% identity with *sc*, suggesting that *ato* and *asc* genes belong to distinct families (Jarman et al., 1993). *Ato* is involved in the determination of neural progenitors in the PNS, including internal sensory receptors (chordotonal organs, *cho*), photoreceptors in the eye and some multidendritic neurons. No proneural activity was established for *ato* in the CNS, but a function of this gene in axonal arborization was demonstrated, a process arising later during the neuronal differentiation (Hassan et al., 2000). Subsequent search of *ato* related genes permitted the identification of two additional genes: *amos* (*absent multidendritic neurons and olfactory sensillia*) and *cato* (*cousin of atonal*). *Amos* has a proneural role in the determination of most multidendritic neurons and olfactory sensillia in the fly PNS (Goulding et al., 2000b). The gene *cato* is involved in neural commitment after selection of neuroblasts (Goulding et al., 2000a). An additional gene, *biparous* (or *TAP*, *target of poxn*), was isolated. This gene constitutes a distinct family but is still more related to *ato* than *asc* genes in term of sequence similarity in the bHLH domain. *Biparous* is expressed in neuroblasts after commitment of neural progenitors and has thus no proneural function (Bush et al., 1996).

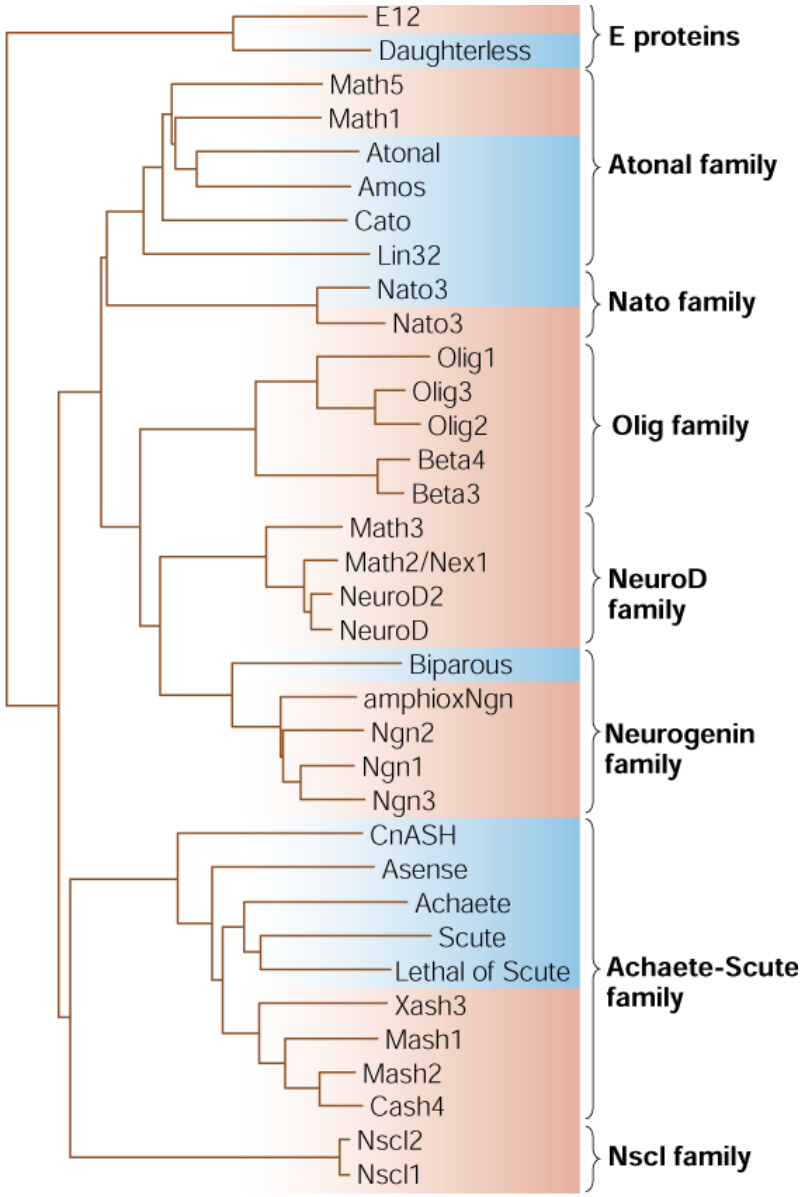


Figure 3: Different sub-families of bHLH proteins. Dendogram of bHLH proteins from vertebrates (pink) and invertebrates (blue) based on sequence similarities in the bHLH domain (from Bertrand et al. 2002).

c Cascade of bHLH leads to neural differentiation

The bHLH genes can be discriminated into two classes based on their function: proneural genes like *ato* and *sc* are expressed before any sign of neural differentiation and are essential to select neural progenitor within the neuroectoderm, whereas neuronal-precursors genes like *cato* and *biparous* are expressed after the selection of neural progenitors and are involved in the process of neural differentiation *per se*. The sequential activation of these two classes of bHLH genes correlates well with the progression of undifferentiated progenitors towards differentiated neural cells. Indeed, there is now strong evidences that sequential activation of the two bHLH classes represent a regulatory cascade in which early expressed determination genes induce a second wave of bHLH differentiation genes (Campuzano and Modolell, 1992; Brand et al., 1993).

The selection of all neuroblasts in the fly PNS relies on the proneural activity of *ato* and *asc* genes. However, genes responsible for proneural activity in the CNS still remain to be identified. Recently, the sequencing of the entire *Drosophila* genome allowed the identification of new putative bHLH genes. Many of those genes are related to known bHLH genes and were shown to be expressed in the nervous system or mesoderm, but none of them were found to have a proneural activity (Peyrefitte et al., 2001; Moore et al., 2000). It is thus possible that other categories of genes (different from the bHLH family) possess a proneural activity and still remain to be identified (Bertrand et al., 2002).

B Identification of vertebrate proneural genes

Identification of neuronal bHLH genes in vertebrates was mostly based on sequence conservation with *Drosophila* genes (figure 3). Orthologues for *Drosophila* proneural genes were obtained either by low stringency hybridization, PCR amplification using degenerated oligonucleotides or yeast two hybrid screen (Zimmerman et al., 1993; Ma et al., 1996; Guillemot and Joyner, 1993; Ferreiro et al., 1993; Johnson et al., 1990; Akazawa et al., 1995; Bartholoma and Nave, 1994; Gradwohl et al., 1996).

a Neurogenins

Sequence comparison with *Drosophila* bHLH genes places the *Neurogenin* (*Ngn*) family as related to *biparous*. In *Xenopus*, *XNgn1* overexpression causes precocious and extensive neuronal differentiation within the neural plate. This phenotype correlates with the ectopic expression of *XNeuroD*, a bHLH gene expressed during neuronal differentiation, after selection of neural progenitor by proneural genes. Moreover XNgn1 activates the expression of *Delta* and is inhibited

by Notch signalling, similarly to proneural genes in *Drosophila* (Ghysen et al., 1993; Ma et al., 1996).

Three *Ngns* genes (*Ngn1* to *Ngn3*) have been identified in the mouse. In the PNS, *Ngn1* and *Ngn2* have been demonstrated to be essential for the determination of cranial sensory lineages (Fode et al., 1998; Ma et al., 1998). In the absence of *Ngn1* or *Ngn2*, complementary sets of cranial sensory ganglia are missing due to a defect in neuronal determination. In addition, loss of *Ngns* expression leads to a lack of sensory neurons in the dorsal root ganglia and most of the ventral neurons in the spinal cord. The absence of these three neuronal lineages (sensory cranial ganglia, sensory neurons in the dorsal root ganglia and spinal neurons) was shown to be the consequence of the loss of neuronal progenitors associated to a defect in the process of lateral inhibition (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999; Scardigli et al., 2001). In the CNS, *Ngn1* and *Ngn2* are expressed in progenitors of the cerebral cortex (dorsal part of the telencephalon) (see, Specification of neuronal subtype, p17). Loss of *Ngns* expression in this tissue does not lead to a loss of neural progenitors but results in the respecification of cortical progenitors toward a GABAergic fate due to the ectopic expression of the *asc* homologue gene *Mash1* in the cortex (Fode et al., 2000). The last member of the *Ngn* family characterized in the mouse is *Ngn3*. This gene is expressed in the spinal cord were it is involved in oligodendrocyte differentiation (Lee et al., 2003a). *Ngn3* is also expressed in the pancreas were it promotes the endocrine fate in pancreatic islets (Gradwohl et al., 2000). The acquisition of an endocrine fate involves the selection of endocrine progenitors through lateral inhibition process mediated by the Delta Notch system (see, lateral inhibition and neurogenic genes, p12) and by activation of the downstream bHLH differentiation factor *NeuroD*, similarly to the determination of neuronal precursors in the nervous system (Gradwohl et al., 2000; Huang et al., 2000). Thus it seems that the molecular mechanisms involved in the selection of progenitors, as well as the cascade of bHLH genes leading to cell differentiation exhibit remarkable similarities during development of two tissues as distinct as the brain and the pancreas.

b *Asc* related genes

The gene *Mash1* (Mouse *achaete scute homologue 1*) was identified in a screen searching for genes sharing similarities with the bHLH domain of *asc* genes (Guillemot and Joyner, 1993). Forced expression of *Mash1* in embryonic carcinoma derived P19 cells induces a neuronal differentiation program (Farah et al., 2000). Gain-of-function studies in *Xenopus* showed that overexpression of *Xash3* (Xenopus *achaete scute homologue3*) causes an expansion of the neural plate and ectopic neuronal differentiation at the expense of the epidermis (Ferreiro et al., 1994). In the mouse, loss of *Mash1* expression causes reduction of neuronal progenitors in both CNS and PNS, and leads to lethality around birth. In *Mash1* knock out mice, generation of neuronal progenitors is diminished in ventral telencephalon, hindbrain, olfactory sensory neurons, sympathetic, parasympathetic and

enteric nervous systems (Pattyn et al., 2004; Casarosa et al., 1999; Hirsch et al., 1998; Cau et al., 1997; Lo et al., 1994). In the olfactory epithelium, the hindbrain and ventral telencephalon, *Mash1* has a proneural function associated in the mutant with a loss of neuronal progenitors and a failure to initiate Notch signalling (Cau et al., 1997; Guillemot et al., 1993; Pattyn et al., 2004; Casarosa et al., 1999). In contrast, in the autonomic system, *Mash1* is required for terminal differentiation of noradrenergic neurons (Sommer et al., 1995). Another orthologue of *asc*, *Mash2*, was found in the mouse. This gene is not expressed in the nervous system, but is required during the development of trophoblastic progenitors in the placenta (Cross et al., 2003; Guillemot et al., 1995; Tanaka et al., 1997).

c *Ato* related genes

In term of sequence conservation in the bHLH domain, the mouse atonal homologues *Math1* and *Math5* are the closest genes related to the *Drosophila* gene *ato*. Moreover, swapping experiment replacing the coding sequence between *ato* and *Math1* showed that these two genes have interchangeable function: *ato* can compensate for the loss of *Math1* in the mouse, and *Math1* can assume the proneural function of *ato* in the fly (Wang et al., 2002). Loss-of-function studies in the mouse showed *Math1* to be necessary for the generation of granular neurons in the cerebellum, and the development of the sensory epithelium of the inner hear (Ben Arie et al., 1997; Chen et al., 2002; Woods et al., 2004). *Math5* is essential for retinal ganglion cells production (Wang et al., 2001). However it is still not clear if these two genes have proneural activity in a wild-type context. Close members of the *ato* family were also found in *Xenopus*, *Xath1* and *Xath5* in which they have been shown to induce a neural fate (Takebayashi et al., 1997; Kim et al., 1997; Kanekar et al., 1997).

Genes more distantly related to *ato* have also been identified in vertebrates. These genes form the *NeuroD* family composed of four members in the mouse, *NeuroD1*, *NeuroD2*, *Math2* and *Math3*. Ectopic expression of *XNeuroD* in *Xenopus* neural plate leads to enhanced neuronal differentiation in a way similar to *Xash3* and *XNgn1*, but this gene (as *Math2* and *Math3*) is expressed in differentiating postmitotic neurons (Lee et al., 1995). Genetic studies in both mouse and *Xenopus* suggest that the *NeuroD* family is more involved in neuronal differentiation than in neuronal determination (Lee et al., 1995; Ma et al., 1996; Bertrand et al., 2002; Schuurmans and Guillemot, 2002). Finally, a last member of the atonal familly, *Nato3*, is expressed in the developing nervous system of the mouse but its function is still not known (Segev et al., 2001; Verzi et al., 2002).

d *Nscl* and *Olig* subgroup

Two additional bHLH groups were isolated in the mouse, the *Nscl* and the *Olig* related genes (figure 3). The *Nscl* family comprises two highly related genes, *Nscl1* and *Nscl2*, both expressed in

a largely overlapping pattern in the CNS and PNS (Kruger and Braun, 2002). Recent analysis of *Nscl1/2* double mutant mice demonstrated a role of these two genes in the differentiation of a subset of neurons in the hypothalamus (gonadotropin releasing hormones neurons or GnR-H1 neurons) (Kruger et al., 2004). In the mouse cortex, *Nscl* genes are likely to act downstream of proneural genes in the process of neuronal differentiation (Schuurmans et al., 2004). Members of the *Olig* subfamily comprise *Olig1*, *Olig2*, *Olig3* and the Olig-related genes *Beta3* (*bhlhb5*) and *Beta4* (*bhlhb4*). The genes *Olig1* and *Olig2*, are implicated in the generation of oligodendrocytes in the brain and in the spinal cord respectively (Zhou and Anderson, 2002; Lu et al., 2002). *Olig3* is also expressed in the mouse neural tube early in development (from the midbrain/hindbrain boundary to the spinal cord), but its function is not established yet (Takebayashi et al., 2002). The two Olig-related gene *Beta3* and *Beta4* are also expressed in the nervous system but their function is still largely unkown (Brunelli et al., 2003; Bramblett et al., 2002).

There is now good evidence that *Olig* genes (at least *Olig1* and *Olig2*) function as repressors, in contrast to other bHLH transcription factors. Indeed, fusion of *Olig2* DNA binding domain with the repressor *Engrailed* does not interfere with the production of oligodendrocytes. In contrary, experiment using the *VP16* activator domain fused to *Olig2* blocks oligodendrocyte formation (Novitch et al., 2001; Zhou et al., 2001). bHLH genes have been implicated in the generation of neurons (*Ngns* and *Mash*) and oligodendrocytes (*Olig* genes). Common progenitors for both class of cells were characterized in the mouse forebrain as well as in the spinal cord (Zhou and Anderson, 2002; Lu et al., 2002; He et al., 2001). In contrast, the determination of the third classes of neural cells (the astrocytes) seems to be independent from the activity of bHLH genes.

Recently, Lee et al. studied the molecular mechanisms leading to the sequential generation of first motoneurons and later oligodendrocytes by a common progenitor cell population in the spinal cord (Lee et al., 2005). The authors showed that Olig2 antagonizes Ngn2 activity by competing with Ebox fixation on the promoter of target genes involved in motoneuron differentiation and also by sequestering E47 away from Ngn2. The downregulation of *Olig2* in progenitors is thus essential to allow Ngn2 accumulation and subsequent motoneurons differentiation (Lee et al., 2005) In the mouse brain, Carlos Parras in the laboratory, showed that *Mash1* is required for the generation of both neurons and oligodendrocytes in the olfactory bulb of postnatal pups (Parras et al., 2004). This paper suggests that a population of neural progenitors in the basal ganglia, able to produce both neurons and oligodendrocytes, is lost in *Mash1*KO embryos. These data are based from *in vitro* cell culture. If *Mash1* is required *in vivo* for the specification of these bipotenital progenitors is currently not known.

In summary, there are now strong evidences that *Ngn1*, *Ngn2* and *Mash1* have a proneural function in the nervous system of vertebrates and that at least some aspects of the molecular mechanisms

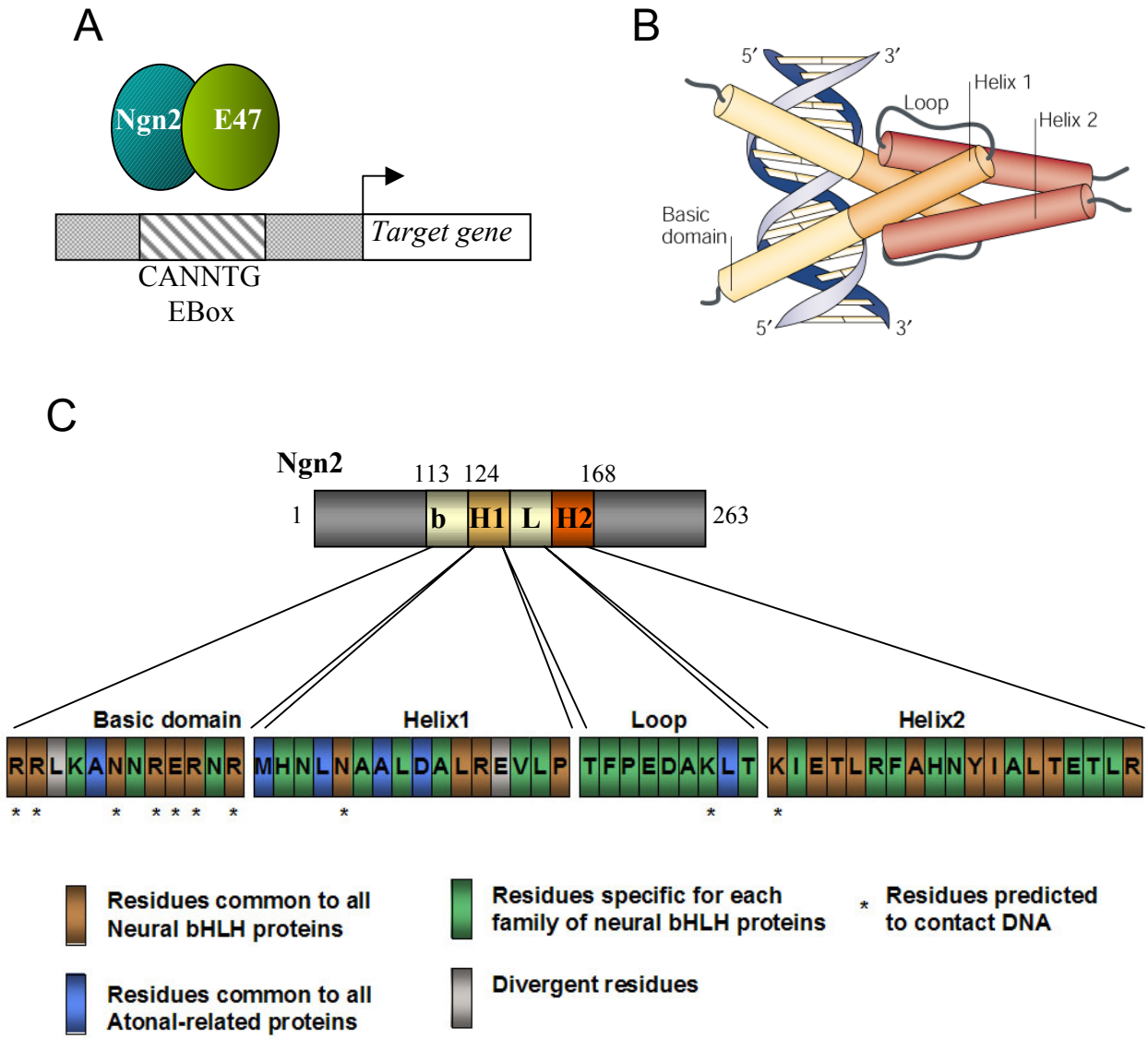


Figure 4: **Structure and dimerization of bHLH proteins.** A) Ngn2 heterodimerize with E protein to bind to the EBox and activate the transcription of target genes. B) Schematic representation of the structure of a bHLH dimer complexed to DNA. The basic region fits in the main groove of the DNA. C) Sequence of Ngn2 protein and detailed of the bHLH region. In total 10 amino acids, mostly from the basic region, are predicted to contact DNA. Start and end position of the motif are indicated (from Bertrand et al. 2000).

leading to neural differentiation are conserved during evolution. Genetic studies in *Xenopus* and in the mouse have demonstrated that vertebrates proneural genes are regulated by neurogenic genes and are able to transactivate a cascade of bHLH genes leading to neuronal differentiation, like in *Drosophila* (Bertrand et al., 2002; Lee, 1997; Kageyama and Nakanishi, 1997). However, it is also important to note the differences. The cellular context is different in vertebrates as the Delta-Notch pathway is activated in the neuroepithelium which is already specified to produce neural tissue, whereas in *Drosophila* lateral inhibition is involved in the selection of neural versus epidermal cells. Proneural genes are thus involved in neural *versus* epidermal fate in *Drosophila*, whereas in vertebrates, proneural genes induce a neuronal fate and inhibit the glial fate.

2 Mechanisms of proneural activities

A Regulation of the activity of proneural genes

Transcription factors of the bHLH family bind to specific DNA sequence as heterodimers composed of one tissue specific and one ubiquitously expressed bHLH protein. In *Drosophila,* the widely expressed protein daughterless (da) heterodimerizes with sc or ato. Vertebrates homologues of *da* constitute the *E* family: *E2A* (*E12* and *E47*), *HEB* and *E2-2* (Cabrera and Alonso, 1991; Johnson et al., 1992). The bHLH domain is composed of the basic motif, which mediates the interaction with a specific DNA sequence called the E-Box (CANNTG), and the HLH region which is involved in dimerization with *E* proteins (Murre et al., 1989; Bertrand et al., 2002) (figure 4). Transactivation of target genes is mediated by the recruitment of coactivators such as p300/CBP (CREB Binding Protein) which facilitate transcription by acetylating histones (Koyano-Nakagawa et al., 1999).

a *Id* proteins

Formation of the proneural bHLH-E protein heterodimer is a prerequisite for DNA binding (figure 4). Factors that compete with the dimerization between proneural proteins and E proteins are thus potent inhibitors of proneural activity. Genes of the *Id* family (*extra macrochaetae* in *Drosophila*) encodes proteins composed of the HLH domain but lacking the basic DNA binding motif. Heterodimers formed with *Id* and *E* proteins are thus unable to bind DNA and indirectly inhibit proneural activity by sequestring *E* proteins (Yokota, 2001; Cabrera and Alonso, 1991). By this process bHLH transcription factors like *NeuroD*, *Mash1* and *Ngns* are unable to bind to DNA and activate neuronal differentiation. Four *Id* genes (*Id1* to *Id4*) have been identified in the mouse. They display partial overlapping pattern of expression and functional redundancy as shown by the analysis of single and double knock out mice (Lyden et al., 1999; Yokota, 2001). For example, loss of both *Id2* and *Id3* genes lead to premature differentiation of neurons due to the precocious

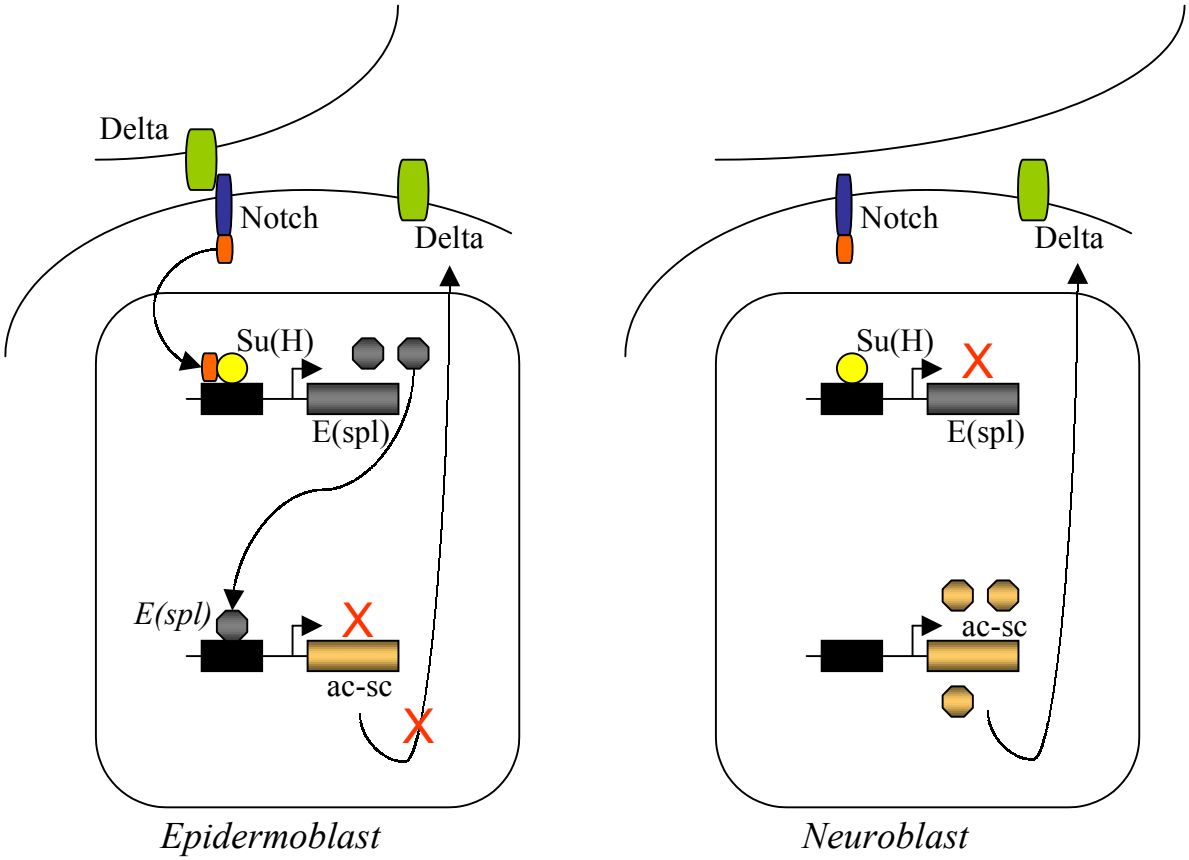


Figure 5: **The Notch signalling pathway in Drosophila.** Simplified representation of the Notch signalling pathway in *Drosophila*. Upon binding with Delta (left), the intracellular domain of Notch receptor (N^{icd}) is cleaved and translocated into the nucleus. N^{icd} forms a complex with Su(H) (supressor and hairless) and induces the expression of E(spl) (enhancer of split) which represses proneural expression and thus inhibits neuronal differentiation. When Notch is not activated (right), proneural gene expression is not inhibited by E(spl) proteins allowing neural differentiation and expression of Delta at the surface of the neuroblast (from Hatakeyama and Kageyama, 2004).

activation of proneural genes in neuronal precursors (Lyden et al., 1999). Conversely, the forced expression of *Ids* in *Xenopus* blocks neurogenesis (Liu and Harland, 2003). Interestingly, *Id4* has been implicated in the intracellular clock regulating the timing of differentiation of oligodendrocytes (Durand and Raff, 2000; Kondo and Raff, 2000). This assumption has been made by the study of oligodendrocyte precursors (OPCs) isolated from neonatal rat optic nerves. In this *in vitro* system, OPCs stop dividing and start to differentiate after eight rounds of cell divisions, suggesting the presence of a cell-intrinsic timing mechanism that regulates the number of cell division (Barres et al., 1994; Durand and Raff, 2000). It has been shown that this intrinsic timer runs faster when OPCs are cultivated at low temperature (33°C rather than 37°C), and that an accelerated decrease of *Id4* mRNA and protein is observed at the lower temperature, consistent with the hypothesis of *Id4* being part of the timer (Gao et al., 1997; Kondo and Raff, 2000).

b Lateral inhibition and neurogenic genes

In *Drosophila*, selection of neuroblasts within the proneural cluster is a process regulated by cell-cell interactions mediated by the neurogenic genes *Delta* and *Notch* (Artavanis-Tsakonas et al., 1999). Loss of genes in the Notch signalling pathway leads to a neurogenic phenotype characterized by an increased number of neurons and a loss of epidermis (Hartenstein and Posakony, 1990; Hartenstein and Posakony, 1989). The neurogenic genes *Notch* (*N*), *Delta* (*Dl*), *Presenilin* (*Psn*), *Suppressor of Hairless* [*Su(H)*], *mastermind* (*mam*) and *Enhancer of split complex* [*E(spl)C*] have been shown to interact genetically in the process of lateral inhibition. Expression of Delta at the surface of one given cell in the proneural cluster activates the transmembranar receptor Notch in the neighbouring cells. Notch undergoes a Psn dependant processing which results in the release of the Notch intracellular domain (N^{icd}) from the membrane (De Strooper et al., 1999; Struhl and Greenwald, 1999). N^{icd} enters in the nucleus were it binds to Su(H) and mastermind to activate the transcription of *E(spl)* genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Wu et al., 2000). *E(spl)* genes encode bHLH proteins that act as transcriptional repressors in a complex with the co-repressor protein Groucho to downregulate the expression of proneural genes (Heitzler et al., 1996; Fisher and Caudy, 1998). Repression of proneural activity is mediated both by the direct binding of the E(spl)/Groucho complex on the promoter of proneural genes to repress their transcription (the DNA motif recognized is called the N-Box, CACNAG) as well as by interfering directly with the formation of proneural-Da protein heterodimers (Kageyama and Nakanishi, 1997; Chen et al., 1997; Van Doren et al., 1994; Davis and Turner, 2001; Giagtzoglou et al., 2003).

The process of lateral inhibition breaks the original steady state within the proneural cluster, as a slight elevation of proneural activity in one cell in the cluster leads to the repression of proneural expression in neighbouring cells. Additional regulatory loops under the control of proneural genes lead to the selection of one neuroblast in the proneural cluster expressing high level of Delta and

proneural proteins, whereas neighbouring cells will develop as epidermoblasts (Bertrand et al., 2002; Bray, 1998; Simpson, 1998) (figure 5).

Genes homologous to *Delta* (*Dll1* to *Dll4*, *Serrate1* and *Serrate* 2), *Notch* (*Notch1* to *Notch4*), *E(spl)* (*Hes*, *Her*, *Hey* related genes) and *Su(H)* (*RBP-Jk*) have been found in the mouse and, like in *Drosophila*, are implicated in the regulation of proneural genes activity (Akazawa et al., 1992; Bae et al., 2000; Bettenhausen et al., 1995; Lindsell et al., 1995; Lindsell et al., 1996; Sasai et al., 1992; Hatakeyama and Kageyama, 2004). Genetic studies in the mouse, *Xenopus* and *Zebrafish* confirm that lateral inhibition participates to the selection of progenitors in many tissues, including the nervous system (Davis and Turner, 2001; de la Pompa et al., 1997). Mutant mice for *Notch1* or *RBP-Jk* show an enhanced and precocious neurogenesis, as indicated by the expanded expression of *NeuroD*, *Ngn1* and *NSCL1* (de la Pompa et al., 1997). Similarly, disruption of *Hairy enhancer of split* genes, *Hes1* and *Hes5*, (the transducer of *Notch* signalling) leads to a constitutively active *Notch* signalling resulting in precocious neurogenesis (Cau et al., 2000; Ohtsuka et al., 2001; Zine et al., 2001; Ohtsuka et al., 1999; Ishibashi et al., 1995). The process of lateral inhibition is thus an evolutionary conserved mechanism in *Drosophila* and higher vertebrates, implicated in singling out neural progenitors and in the maintenance of neural progenitors population (Bertrand et al., 2002; Artavanis-Tsakonas et al., 1999).

c Homeobox transcription factors

Regulators of proneural genes are still largely unknown besides the lateral inhibition process. However some lines of evidence suggest that transcription factors of the homeodomain family could be regulators of neural bHLH genes in *Drosophila* and in vertebrates. Members of the *Iroquois* family, *araucan* and *caupolican*, were first shown to regulate the expression of *ac* and *sc* genes in *Drosophila* (Gomez-Skarmeta et al., 1996). Absence of those genes downregulates expression of *ac-sc* genes whereas overexpression of *ara* induces their ectopic expression (Gomez-Skarmeta et al., 1996; Leyns et al., 1996). Overexpression of *Iroquois* in *Xenopus* (*Xiro1* or *Xiro2*) induces an expansion of the neural plate (in a similar way to what its observed upon *Xash3* overexpression) and the expression of *Xash3* and *Xngn1* (Gomez-Skarmeta et al., 1998). In the mouse, the homeobox gene *MBH1* (*mouse BarH homologue 1*) is expressed in a complementary manner to *Mash1*. Forced expression of *MBH1* in P19 cells downregulates *Mash1* and upregulates *Ngn2* expression (Saito et al., 1998). Finally, the homedomain protein *Pax6* has been shown to regulate directly the expression of *Ngn2* in the spinal cord and in the telencephalon (Scardigli et al., 2001; Scardigli et al., 2003).

d Extrinsic signalling pathways

Beside the essential role of intrinsic factors, excreted molecules have also been identified to stimulate or inhibit the production of neurons. Growth factors like fibroblast growth factors (FGFs), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), Sonic hedgehog (SHH) and insulin-like growth factors (IGFs) have been implicated in neural progenitor proliferation. Some factors like TGF-β can either stimulate or inhibit proliferation depending on the conditions (Anchan and Reh, 1995; Kane et al., 1996). However, growth factors are not only mitogenic. *In vitro* studies suggest that they are also able to instruct the fate of neural progenitors. FGF2 and neurotrophin-3 instruct progenitor cells to differentiate primarily as neurons (Ghosh and Greenberg, 1995; Qian et al., 1997). The Wnt-β catenin pathway is also required for proper neural differentiation (Otero et al., 2004; Patapoutian and Reichardt, 2000). On the other hand EGF, ciliary neurotrophic factor (CNTF) or leukemia inhibitory factor (LIF), cause progenitors to differentiate as astrocytes (Nakashima et al., 1999; Bonni et al., 1997; Qian et al., 1997; Kuhn et al., 1997). Depending on the time of development, members of the TGF-β superfamily can either promote neuronal or glial fate (Gross et al., 1996; Li et al., 1998). Importantly, CNTF-LIF has been shown to instruct astrocytic differentiation via the JAK-STAT pathway (janus kinase–signal transducer and activator of transcription) and the activation of the glial fibrillary acidic protein (GFAP) promoter (Sun et al., 2001; Bonni et al., 1997).

The role of extrinsic factors on the regulation of proneural genes is not well understood. In the mouse, BMP2 and BMP4 (two members of the TGF-β superfamily), were demonstrated to induce the expression of *Mash1* and to promote autonomic neuronal differentiation of neural crest stem cells (Lo et al., 1997). Another example is sonic hedgehog (SHH) in zebrafish which is essential for the activation of *Ngn1* in dorsal root ganglia. SHH is a secreted molecule acting as a morphogen for the patterning of axial mesoderm and specification of neurons in the ventral part of the spinal cord (Briscoe and Ericson, 2001; Johnson et al., 1994; Fan and Tessier-Lavigne, 1994). Loss of SHH results in the absence of sensory neurons in zebrafish embryos concomitant with the loss of *Ngn1* expression in the dorsal root ganglia (DRG) (Ungos et al., 2003). The defect of sensory neuron formation in the DRG seems not to be a consequence of a defect in neural crest specification, as suggested by the normal expression of the neural crest marker *crestin*, or a secondary effect due to a defect in axial mesoderm patterning. The authors suggested rather that neurons in the DRG require directly SHH signalling and this process works through the activation of *Ngn1* (Ungos et al., 2003). Finally, a recent paper from Zur Lag *et al.* investigated the relation of proneural transcription factors and EGFR signalling in *Drosophila*. The authors showed that sensory organ precursors selected by the classical way of lateral inhibition, are able to recruit other sense organ precursors from the surrounding ectoderm through the activation of *atonal* by EGFR signalling (Zur Lage et

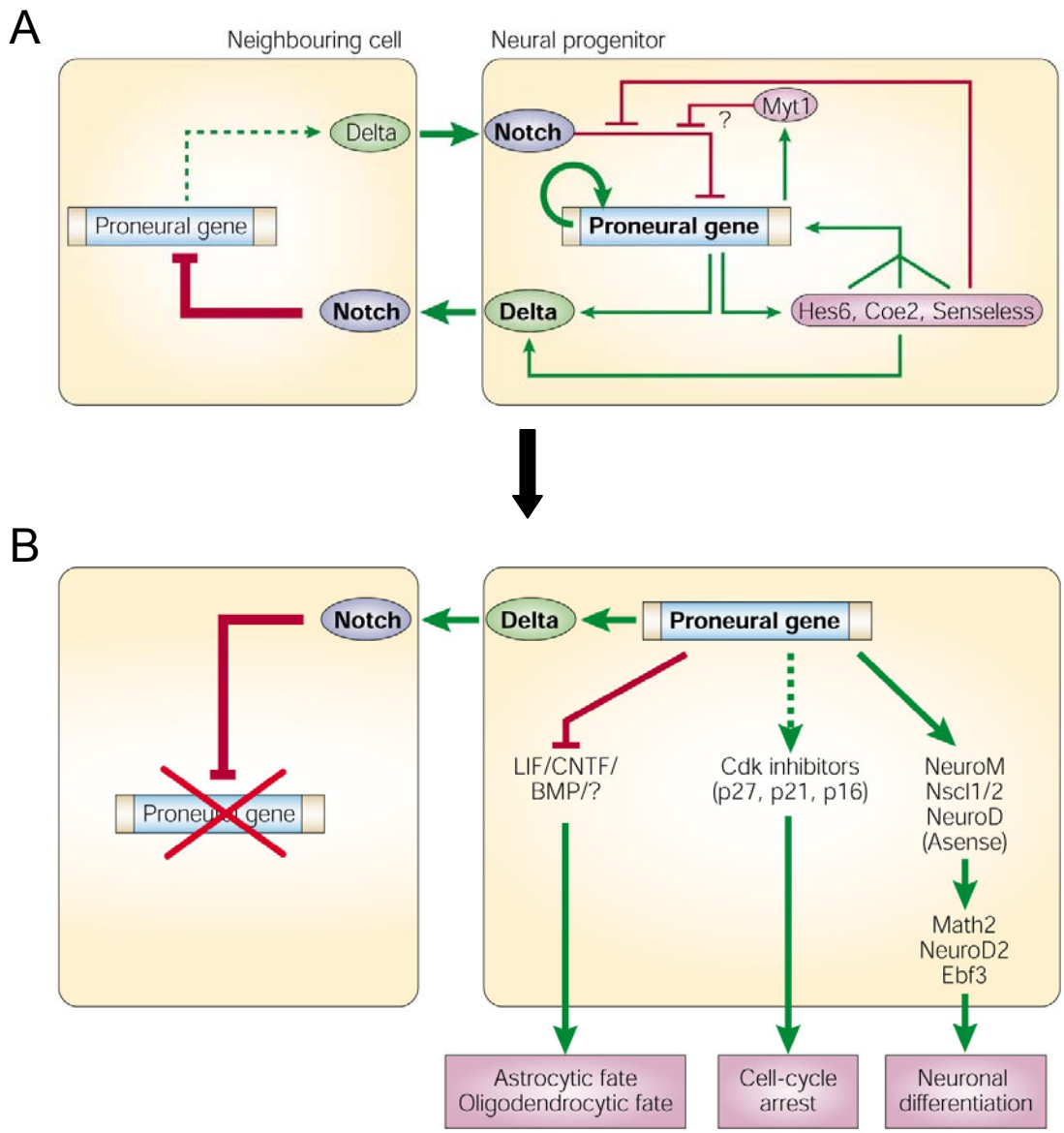


Figure 6: **Positive feedback loops and neuronal differentiation.** A) After singling-out of the neural precursor, positive feedback loops between proneural proteins and others genes such as *Hes6* and *Coe2* increase the level of proneural genes expression in the future neural progenitor, while proneural genes expression is inhibited in neighbouring cells. B) Accumulation of proneural proteins allows the activation of neuronal differentiation programs and cell cycle arrest. At this stage high proneural genes expressing cells are irreversibly domed into the neuronal fate (from Bertrand et al. 2002).

al., 2004), showing thus that local EGFR signalling can activate *atonal* transcription and determines neural fate in the recruited cells.

B Process of neuronal differentiation

a Positive feedback loop

Before singling out of neural progenitors by the process of lateral inhibition, proneural genes are expressed at low levels in all precursors. At this stage, progenitors are not committed to differentiate towards the neural fate. It is only when proneural genes reach high level of expression that progenitors are irreversibly doomed for neural differentiation (Sun et al., 1998; Van Doren et al., 1992). The initial upregulation of proneural gene expression is controlled by the process of lateral inhibition, but other positive feedback mechanisms are required for the maintenance of high levels of proneural genes expression (figure 6). Overexpression experiments in vertebrates indicate that *Xcoe2* and *Hes6* can stimulate proneural gene expression and are themselves induced by proneural genes (Koyano-Nakagawa et al., 2000; Dubois et al., 1998; Bae et al., 2000). Similarly, a zinc finger transcription factor, *sensless*, has been shown to interact synergistically with proneural genes in a positive feedback loop in *Drosophila* (Nolo et al., 2000). In *Xenopus*, the related zinc finger factor *Myt1* is induced by *XNgn1* and renders selected progenitor insensitives to *Notch* signalling (Bellefroid et al., 1996). Proneural genes can also regulate positively their own expression, at least in *Drosophila* (Sun et al., 1998; Van Doren et al., 1992).

b Inhibition of glial fate

In the mouse, neural stem cells in the neuroepithelium produce restricted progenitors at the origin of neurons in the early phase of development and glia (astrocytes and oligodendrocytes) in the later phase. This stereotyped pattern of production is also strikingly observed *in vitro*, without any environmental cues, suggesting that an internal molecular clock directs, at least in part, the generation of neurons first and glial cells later in development (Qian et al., 2000; Sauvageot and Stiles, 2002). There are now strong evidences that proneural genes are intrinsic regulators of this sequential fate decision. In mice lacking both *Ngn2* and *Mash1*, a premature astrocytic differentiation is observed at the expense of neuron production (Fode et al., 2000; Nieto et al., 2001). Sun *et al.* proposed a model where *Ngn1* inhibits the astrocytic fate by two independent mechanisms (Sun et al., 2001). The first mechanism involves the coactivator complex p300/CBP, which is recruited on the promoters of both neuronal and astrocytic target genes. As the level of this complex is limited in cells, there is competition among the different transcription factors for p300/CBP binding (Goodman and Smolik, 2000). *In vitro* studies suggest that *Ngn1* inhibits the astrocytic fate by sequestering the p300/CBP complex away from glial-specific genes (glial

fibrillary acidic protein, GFAP). The second mechanism characterized by the authors is the direct inhibition by *Ngn1* of the JAK/STAT signalling pathway, which has been shown to activate astrocytic differentiation (Bonni et al., 1997; Nakashima et al., 1999).

c Cell cycle regulation and proneural genes

Experimental evidences in both vertebrates and invertebrates indicate that neural fates may be instructed during the final cell division of progenitors (Isshiki et al., 2001; Ohnuma et al., 2002). Cell cycle arrest and regulation of the cell cycle are thus critical events during neurogenesis. In cell culture assays, *Ngn2* ectopic expression decreases the proportion of proliferating progenitors, suggesting that *Ngn2* can promote cell cycle arrest (Lo et al., 2002). Overexpression of *Ngns* or *Mash1* in P19 cells, activates the expression of the cyclin-dependent kinase inhibitor p27Kip1. This elevated level of p27Kip1 precedes neuronal differentiation and is correlated with cell-cycle withdrawal (Farah et al., 2000). Recent experiments made *in vivo* in *Xenopus* have provided further evidence that proneural proteins link neuronal differentiation to cell cycle regulation (Vernon et al., 2003; Ohnuma et al., 1999). In these experiments, the related cyclin-dependent kinase inhibitor p27(Xic1) is required for the proper differentiation of neural progenitors. Indeed, inhibition of p27(Xic1) expression by injection of specific morpholino prevents differentiation of primary neurons, while p27(Xic1) overexpression promotes ectopic neuron formation and stabilizes the proneural protein X-NGNR-1 (Vernon et al., 2003).

3 Specification of neuronal subtype

A Neuronal subtype specification induced by proneural genes

a In *Drosophila*

A striking observation in *Drosophila* is the restriction of particular proneural genes in different sensory organs. *Asc* is involved in the determination of neuroblasts in external sense organs, *atonal* in chordotonal organs and *amos* for the formation of multidendritic neurons and olfactory sensillae (see, Characterization of proneural genes, p6). This observation raises the possibility that proneural genes may not be only involved in the activation of a generic program of neural determination (epidermis *versus* neuroblast) but also in the specification of neural identity (multidendritic neurons *versus* chordotonal organs for instance). Support for this hypothesis comes from gain-of-function experiments in the fly. Indeed, the ectopic expression of *asc* induces the formation of ectopic external sense organs while ectopic expression of *amos* leads to ectopic multidendritic neurons and olfactory sensilliae (Rodriguez et al., 1990; Goulding et al., 2000b; Chien et al., 1996; Parras et al.,

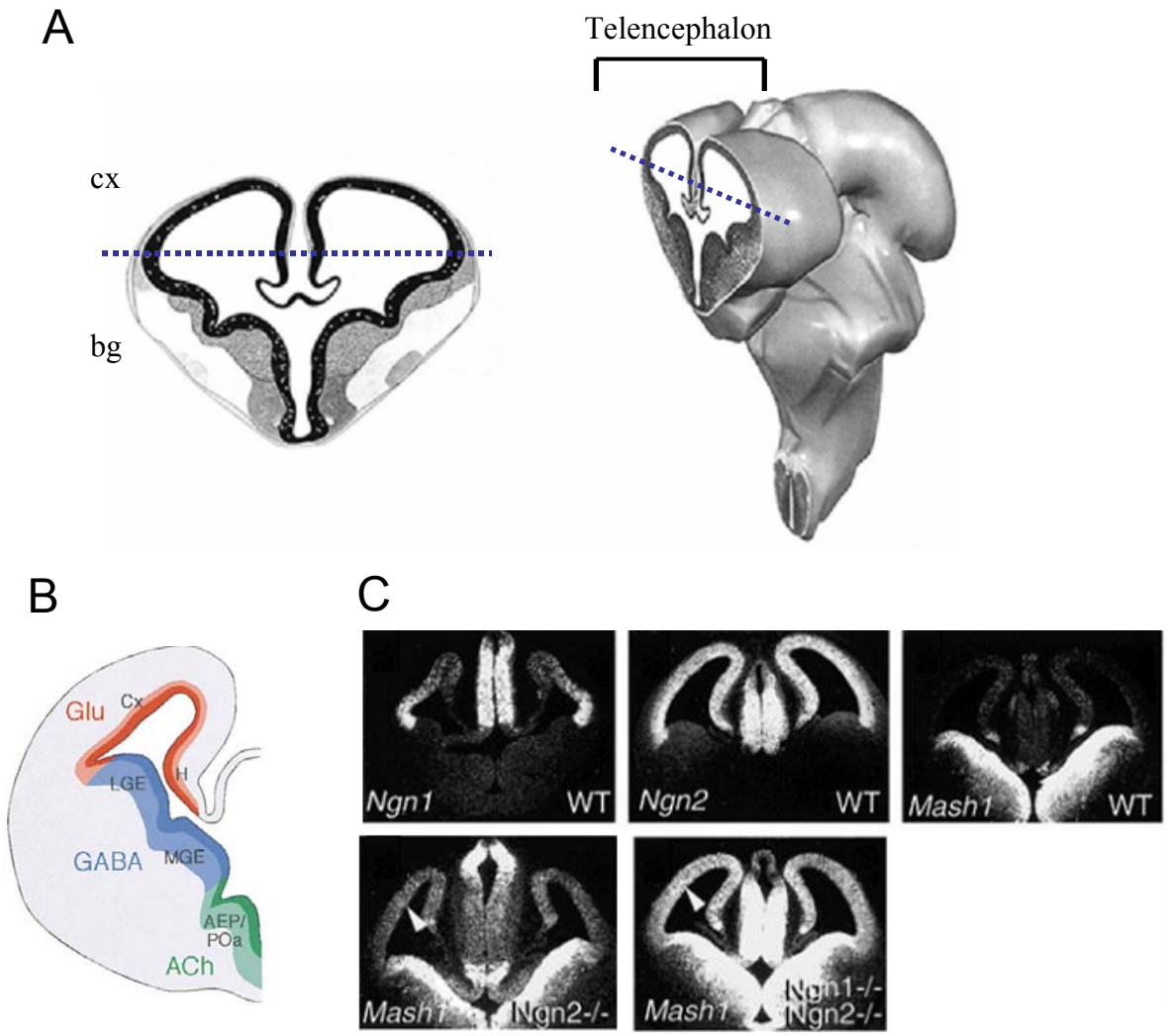


Figure 7: **Structure of the embryonic mouse telencephalon**. A) Structure of the mouse embryonic telencephalon at embryonic stage E12.5. The telencephalon (or forebrain) is formed by the cerebral cortex dorsally and the basal ganglia in the ventral part (separated by the dashed blue line). B) Progenitors in the cortex produce glutamatergic excitatory neurons, whereas progenitors in the basal ganglia produce GABAergic interneurons. C) In the wild-type situation, *Ngn1* and *Ngn2* are specifically expressed in the cortex, while *Mash1* is expressed at high levels in the basal ganglia. Mutations of *Neurogenin2* or *Neurogenin1;2* lead to the ectopic expression of *Mash1* in the cortex and the respecification of cortical neurons into a GABAergic phenotype (see text) (from Fode et al. 2000). cx: cortex, bg: basal ganglia, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, H: heme, AEP/POa: anterior antopeduncular and preoptic area, Glu: glutamatergic neurons, GABA: gamma amino butyric acid, Ach: Acetyl choline).

1996; Skeath and Doe, 1996). This shows that *ato* and *sc* are involved in the specification of neuronal-subtype characteristics.

b In the mouse

In the mouse *Mash1* and *Ngns* are expressed in distinct areas of the nervous system. In the telencephalon (the more rostral part of the CNS), *Neurogenin1* and *Neurogenin2* are expressed in dorsal progenitors of the cerebral cortex while *Mash1* is expressed in ventral progenitors located in the basal ganglia (striatum and pallidum). Interestingly, progenitors in the dorsal telencephalon differentiate into glutamatergic projection neurons of the cerebral cortex, while progenitors in the ventral telencephalon differentiate into GABAergic neurons, raising the possibility that proneural genes have also a role in neuronal subtype specification in the mouse, similarly to their *Drosophila* counterparts (figure 7).

The role of *Mash1* in the specification of the GABAergic neuronal sub-type has been demonstrated in mutant mice lacking the *Ngns* genes. In *Ngn2* mutant mice, and in a more dramatic manner in *Ngn1 ;Ngn2* double mutant mice, cortical progenitor are respecified towards a ventral GABAergic fate as the consequence of *Mash1* ectopic expression in the cortex. (Fode et al., 2000; Schuurmans et al., 2004). This phenotype is not due to migration or patterning defect as normal numbers of neurons are still produced and markers of cortical progenitors are expressed normally. A role of *Mash1* was also demonstrated in the process of subtype specification of the noradrenergic phenotype in the autonomous nervous system. Genetic studies demonstrated that *Mash1* activates the downstream effector gene *Phox2a* and is required for the generation of noradrenergic neurons as determination factor (Hirsch et al., 1998; Lo et al., 1998).

A role of *Ngn2* in subtype specification has been shown for the generation of sensory neurons in the PNS and motor neurons in the spinal cord (Mizuguchi et al., 2001; Ma et al., 1999). The specificity of *Ngn2* to generate either of those two fates is restricted by the local environment. In dorsal root ganglia, generation of sensory neurons by *Ngn2* is dependent on the BMP2 level excreted by the roof plate of the neural tube (Lo et al., 2002). In the spinal cord, *Ngn2* requires the activity of *Olig2* to specify the identity of motor neurons (Novitch et al., 2001; Mizuguchi et al., 2001).

The role of *Mash1* and *Ngn2* in neuronal subtype specification were investigated nicely by swapping the coding sequence of *Mash1* and *Ngn2* in their respective genomique locus (Parras et al., 2002). In the *Mash1*KI^{Ngn2} embryos, the locus of *Mash1* is replaced by the coding sequence of *Ngn2*. The expression of *Ngn2* is thus controlled by endogenous *Mash1* regulatory elements. Analysis of such *Mash1*KI^{Ngn2} mouse showed that neuronal progenitors still differentiate normally but do not show signs of respecification. Conversely, in *Ngn2*KI^{Mash1} embryos, ectopic expression of *Mash1* in *Ngn2* domains of expression, leads to a respecification of cortical neurons towards a GABAergic fate in the telencephalon and a conversion of motoneurons into interneurons in the

spinal cord (Parras et al., 2002). These results suggest that *Ngn2* is more dependent on the presence of locally expressed cofactors than *Mash1* for the determination of neuronal subtype specification.

B Molecular basis of neuronal subtype specification

Proneural genes are able to activate a generic program of neuronal development together with neuronal subtype specific programs restricted to particular regional context (Bertrand et al., 2002). Proneural factors must therefore regulate both a common and a divergent panel of target genes. The molecular mechanism by which this is achieved is still largely unknown, but two levels of action can be distinguished. The first is the specific binding of certain category of proneural proteins to particular Ebox DNA sequence. The second is the presence of specific cofactors that modulate the activity of proneural proteins.

a Specificity of the Ebox

The Ebox recognized by bHLH proteins is a hexanucleotidic motif degenerated to a large extent (CANNTG). The detailed analysis of Ebox in the promoter of various target genes raises the possibility that certain motifs are preferred for the binding of proneural proteins and suggest that functional specificity could be driven, at least in part, at the level of these Eboxes. Recently, *Powel et al.* investigated the specificity of regulation of *Bearded*, a common target of *ato* and *sc* in *Drosophila* (Powell et al., 2004). The authors showed that the promoter of *Bearded* is regulated by *ato* and *sc* through two distinct EBoxes and defined distinct Ebox consensus sequences sufficient to confer *ato* and *sc* specificity *in vivo*. Consensus sequence extends even outside of the EBox motif. The consensus sequence A(A/T)CA(G/T)GTG(G/T) can be proposed for *ato*, and GCAG(C/G)TG(G/T) for *sc* (from 5' to 3') (Powell et al., 2004). Significantly, *ato* can rescue mutations of its mouse orthologue *Math1* and vice versa, suggesting that EBox motif preferences are conserved in vertebrates (Ben Arie et al., 2000; Wang et al., 2002).

b Importance of cofactors

Indication of the importance of cofactors in cell type specification was obtained in *Drosophila*. Chien *et al.* produced transgenic flies expressing ectopically proneural genes chimeras composed of the basic region of *scute* and the HLH region of *ato*, and *vice versa* (Chien et al., 1996). Proneural proteins chimeras composed of *ato* basic domain and HLH *scute* domain induce ectopic chordotonal (*cho*) organs and rescue the *ato*-null mutation, demonstrating that the basic domain alone is sufficient for the specification of *cho* fate. Bioinformatic analysis based on *MyoD* crystal structure predict that residues differing between *ato* and *scute* are located opposite to the surface in contact with DNA, making them available for interactions with potential cofactors (Chien et al., 1996). Such cofactors have been identified in *Drosophila*. The GATA transcription factor *pannier*

is an essential coactivator of *scute* for the determination of *es* organs in the fly. *Pannier* has been shown to recognize a specific enhancer near the EBox enabling this factor to synergize with *scute* (via the bridging factor *chip*) and activate transcription of *scute*'s target genes. As the synergy with *pannier* is a prerequisite for activation of target genes, the expression of this GATA transcription factor spatially restricts the activity of *scute* (Ramain et al., 2000). Similarly the Ets transcription factor Pointed synergize with atonal to induce atonal expression in an autoregulatory loop for the selection of sensory organ precursors (Zur Lage et al., 2004).

A role of cofactors in the modulation of proneural genes activity has also been demonstrated indirectly in vertebrates. Depending on the tissue where they are expressed, *Mash1* and *Ngn2* are implicated in the generation of distinct neuronal types (see, specification of neuronal subtype, p17). Such functional diversity can not be attributed solely to different affinity for distinct DNA motifs, which shows again the importance of the cellular context. Interestingly, gain-of-function studies in the mouse demonstrated a difference of context susceptibility between *Mash1* and *Ngn2* in the central nervous system. Indeed, in the telencephalon of *Mash1*KI^{Ngn2} mouse (*Ngn2* replace the locus of *Mash1*), *Ngn2* is not sufficient to override the endogenous differentiation program and respecify progenitors whereas, in the converse experiment, *Ngn2*KI^{Mash1} (*Mash1* replace the *Ngn2* locus), the ectopic expression of *Mash1* changes the fate of cortical progenitors towards a GABAergic fate (Parras et al., 2002; Fode et al., 2000).

In the spinal cord of the mouse, transcription factors of the LIM-homeobox (LIM-HD) familly were shown to interact with bHLH transcription factor to induce the expression of the motoneuron differentiation gene *Hb9* (Lee and Pfaff, 2003). Indeed it was shown that Isl1/Lhx3 interacts synergistically with the bHLH transcription factor NeuroM to activate the *Hb9* promoter. The authors showed also that the bridging LIM factor NLI facilitates interaction between LIM-homeobox and bHLH transcription factors, in a way similar to the interactions with Pannier, Chip and achaete/scute-daughterless heterodimers in *Drosophila* (Lee and Pfaff, 2003; Ramain et al., 2000).

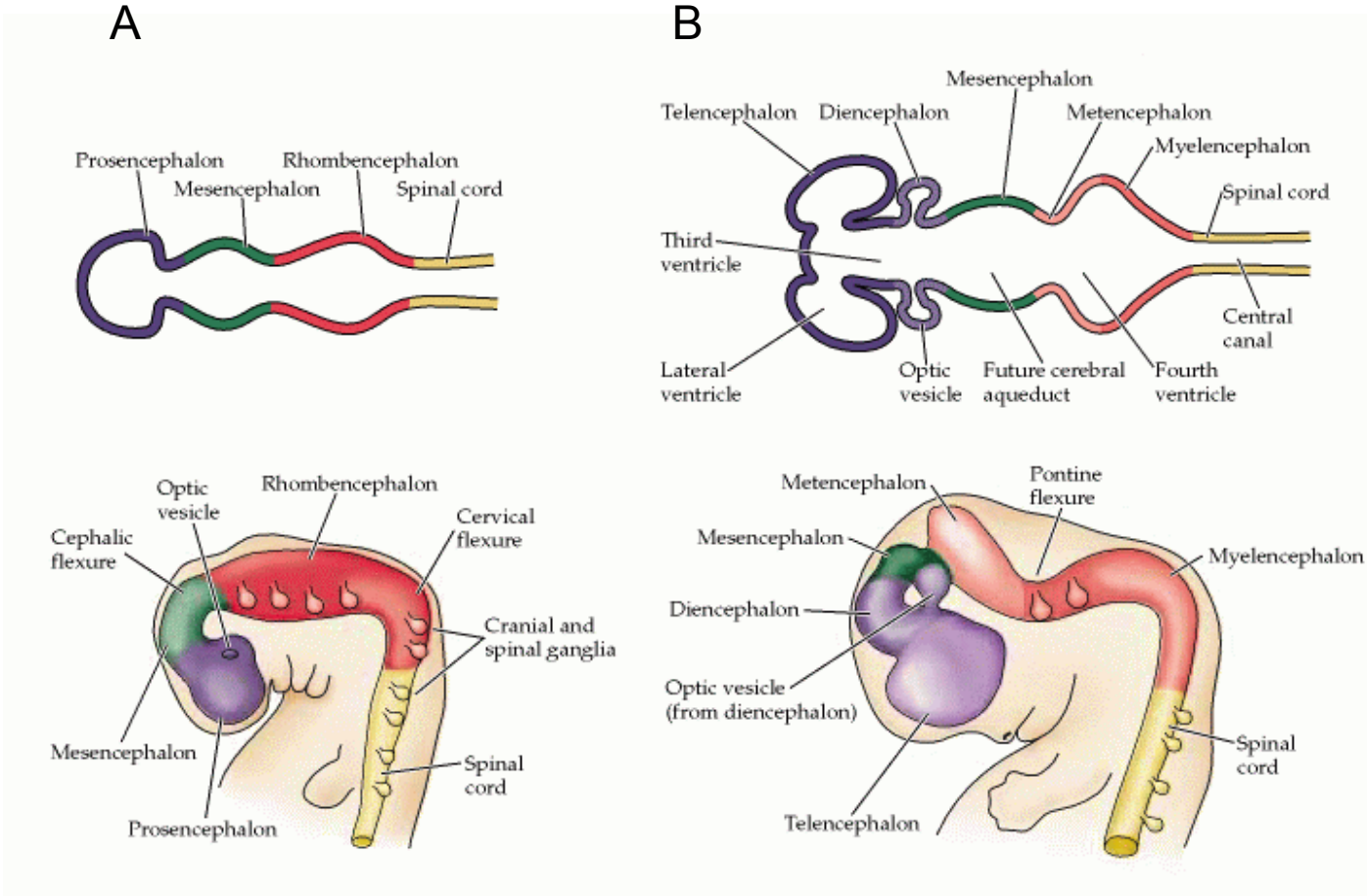


Figure 8: **Development of the mammalian neural tube**. A) After neurulation, the neural tube becomes subdivided into the three vesicles: the prosencephalon (at the anterior end of the embryo), the mesencephalon, and the rhombencephalon. The spinal cord differentiates from the more posterior region of the neural tube. (B) Later in development (five vesicles stage), the prosencephalon subdivides into the telencephalon and the diencephalon, and the rhombencephalon into the metencephalon and the myelencephalon (from Neuroscience. 2nd ed., Sinauer Associates, Inc. 2001).

II Presentation of the model system

1 Neurogenesis in the mouse CNS

The entire CNS derives from a single layer of neuroepithelial cells, the neural plate, which folds into the neural tube by a developmental process called neurulation. During early development, the neural tube can be subdivided along the anteroposterior axis into vesicles, from which will derive the whole CNS. The most anterior part of the early mammalian neural tube will eventually form the forebrain, subdivided into the telencephalon (giving rise to the cerebral hemispheres) and the more caudal diencephalon (thalamus and hypothalamus) (see figure 8).

I have focused my studies on the development of the telencephalon, the most rostral region of the CNS. Higher cognitive functions of the human brain (associative area, behaviour, memory), control of voluntary movement, interpretation of sensitive informations (view, audition, smell, etc), all those essential functions reside in the telencephalon. The adult telencephalon is the more complex structure of mamalian brain both in term of cellular diversity and connectivity. Embryonic neural stem cells at the origin of the large diversity of neural cells in the adult telencephalon have been isolated and are the subject of intensive studies aiming to understand the mechanisms of neurogenesis (Temple, 2001; Temple, 2003; Cattaneo and McKay, 1990; Reynolds et al., 1992). A great hope is to use this knowledge for cellular therapy of neurodegenerative disease like Parkinson and Alzheimer's diseases in the future (Conti et al., 2003; Temple, 2001).

A Structure and functions of the mouse telencephalon

The telencephalon can be divided in two parts, the dorsal pallium forming the cerebral cortex, and the ventral subpallium giving rise to the basal ganglia (Figure 9A and see, Specification of neuronal subtype, p17).

a Basal ganglia and voluntary movement

The basal ganglia receives excitatory inputs from virtually all cortical areas and plays major role in normal voluntary movements . Early in development of the mouse, the basal ganglia can be divided into the medial (MGE) and the lateral ganglionic eminences (LGE), which will give rise in the adult to the pallidum and the striatum respectively (Schuurmans and Guillemot, 2002). The vast majority of neurons in the basal ganglia are inhibitory GABAergic projection neurons. Cholinergic neurons from the septum and the MGE migrate also in the striatum where they constitute a subpopulation of interneurons. Movement disorders observed in Huntington and Parkinson diseases implicate pathologies of the basal ganglia (striatum, pallidum).

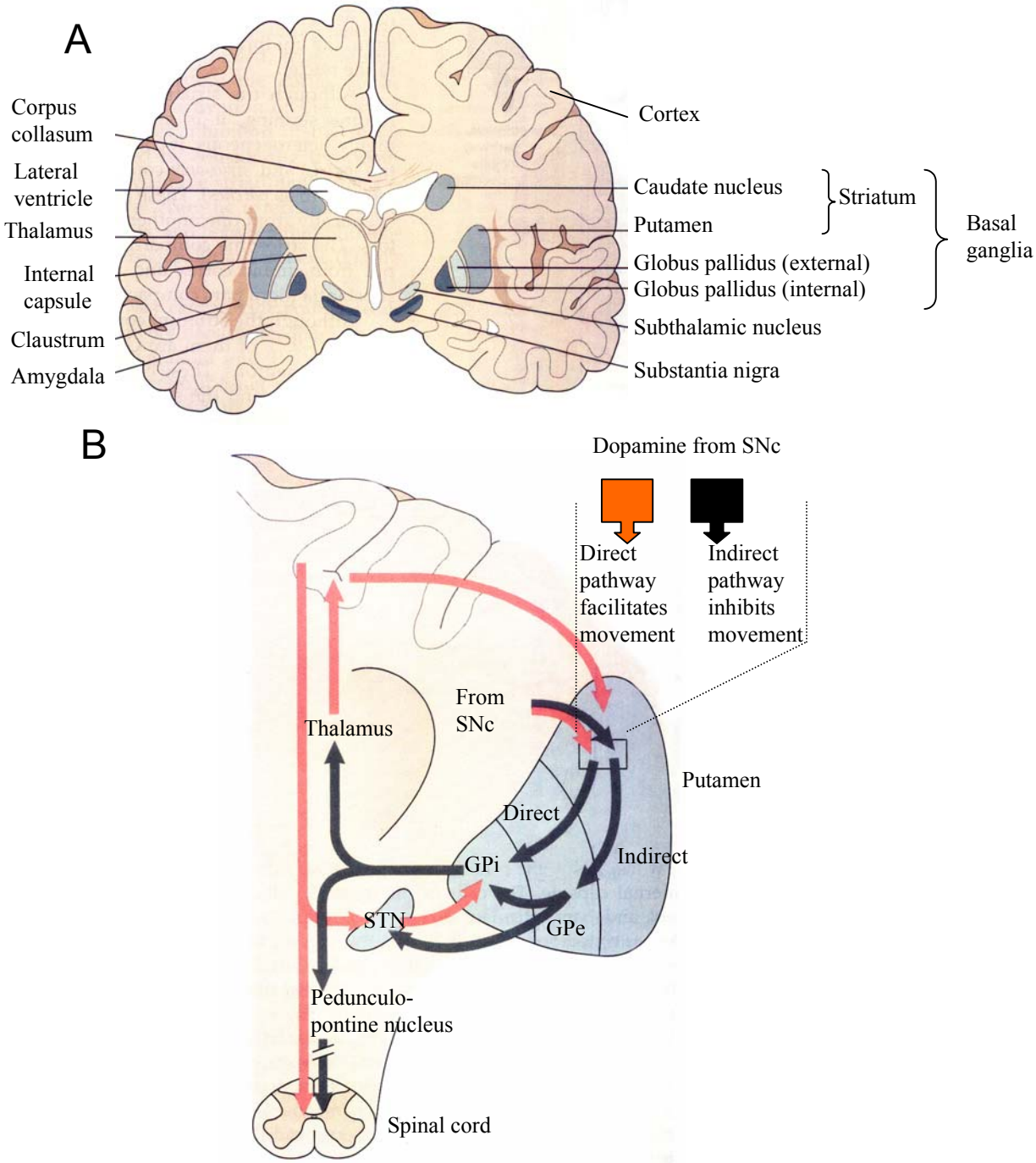


Figure 9: **Structure of the human brain and control of voluntary movement.** A) Coronal section of adult human brain and structure of the basal ganglia. B) Connection between the cortex, basal ganglia and thalamus controlling voluntary movement. Excitatory pathways are in red and inhibitory pathways in black. In the putamen (part of the striatum), dopamine from the SNc activated two types of dopamine receptors located on different sets of output neurons. One population of neurons is activated by dopamine and give rise to the direct pathway, which facilitate movement. The other population is inhibited by dopamine and gives rise to the indirect pathway which inhibits movement. SNc: substantia nigra pars compacta, STN: subthalamic nucleus, GPe: globus pallidus (external), GPi: globus pallidus (internal) (from Principles of Neural Science, Kandel et al., McGraw-Hill edition, 2000).

The striatum is the major input nucleus of the basal ganglia. Glutamatergic cortical neurons in the cortex, dopaminergic neurons from the midbrain, serotonergic neurons from the raphe nuclei and projection neurons from the intralaminar nuclei of the thalamus send excitatory projections to specific portions of the striatum. The striatum is not a homogenous structure. It consists of two separate parts, the matrix and the striosome (or patches) compartments (Joel and Weiner, 2000; Redies et al., 2002). These compartments can be distinguished on the basis of immunoreactivity with several markers (acetyl choline transferase, enkephalin, substance P, dopamine receptor, opiate receptors and calcium binding protein), connectivity and birthdates (the time at which the progenitor undergoes its last mitotic division to produce a neuron) (Murrin and Ferrer, 1984; Foster et al., 1987; van der and Fishell, 1987). The vast majority of early born neurons end up in the patch compartment and are acetyl choline esterase positive, whereas late born neurons end up in the matrix compartment and express dopaminergic receptors (DARPP32).

Striatal GABAergic neurons project to the pallidum and substantia nigra (a nuclei in the midbrain composed of dopaminergic neurons). The neurons in those two nuclei discharge tonically at high frequency. Pallidal and substantia nigra neurons project in the thalamus and brain stem were they inhibit their target nuclei. This inhibitory output is thought to be modulated by two parallel pathways (figure 9B). In the first pathway (the direct pathway) tonically active neurons in the pallidum are suppressed by striatal projections, permitting the thalamus and ultimately the cortex to be activated. In other words striatal activity inhibits the inhibitory action of the pallidum on the thalamus. In the second pathway (or indirect pathway), the signal arising from the striatum is relayed in the external pallidal segment, from there to the subthalamic nucleus which project excitatory glutamatergic signals back into the internal pallidal segment leading to an increased inhibition of the thalamus. On the top of these two pathways, dopaminergic projections from the substantia nigra pars compacta affect the striatum in different ways. Indeed, dopaminergic inputs facilitate transmission of the direct pathway and in contrast inhibit the indirect pathway. As a result dopamine inputs in the striatum reduce the inhibition of thalamocortical neurons and thus facilitate voluntary movement: i) in the direct pathway, dopamine activates the striatum which inhibits the pallidum and thus release the activity in the thalamus, ii) in the indirect pathway, dopamine inhibits the striatum, the tonic signal of the pallidum is then not inhibited by the striatum, active output of the pallidum inhibits the substantia nigra pars compacta which do not inhibit back the internal segment of the pallidum and release then thalamocortical activity (figure9B) (Joel and Weiner, 2000; Redies et al., 2002).

b The cerebral cortex

The cerebral cortex is the most complex structure of the human brain both in terms of cellular diversity and cell-cell interactions. It is in the cerebral cortex that the higher functions of the brain

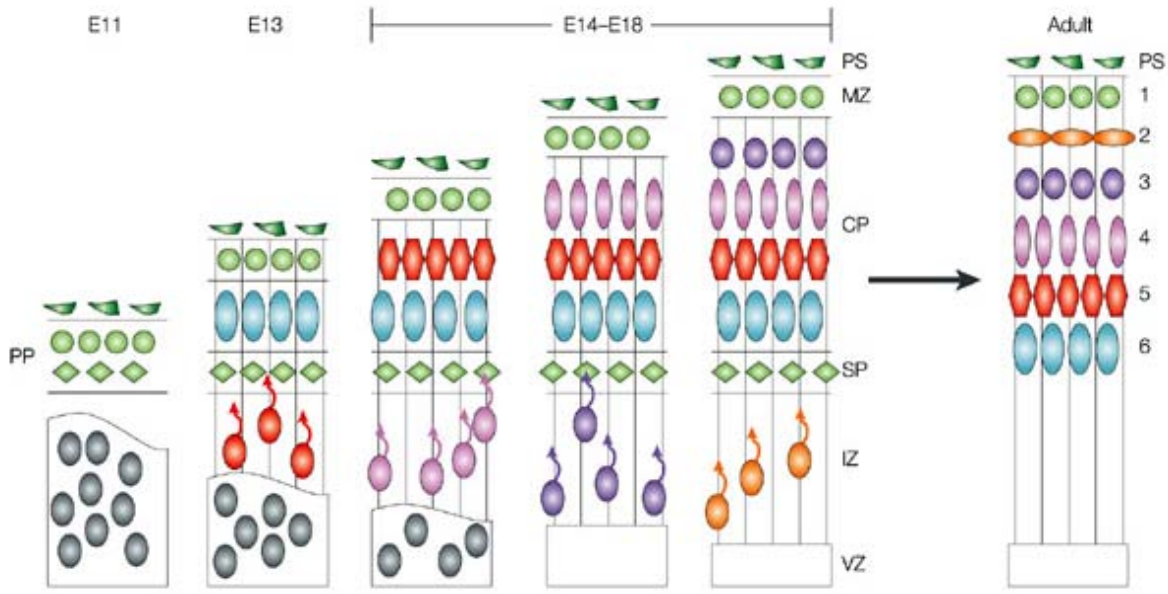


Figure 10: **Layers formation in the mouse cortex.** The adult mammalian cortex is organized into six distinct cellular layers (from I to VI). At E11 in the mouse, the PP constitute the first layer of postmitotic neurons. By E13, a second wave of neurons form the CP by splitting the PP into the upper MZ and the deeper SP. Subsequent waves of neurons bypass the earlier generated layer to form the more superficial layers of the cortex in an inside-out manner. Early born neurons form the deeper layers of the cortex, while later born neurons form the more superficial layers of the cortical plate. VZ: ventricular zone, IZ: intermediate zone, PP: preplate, SP: subplate, CP: cortical plate, MZ: mantle zone, PS: pial surface (from Gupta et al. 2000).

reside and are thought to give rise to human consciousness. The cortex receives inputs from the thalamus and from other regions of the cortex. Outputs of the cortex is directed toward the basal ganglia, others regions of the cortex, the thalamus, the spinal cord and the pontine nuclei. The cerebral cortex is organized into areas that regulate anatomically and functionally distinct processes (visual area, somatosensory area, auditory area, etc).

Neuronal and glial cells in the cortex are arranged in six distinct layers (from I to VI) different in terms of cellular density, morphology and axonal projection pattern (figure 10) (Gupta et al., 2002). During neurogenesis, neurons are produced in proliferative regions lining the ventricular wall (the ventricular zone, VZ), and migrate away toward the pial surface (Luskin and Shatz, 1985). The laminar organization of the adult cortex is an inside-first outside-late mechanism: neurons generated first are located in the deep part of the cortex whereas later born neurons populate more superficial layers (Gillies and Price, 1993). At embryonic day 11 (E11), the first wave of neuron migrates away from the ventricle and settles as a single layer just beneath the pial surface. This first layer of neurons constitutes the primordial plexiform layer, also called the preplate. The preplate is constituted of stellate-shaped cells, the Cajal-Retzius cells, which are essential for the proper radial migration of later born neurons (Luskin and Shatz, 1985; Gupta et al., 2002). A second wave of neurons (at E13) splits the preplate into two layers, the upper constitute the marginal zone (future layer I) and the deeper subplate. Between the marginal zone and the subplate resides the first layer of the cortical plate (the latter called layer VI). Subsequent waves of neurons bypass earlier generated neurons in the cortical plate and migrate onto the top of it to constitute more superficial layers of the cortex (layer V to II) (Gillies and Price, 1993; Luskin and Shatz, 1985) (figure 10). As the cortex thickens during neurogenesis, an additional layer develops above the ventricular zone: the intermediate zone. This zone of low cell density is non proliferative and will eventually contain the major axonal tract (the white matter) in the adult cortex. At the end of neurogenesis, the cortical layers comprise neurons with distinct morphological and connective properties. An exception is the layer I, which is occupied by dendrites of cells located deeper in the cortex or axons that travel through or form connections in this layer.

The function of the layered organization of the cortex can be envisaged under the light of cortical connectivity. Indeed the different cortical areas do not have the same laminar organization. For instance, the primary motor cortex has a very thin layer IV. In contrast the primary visual cortex has a very thick layer IV that is typically further subdivided into three sublayers. These different laminar structures can be correlated with different connectivity with the thalamus. Indeed, the layer IV is the main laminar target of sensory information arriving from the thalamus. In contrast, the motor cortex, is primarily an output of the cortex and receives little sensory information directly from the thalamus.

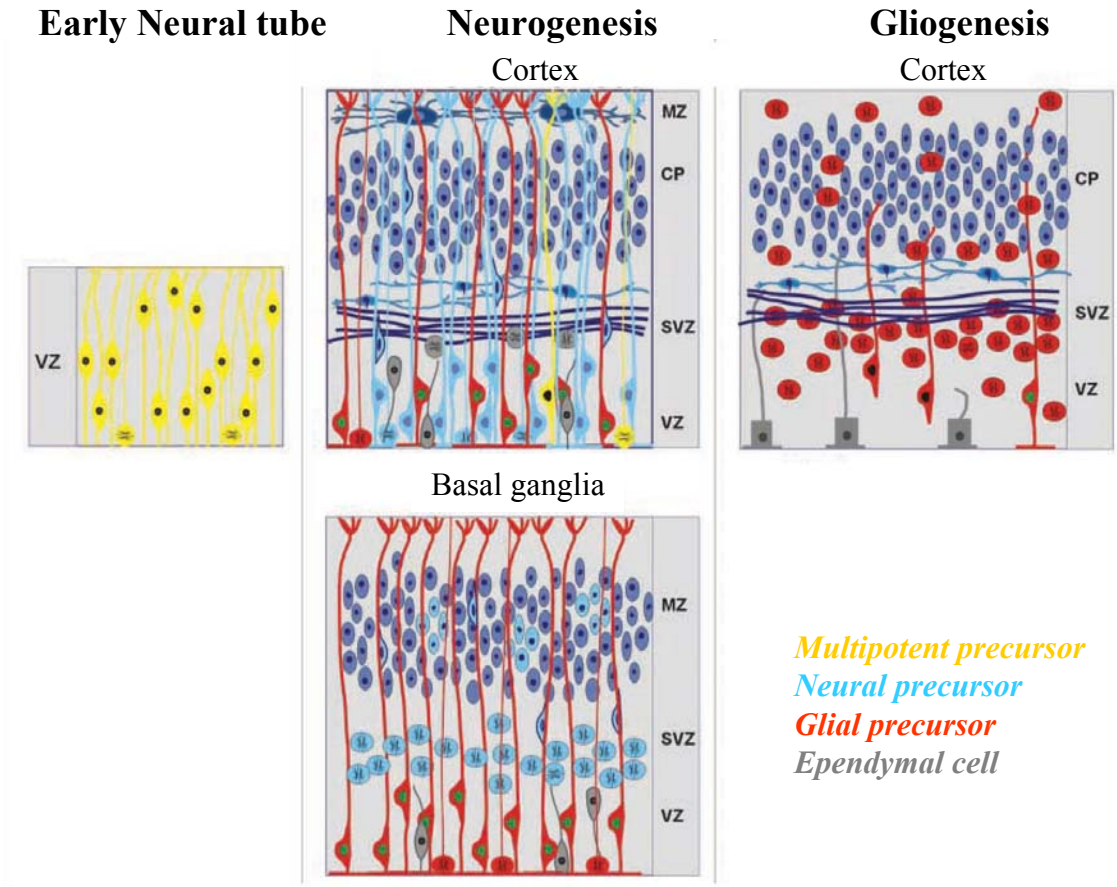


Figure 11: **Diversity of neural progenitors in the VZ and SVZ.** The early neuropeithelium (E11) is composed of multipotent progenitors (yellow). During neurogenesis (E13) a second type of progenitor arise: the radial glia. In this model a sub-population of radial glia progenitors produces only neurons (blue), while another sub-population (red) will produce astrocytes at later stage. Only a small fraction of the progenitor are still multipotent during neurogenesis (yellow). VZ: ventricular zone, IZ: intermediate zone, PP: preplate, SP: subplate, CP: cortical plate, MZ: mantle zone (from Gotz 2003).

B Cell proliferation and migration

a Ventricular and subventricular zone

The three types of cells found in the adult CNS, neurons, astrocytes and oligodendrocytes, are generated in the ventricular zone (VZ), an epithelial layer of progenitor cells that lines the ventricle (figure 11). Neuroepithelial cells in the VZ are multipotent and highly mitotic (Temple, 2001; Okano, 2002). These progenitors are considered as embryonic neural stem cells and are found in the VZ during the early phase of neurogenesis (E11). Morphologically, these cells have the particularity to extend long radial processes from the ventricular surface to the pial surface. During cell cycle, the nuclei of neuroepithelial cells move within the whole thickness of the VZ, a process known as interkinetic movement. The nuclei of cells in G1 phase of the cell cycle are located in the middle of the VZ. Nuclei move upward to the top of the VZ with the initiation of DNA synthesis in the S phase and descend to the ventricular surface in G2. During mitosis, nuclei are lining the ventricle to generate the two daughter cells (Takahashi et al., 1995).

At E13, an additional population of multipotent progenitor cells is found in the VZ, the radial glia cells. This population shares morphological properties with the earlier neuroepithelial cells as they also possess radial fibers that connect the pial and ventricular surface. However, radial cells possess hallmarks of astrocytes, as they express the glia-fibrillary acidic protein (GFAP), the astrocyte-specific glutamate transporter (GLAST) and the brain lipid binding protein (BLBP) (Campbell and Gotz, 2002; Malatesta et al., 2003). Radial glial cells were considered as support structure for a long time, mainly because of their astrocytic traits and the observation that they produce astrocytes in postnatal stage of development. However, lineage analysis showed the capacity of radial cells to generate neurons both *in vitro* and *in vivo* (figure 11) (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Heins et al., 2002).

A second proliferative zone has been defined during the generation of neurons in the telencephalon: the subventricular zone (SVZ), immediately adjacent to the VZ (Smart, 1976). The murine SVZ is established around E13.5, although mitotic cells have been identified under the VZ as early as E10.5 (Ishii et al., 2000). Progenitors in the SVZ do not show interkinetic movement of the nucleus and do not have radial fibers. Interestingly, the proportion of neurons produced by the ventricular or the subventricular zone is different in the cortex and in the basal ganglia. Indeed, the basal ganglia SVZ is much bigger than the SVZ in the cortex. This size difference can be correlated with the proportion of progenitors found in the VZ compared to the SVZ in the two tissues. In the cortex, the main source of neurons is the radial glia, located in the VZ, whereas in the basal ganglia the SVZ produces the vast majority of neurons populating the striatum (Malatesta et al., 2003; Gotz, 2003).

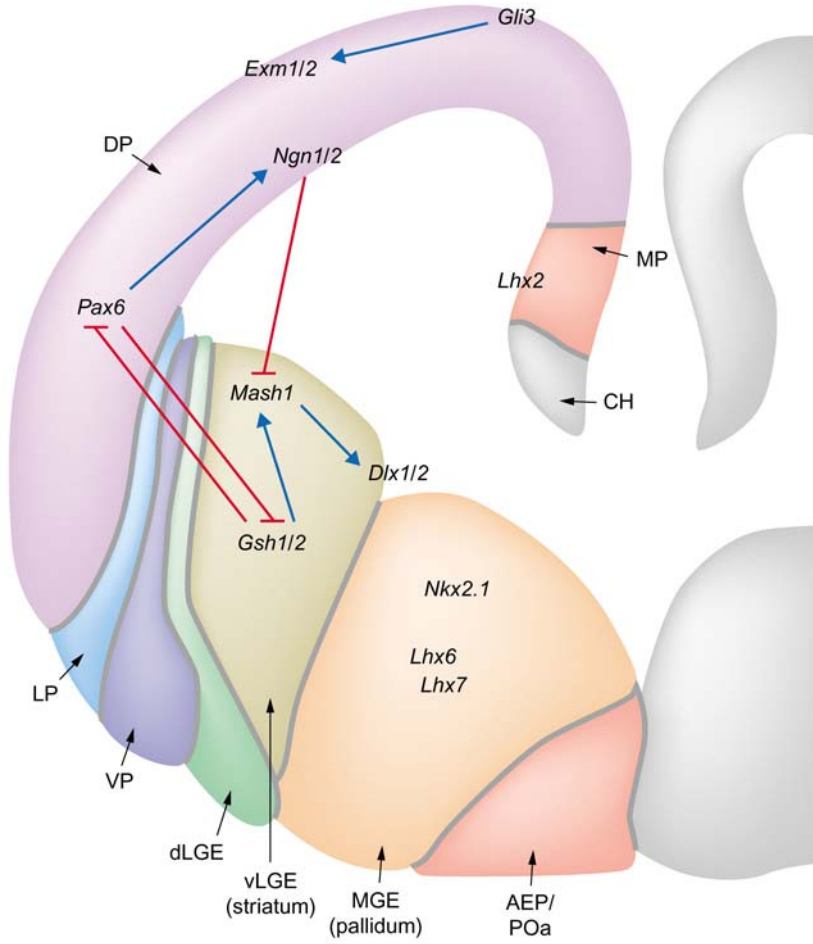


Figure 12: **Expression of dorsal and ventral regionalization markers and their genetic interaction in the mouse telencephalon at E12.5.** Cortical progenitors express high levels of *Ngns*, *Emx1/Emx2* and *Pax6*. Ventral progenitors express *Mash1*, *Dlx1/Dlx2* and *Gsh1/Gsh2*. In the basal ganglia, LGE and MGE progenitors are distinguished by the expression of *Nkx2.1* and *Lhx6/Lhx7* in the LGE. DP: dorsal pallium, LP: lateral pallium, VP: ventral pallium, dLGE: dorsal LGE, vLGE: ventral LGE, AEP/POa: anterior antopeduncular and preopotic area. CH: cortical hem, MP: medial pallium (from Schuurmans and Guillemot, 2002).

b Cell migration

Neurons generated in the VZ migrate radialy along the fiber of radial glia to reach their final position and complete their differentiation (Hatten, 1999). This type of cellular migration is called locomotion. Interestingly the mechanisms of migration of cortical neurons seem to differ in the early phase of neurogenesis. Between embryonic days E12 and E13, newly formed neurons in the VZ extend a leading process which attaches to the pial surface. The progressive shortening of the leading process allows the entire cell to migrate toward the pia, independently from the radial glia. This mechanism of cellular migration is called somal translocation (Gupta et al., 2002).

In addition, some neuronal progenitors produced in the basal ganglia migrate tangentially to populate the cortex. Indeed it has been demonstrated that the vast majority of GABAergic interneurons found in the mouse cerebral cortex are generated in the basal ganglia (Lavdas et al., 1999; Anderson et al., 1997a). These interneurons are born as early as E11 in the medial ganglionic eminence and migrate toward the dorsal telencephalon. In contrast, in humans and primates, interneurons are mainly generated in the cortex and only a small fraction in the subpallium (Letinic et al., 2002).

2 Patterning of the telencephalon

A Transcription factors define the regional identity of the telencephalon

Genetic analyses have demonstrated that regional expression of transcription factors was involved in the specification of regional identity in the telencephalon. Indeed, expression of many transcription factors (mainly of the homeodomain family) is restricted to specific regions of the telencephalon. For instance *Pax6* and *Emx1/2* are expressed in the developing cortex whereas *Nkx2.1*, *Lhx6* and *Gsh1/2* are specifically expressed in progenitors of the basal ganglia (figure 12) (Toresson et al., 2000b; Sussel et al., 1999; Schuurmans and Guillemot, 2002; Mallamaci et al., 2000; Casarosa et al., 1999; Bishop et al., 2003; Stoykova et al., 2000).

Pax6 and *Emx2* are expressed in a complementary graded manner in the cortex: *Emx2* is expressed in low rostral to high caudal and low lateral to high medial gradients, whereas *Pax6* is expressed in low caudal to high rostral and low medial to high lateral gradients. Genetic analysis showed that these two genes are involved in the regional identity of the cortex. Mutation of both *Pax6* and *Emx2* leads to a loss of cortical area and the expansion of basal ganglia dorsally (Muzio et al., 2002) (Toresson et al., 2000b; Stoykova et al., 2000). In addition to a function of *Pax6* and *Emx2* in the morphogenesis of the cortex, those two genes have been shown to regulate arealization of the cortex. Indeed, cortical areas (the authors focused on motor somatosensory area) are shifted toward more caudal or rostral part of the cortex when *Pax6* or *Emx2* are mutated, suggesting that those two

genes are directly involved in the control of area identity (Bishop et al., 2000; Hamasaki et al., 2004). Regional identity transcription factors were also identified in the basal ganglia. Mutation of *Nkx2.1* leads to the respecification of the MGE into LGE (Sussel et al., 1999). Another gene, *Gsh2*, is required to maintain the identity of LGE. In *Gsh2* knock out mice, striatal genes like *Mash1* and *Dlx1* are lost in the LGE and cortical genes (*Pax6*, *Ngn1*) are expressed ectopically (Toresson et al., 2000b).

These genetic studies strongly suggest that identity of the different regions of the telencephalon is controlled by the expression of specific patterning genes. In addition, the molecular events linking regional patterning and neuronal specification begin to be investigated. It has been demonstrated that *Pax6* activates directly the proneural gene *Ngn2* in cortical progenitors, providing thus a mechanisms in which expression of patterning genes induce neurogenesis (Scardigli et al., 2003). In the basal ganglia the molecular events leading to GABAergic differentiation in the striatum seems to involve the sequential activation of *Mash1*, *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) and glutamic acid decarboxylase enzymes (*GAD65* and *GAD67*) (Stuhmer et al., 2002a; Letinic et al., 2002; Casarosa et al., 1999).

B Patterning centers

Recent studies have started to identify key regulators of the initiation of telencephalon regionalization. The current model proposes the existence of patterning centers that set up the graded expression of regulatory genes controlling regional identity of the telencephalon (see above) (O'Leary and Nakagawa, 2002; Wilson and Rubenstein, 2000). For example, *FGF8* is excreted by the anterior neural ridge (located rostrally, between the two telencephalic vesicules) and is a very good candidate to set up the expression of the regional identity gene *Emx2* in the cortex. In the chick, *FGF8* inhibits the expression of *Emx2*. *FGF8* overexpression in the mouse cortex results in the caudal shift of cortical areas in a way similar to what is observed in *Emx2* knock out mice (Bishop et al., 2003; Mallamaci et al., 2000; Fukuchi-Shimogori and Grove, 2001; Crossley et al., 2001). Conversely, a decrease of endogenous *FGF8* expression results in a rostral shift of cortical areas (Fukuchi-Shimogori and Grove, 2001). In the ventral part of the telencephalon, the prechordal plate (an axial mesoderm derivative of the node) expresses SHH which is crucial for the correct specification of the whole ventral forebrain (Chiang et al., 1996; Kohtz et al., 1998). In addition SHH has been shown to induced the expression of the MGE specific gene *Nkx2.1* (Kohtz et al., 1998; Dale et al., 1997; Ericson et al., 1995). Finally, BMPs (BMP2, 4, 5 ,6, 7) and Wnts (Wnt2b, 3a, 5a) are expressed by the cortical hem (the most medial part of the cortex) and has been proposed to control specification of the dorsal telencephalon (Grove et al., 1998; Furuta et al., 1997) (figure 12). Comforting this hypothesis is the analysis of conditional mutation of BMPR1a in the mouse

telencephalon which leads to serious defect in morphogenesis of the choroid plexus (a structure arising from the cortical hem (Hebert et al., 2002).

C Neural subtype specification along the dorsoventral axis of the CNS

Progenitors in the CNS produce different type of neural cells along the dorsoventral axis of the neural tube (Briscoe and Ericson, 2001; Zaki et al., 2003; Helms and Johnson, 2003; Lee and Jessell, 1999). The best studied model is the chicken spinal cord in which graded signalling molecules (mainly SHH and BMPs) control distinct set of transcription factors which regulates the appearance of distinct cell types at defined positions in the neural tube (Briscoe and Ericson, 2001; Lee and Jessell, 1999). In the ventral spinal cord, progenitors born in ventral regions generate first motoneurons and later oligodendrocytes, while interneurons are generated from a more dorsal domain that later generates astrocytes (Lu et al., 2002; Gabay et al., 2003; Novitch et al., 2001; Lee and Jessell, 1999). The sequential generation of motoneurons and oligodendrocytes in the ventral spinal cord depends on the activity of the bHLH transcription factor *Olig2*, which is necessary and sufficient for the production of both cell types (Lu et al., 2002; Gabay et al., 2003). A similar mechanism could also be implicated in the generation of neurons and oligodendrocyes in the ventral telencephalon. Indeed, a common progenitor for GABAergic interneurons and oligodendrocytes has been identified in the mouse ventral telencephalon and it has been suggested that mutation of *Olig2* and its paralogue *Olig1* leads to a complete loss of oligodendrocyte lineage in the brain (He et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002). In contrast it has been proposed that cortical progenitors produce first glutamatergic neurons and later astrocytes (Gotz, 2003; Malatesta et al., 2003).

III Functional Genomic

The purpose of functional genomic is to study the expression, regulation and function of genes and to understand how they work together in a living organism, ultimately in humans. Data from genome sequencing projects such as *Drosophila melanogaster*, *Caenorhabditis elegans*, the mouse and recently the human genome, provide a huge amount of information allowing researchers to investigate regulatory pathway in a whole-genome scale. Raw data from genome sequencing allows then the prediction *in silico* of new Open Reading Frame (ORF), splicing sites, promoter and enhancer location, etc. This fastidious and difficult process of genome annotation is essential to give functional relevance to the succession of nucleotides provided by genome sequencing projects.

1 DNA microarray

The technology of DNA microarray (or DNA chips) allows to measure levels of gene expression (messenger RNA abundance) for ten of thousands of genes simultaneously and ultimately complete transcriptional program in any tissue or cell type and at any time of development or pathological process. Initial studies in the yeast proved the feasibility and reliability of DNA microarray to provide access to entire regulatory pathways and networks by revealing genome-wide transcriptional changes. By making the statement that coregulation of genes is a good indication that they respond to the same regulatory pathways, the study of gene expression profiling by microarray allowed to propose a role to previously uncharacterized genes (Chu et al., 1998; Lockhart and Winzeler, 2000; Wen et al., 1998; Cho et al., 1998; Rabitsch et al., 2001; Iyer et al., 1999; Hughes et al., 2000).

In a typic microarray experiment, RNA is first isolated from different tissues (developmental stages, disease states or samples subjected to appropriate treatments). The messenger RNA is then reverse transcribed, labeled with fluorochrome (usually Cy3 or Cy5) and hybridized to the arrays using an experimental strategy that allows expression to be assayed and compared between appropriate sample pairs. The underlying principle of DNA microarray is that the measured hybridization signal for each gene on the chips is proportional to the quantity of mRNA present in the original biological sample. Two different technologies of DNA microarray coexist: i) cDNA microarrays which use spotted cDNA (usually PCR product from 300pb to 1.5kb), ii) oligonucleotide microarrays which use short single stranded DNA spotted on slides (25 bp in the case of Affymetrix microarray). In the case of oligonucleotide array, I will only refer to Affymetrix high density microarray. The strategy of hybridization of the biological samples is different between the two types of microarrays. Indeed, a mixture of differentially labeled samples (for instance wild-type tissue labeled in one color and the mutant tissue in another color) is hybridized on one chip in the case of the cDNA microarray, whereas each sample has to be hybridized separately in the case of oligonucleotides microarray

from Affymetrix (one slide for the wild-type and another slide for the mutant tissue) (Lockhart and Winzeler, 2000; Young, 2000). As a consequence, differential expression between two samples can be directly determined on one slide with cDNA chips, whereas in the case of oligonucleotide microarrays, expression profile is performed *in silico* after scanning and normalization of the two slides.

High density oligonucleotide and cDNA microarrays also differ in the genes spotted on the slides. Indeed, cDNA microarray can contain genes from specific regulatory pathways or tissues, whereas oligonucleotide arrays are directly derived from whole genome data. In consequence oligonucleotide microarrays provide information at the genome-wide scale, but are nevertheless limited by the quality of genome annotation. Uncharacterized genes (or newly characterized genes) are thus probably not present on Affymetrix microarrays. This limitation (which decreases with time and improvement of genome annotation) can be bypassed with cDNA microarrays derived from subtractive libraries which are not dependent on knowledge of the genome.

2 Treatment of data

Microarrays provide a huge amount of data. A critical step is thus to handle efficiently the data to provide an answer as clear as possible to the biologic question.

A Normalization

Before any data analysis, the raw data obtained from hybridization must be normalized to remove any systematical errors and make the data from multiple arrays comparable (Brazma and Vilo, 2000). Different methods of normalization can be used. A first classical method is to normalize the data against all genes on the array. This "global" normalization method use the principle that most of the genes on the chips should not change between the different conditions tested. A second normalization method uses expression of housekeeping genes (usually genes in metabolic pathways) that are supposed to stay at constant level in the different experiments. Finally, "spiked" DNA can be added during hybridization to work as internal control between the different experiments (Quackenbush, 2002; Leung and Cavalieri, 2003).

B Data analysis

Once normalized, microarray data can be compared to one another to provide ratio of expression for a particular gene in two experimental conditions (for instance expression of a gene is upregulated 3 times in the wild-type tissue compared to the knock out tissue). The determination of the threshold for genes with significant increase or decrease level of expression can be determined by statistical analysis of the data. Methods like student's t test, ANOVA, Wilcoxon or Mann-Whitney test, compare data from replicates to rank the genes into their probability (p value) that a difference in

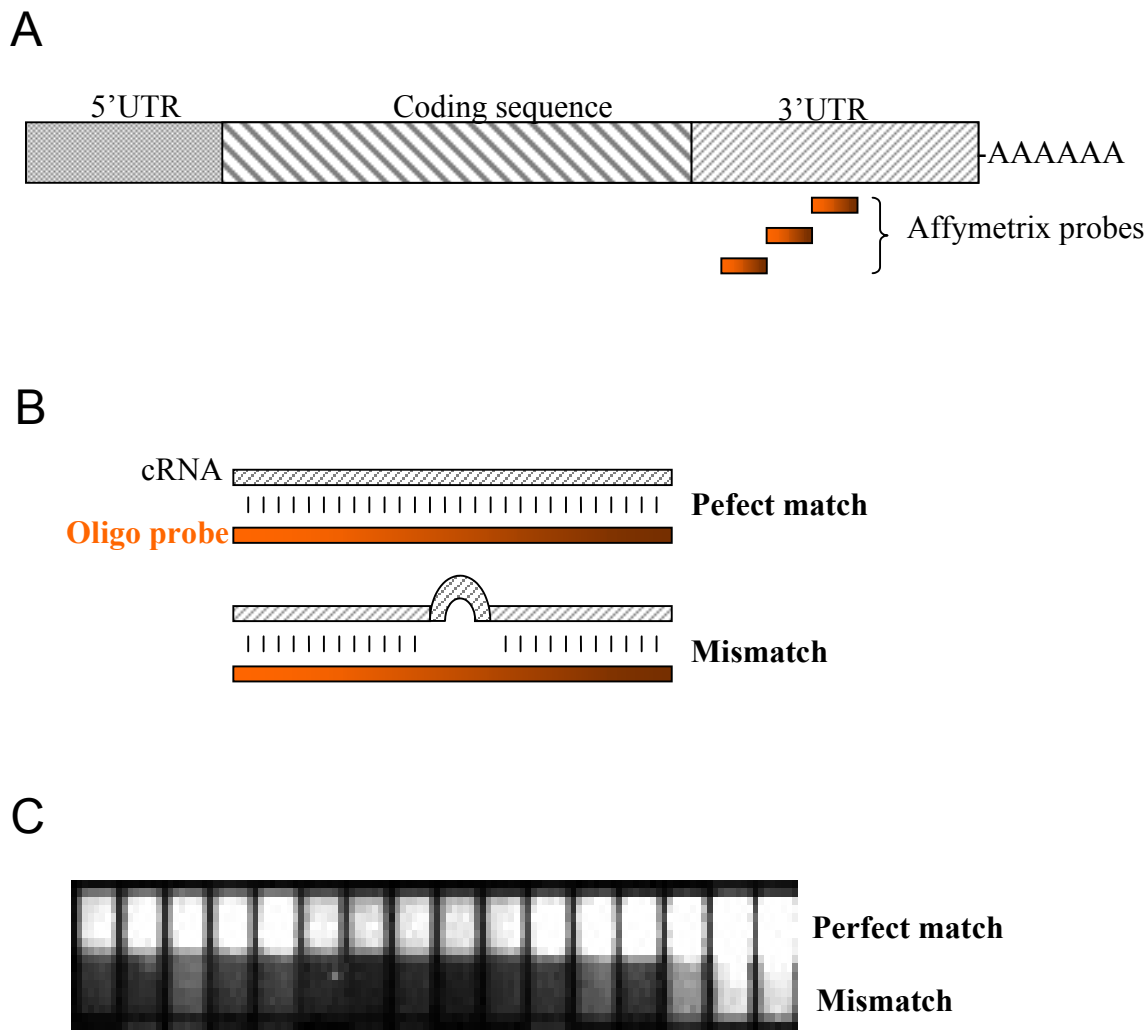


Figure 13: **Schematic representation of the design of Affymetrix probes.** A) Probes on Affymetrix chips (red) are 25 mers oligonucleotides complementary to sequence in the 3' untranslated region (3'UTR) of a given mRNA. 16 different oligonucleotides span the 3'UTR to increase reliability of hybridation data. B) For each oligonucleotide which perfectly match the mRNA sequence (perfect match), an additional oligonucleotide is designed with one central mismatch. In the condition of hybridation the mRNA should not bind to the mismatch oligonucleotide, giving an estimation of the background for each probe. C) Illustration of the signal observed on an Affymetrix microarray with perfect match oligonucleotides on the top and their corresponding mismatch oligonucleotides below.

expression could have been observed by chance (Quackenbush, 2002; Leung and Cavalieri, 2003). These statistical analyses require a significant number of replicates (usually at least three). A common alternative to a statistical approach is to perform only one or two microarrays for each condition and rely on non-statistically motivated criteria such as "2-fold-change" to select genes. This “fold change” approach has the advantage of using very few microarrays, but its sensitivity and reliability are low. A statistical analysis can reveal genes which show small but highly significant changes in expression (Leung and Cavalieri, 2003; Pavlidis, 2003). In the case of Affymetrix microarrays, statistical comparison of test sample with the reference is directly possible without replicate. Indeed, the design of Affymetrix microarray relies on the analysis of 11 to 20 different oligonucleotides for one given gene. The statistical analysis can thus be done between the different oligonucleotides to provide a better confidence of the hybridization signal (see figure 13).
Once the list of genes significantly up or downregulated is established, two strategies of data analysis can be used (Brazma and Vilo, 2000). The first way to analyze this list of candidates is to select genes putatively involved in the biological process of interest, based on gene annotations or level of expression. This supervised analysis is of course limited in the number of genes to analyze, as it relies on the knowledge of the biological process studied (Schulze and Downward, 2001). Another strategy is to use a nonsupervised analysis of the microarray data. In this method, no *a priori* knowledge of the biological system is required. This strategy relies on clustering algorithms that group genes with similar expression profiles (Altman and Raychaudhuri, 2001; Raychaudhuri et al., 2001; Schulze and Downward, 2001; Sherlock, 2000; Brazma and Vilo, 2000; Gaasterland and Bekiranov, 2000). Indeed, if the expression of two genes varies the same way under different experimental conditions, these genes may be regulated by the same signalling pathway and implicated in the same biological process. Two categories of clustering algorithms can be distinguished, the hierarchical and non-hierarchical clustering (Eisen et al., 1998; Tamayo et al., 1999; Tavazoie et al., 1999). Hierarchical clustering works by iteratively joining the two closest gene expression pattern and provides a phylogenetic tree of related clusters (Eisen et al., 1998). In the case of non-hierarchical clustering, the number of cluster has to be determined *a priori*. Expression data are represented as points in a Euclidean space. After the initial partitioning of the vector space into *K* parts (the number of clusters chosen), the algorithm calculates the center points in each subspace and adjusts the partition so that each point is assigned to the cluster which is the closest. This is repeated iteratively until either the partitioning stabilizes or the given number of iterations is exceeded (Brazma and Vilo, 2000; Tavazoie et al., 1999).

3 Microarray and nervous system development

Microarray analysis in neurobiology is without contest one of the most powerful techniques available to explore the molecular mechanisms involved in complex developmental events such as

cell fate commitment, formation of neural connections and behavior. However, the use of microarrays to study the development of the nervous system is also challenging, because of the high cellular heterogeneity of neurological samples and the limited amount of biological material usually available. These practical problems may result in the extreme "dilution" of rare cell type-specific transcript and finally to a lack of sensitivity of the technique. To bypass these difficulties, reductionist approaches can be envisaged. For instance the use of mutant organisms or the study of particular events in cell culture allows to answer to a specific biological question and decreases the complexity of biological systems (Geschwind et al., 2001; Cao and Dulac, 2001; Stathopoulos et al., 2002; Egger et al., 2002; Karsten et al., 2003; Hu et al., 2004). Techniques of cDNA subtraction (for instance, representational difference analysis and suppression subtractive hybridization) can also be envisaged to enrich specific transcripts (Lisitsyn, 1995; Hubank and Schatz, 1994; Diatchenko et al., 1996; Mattar et al., 2004). In addition, these techniques can be used to produce home made cDNA microarrays, in which individual clones from the subtraction are spotted on slides (Geschwind et al., 2001; Dougherty and Geschwind, 2002; Welford et al., 1998). A drastic (and efficient) approach was used by Tietjen *et al* to decrease as much as possible cellular heterogeneity. The authors used microarray to analyse the transcriptome of single neurons by coupling laser-capture of single cells and PCR amplification of cDNA (Tietjen et al., 2003). By this way, the authors investigated the transcriptional program regulated by the proneural gene *Mash1* during the selection of olfactory progenitors by comparing single cell from the developing olfactory epithelium of wild-type and *Mash1-/-* mice. *In situ* hybridization was used to confirm data from microarray and to verify the expression pattern of candidate genes (Tietjen et al., 2003). This last remark raises the importance to confirm microarray data. It is indeed essential to confirm expression data by another technique. Quantitative PCR, western blot and RNA *in situ* hybridization are the main techniques used to confirm microarray data.

IV Problematic

Three proneural genes have been characterised to date in the embryonic mouse telencephalon: *Neurogenin1* and *Neurogenin2* are expressed in progenitors of the cortex, while *Mash1* determines the fate of progenitors in the basal ganglia. In addition, *Mash1* and *Ngns* have been shown to specify neuronal identities in various parts of the nervous system. It seems thus that vertebrate proneural genes have conserved their role in the specification of lineage identity, like in *Drosophila*. How proneural genes regulate these different aspects of neurogenesis is not understood. In addition, the downstream effectors of *Neurogenins* and *Mash1* are basically not known. I focused my thesis on the molecular pathways regulated by *Mash1* and *Neurogenins* during the development of the telencephalon. In particular, the goal of this thesis was to get insights in the following questions:

Do *Neurogenins* have a role in the specification of cortical identity in the telencephalon?

What are the downstream effectors of *Neurogenins* and *Mash1*?

How do proneural genes regulate the activation of a pan-neuronal differentiation program together with neuronal subtype identities?

To answer these questions, I set up a genomic approach to analyse the transcriptome of mouse embryos either lacking or expressing ectopically *Mash1, Ngn1* or *Ngn2*. Five different genotypes were used in the loss-of-function studies: i) *Ngn1* mutant (KO), for which no phenotype was observed in the telencephalon, ii) *Ngn2*KO, which display a respecification of cortical progenitors into a GABAergic fate due to the ectopic expression of *Mash1* in the cortex, iii) *Ngn1*;*Ngn2* double mutant, which have a phenotype in the cortex comparable to *Ngn2* single mutant but exacerbated, iv) *Mash1*;*Ngn2* double mutant (DKO) which show a precocious astrocytic differentiation and a loss of neurons in the cortex, and finally v) *Mash1* single mutant which have lost completely progenitors of the MGE and show premature expression of subventricular zone markers in LGE progenitors.

The cortex of these five mutants was analyzed, as well as the basal ganglia of *Mash1* single mutant. Such strategy has the advantage to decrease the complexity of microarray results, as only particular pathways are analyzed. These different experiments will help to analyze various aspects of the signalling pathways regulated by proneural genes. In the cortex of *Ngn2*KO and *Ngns*DKO we expect to analyze the ectopic role of *Mash1* in the specification of GABAergic specification, a

function that is probably not possible to determine in the basal ganglia of *Mash1*KO embryos, as GABAergic specification still occurs due to compensatory mechanisms. In parallel, the *Ngns*KO should help to determine the molecular pathways implicated in the determination of cortical identity. In the *Mash1;Ngn2*DKO, the general role of proneural genes can be assessed. In addition, these data can help to decipher between genes upregulated in the cortex of *Ngns*KO due to the loss of *Ngn2*, or to *Mash1* ectopic expression in the cortex. I also designed a gain-of-function strategy by electroporation of *Mash1* or *Ngn2* in the telencephalon of mouse embryos. This strategy combining both loss and gain-of-function studies should provide insightful informations on genes regulated either directly or indirectly by proneural genes during neurogenesis of the mouse telencephalon.

The availability of both gain and loss-of-function data should provide a reliable model for the identification of genes regulated by proneural genes in the cortex and in the basal ganglia during the early phase of neurogenesis. In addition, as *Mash1* and *Ngn2* transcriptional programs are investigated, this strategy should help to discriminate between genes involved in pan-neuronal differentiation and neuronal subtype identities.

RESULT S

I Neurogenins are essential for the early phase of cortical specification

Title: Sequential phases of cortical specification involve Neurogenin-dependent and -independent pathways

Published in July 2004 in EMBO Journal

Authors : Schuurmans,C.; Armant,O.; Nieto,M.; Stenman,J.M.; Britz,O.; Klenin,N.; Brown,C.; Langevin,L.M.; Seibt,J.; Tang,H.; Cunningham,J.M.; Dyck,R.; Walsh,C.; Campbell,K.; Polleux,F.; Guillemot,F.

Problematic: Previous studies in the telencephalon showed a proneural role of *Ngns* but there was no evidence that *Ngns* are required for the specification of cortical identity. In this paper we investigated the role of *Neurogenins* in the specification of cortical neurons during neurogenesis by analyzing the cortex of embryos and newborn mice mutant for *Ngns*.

Contribution: We analyzed the role of *Ngn1* and *Ngn2* in specifying the identity of neocortical neurons. In this study, cortex and basal ganglia were dissected from E13,5 proneural mutant and wild-type mice embryos. I prepared total RNA from each dissected tissue and polled three to five basal ganglia or cortex of the same genotype to obtain a minimum of 10µg of total RNA required for hybridization to Affymetrix microarray. Hybridizations to Affymetrix microarrays (U74v2 A and B) were performed as follows: 4 replicates for WT cortex, duplicates for *Ngn1*KO, duplicates for *Ngn2*KO, one hybridization for *Mash1;Ngn2*DKO and one for *Ngn1;Ngn2*DKO embryos. After data normalization and comparative analysis with the GenChip Affymetrix suite, I analysed the genes regulated in the cortex of these various mutants. The microarray analysis was the starting point of this paper which demonstrated that distinct genetic programs operate at different stages of corticogenesis to specify the identity of cortical neurons.

Conclusion of the paper: *Neurogenins* do have a role in specification of the identity of early born cortical neurons. In particular, *Ngns* are essential for the specification of glutamatergic excitatory neurons of cortical layers V and VI.

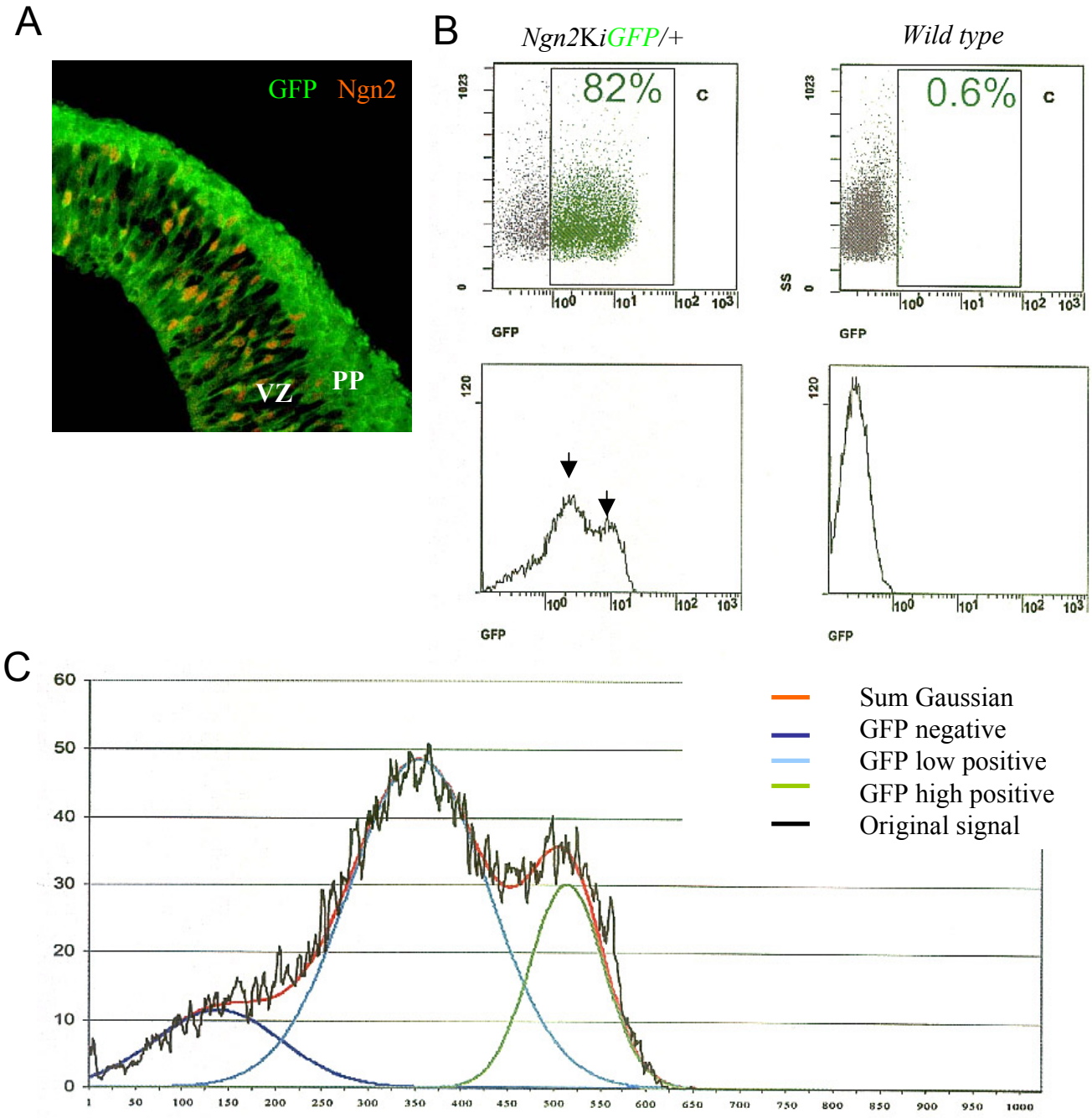


Figure 1: Two populations of *Ngn2*Ki^{GFP} cells in the embryonic cortex. A) Double immunofluorescence on E12.5 sections showing GFP (green) and Ngn2 (red) labelling. All Ngn2 positive cells are also GFP positive in the VZ (yellow), showing that GFP co-localize with Ngn2 protein in the VZ. In contrast, postmitoc neurons in the preplate (PP) are Ngn2 negative and GFP positive, probably as a consequence of the lower turn-over of GFP compared to Ngn2 proteins. B) Flow cytometry on dissociated cortical cells from E12.5 heterozygotes *Ngn2*Ki$^{GFP/+}$ and wild type embryos. Two populations of GFP positive cells are distinguished in heterozygotes embryos (indicated by the black arrow): a low and a high GFP expressing cells. C) The GFP pattern observed (black line) can be mimicked by the sum of three different Gaussian populations (red line) corresponding to GFP negative cells (dark blue), low GFP positive cells (light blue) and high GFP expressing cells (green).

II Analysis of the molecular pathways regulated by proneural genes

Although *Ngns* and *Mash1* are implicated in the specification of neuronal identities in the telencephalon (Fode et al., 2000; Parras et al., 2002; Schuurmans et al., 2004), the downstream effectors of proneural genes are still largely unknown. To address this question, I used a microarray approach to characterize genes regulated by *Ngns* or *Mash1* in the developing telencephalon. I will first expose the preliminary results obtained by the screening of a cDNA subtracted library. Then, I describe data obtained with Affymetrix microarrays in order to highlight transcriptional pathways regulated by proneural genes. Finally, I propose a strategy to identify genes directly regulated by *Mash1* or *Ngn2* during the development of the mouse telencephalon and show evidence that *Dll1* is a direct target of proneural genes.

1 Identification of proneural genes effectors by screening a subtractive cDNA library

To identify genes regulated by *Ngn2* or *Mash1* and find new genes implicated in the acquisition of cortical or sub-cortical fate, I designed a subtractive screen between *Ngn2*KO (*Ngn2* single mutant) and wild-type embryos. In this method, cDNA differentially expressed between two tissues (*Ngn2*KO and wild-type) are selectively enriched, while genes expressed at similar levels are subtracted. In the cortex of *Ngn2*KO embryos at embryonic day 12.5, glutamatergic cortical cells are respecified into a GABAergic phenotype as a consequence of *Mash1* ectopic expression in the cortex. The subtraction between wild-type and *Ngn2*KO cortical cells should thus yield genes implicated in cortical development, while the converse subtraction, *Ngn2*KO minus wild-type, enriched in gene upregulated by the ectopic expression of *Mash1*, should yield genes implicated in GABAergic differentiation.

A Construction of a subtractive library

a Cell sorting by flow cytometry

Together with Olivier Britz and Carol Schurmanns (a former post-doctoral fellow of the laboratory), I dissected E12.5 telencephalic vesicles from heterozygous (i.e. wild-type) and *Ngn1*;*Ngn2* double mutants embryos. I took advantage of the Green Fluorescent Protein expression from the *Ngn2* locus to sort mutant and wild-type cells (hetorozygous $Ngn2^{+/GFP}$) in the *Neurogenins* lineages. At embryonic day E12.5, *Ngn2* proteins and transcripts are detected in the VZ whereas GFP positive cells are located in the VZ and in postmitotic neurons in the preplate (figure 1). The detection of GFP in postmitotic cells that do not normally express *Ngn2* is attributed to a slower turnover of GFP.

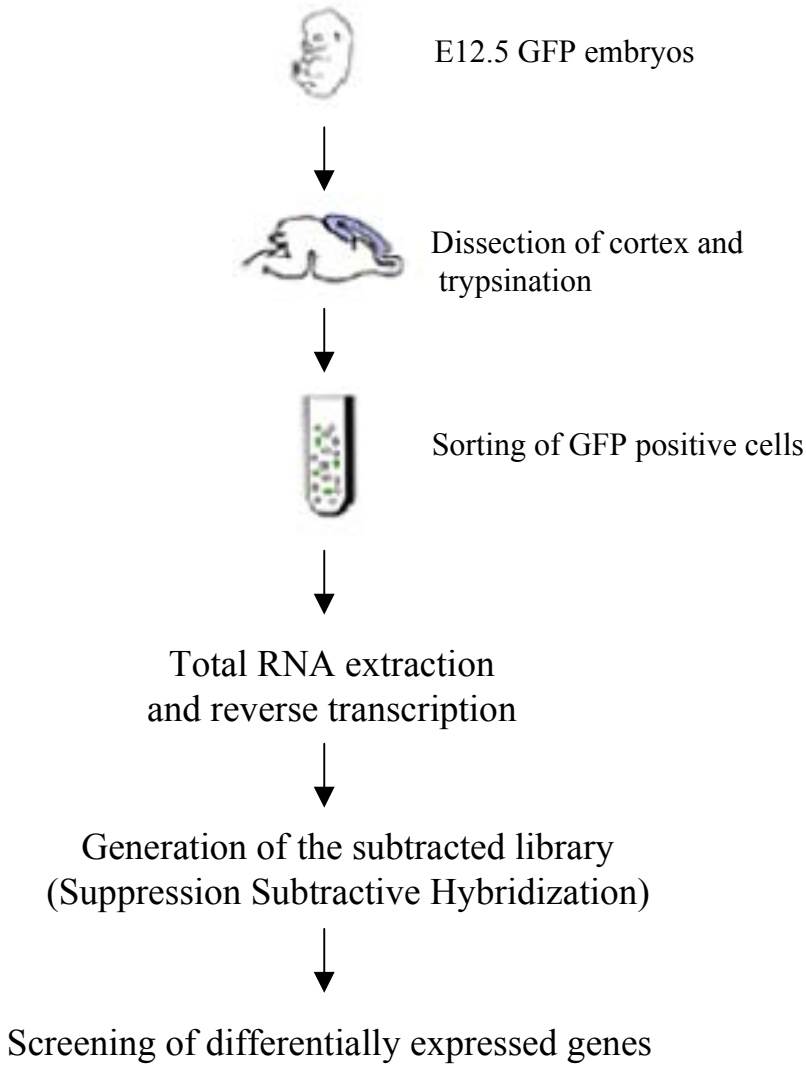
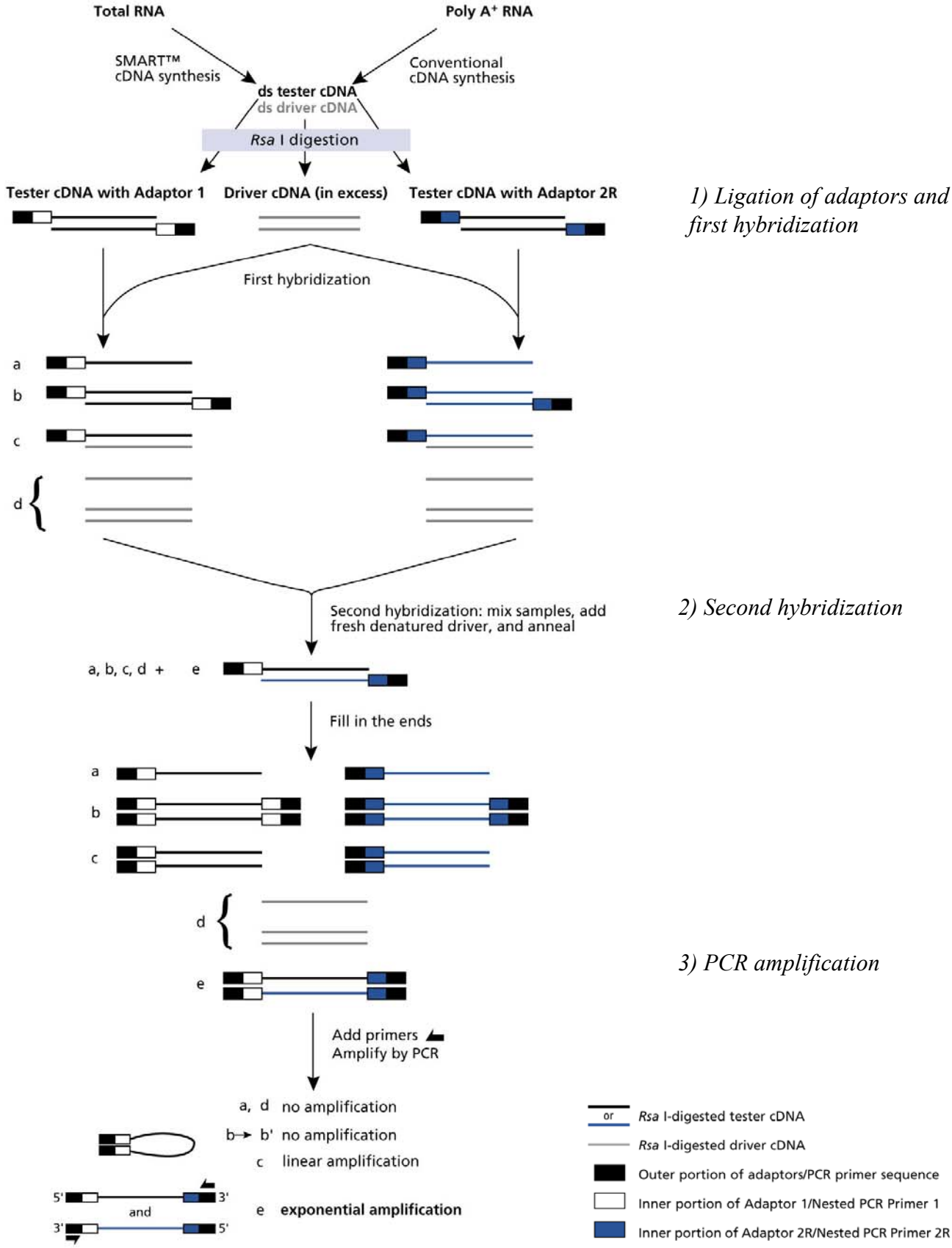


Figure 2 : Construction of the subtractive libraries. GFP positives cells from *Ngn1*$^{-/-}$;*Ngn2*$^{GFP/GFP}$ double mutants (*Ngns*DKO) and *Ngn1*$^{+/+}$;*Ngn2*$^{GFP/+}$ (ie control) were sorted by flow cytometry. Total RNA from 30.000 sorted cells was extracted and reverse transcribed. Two rounds of subtraction between control and *Ngns*DKO were done to generate the subtractives libraries (see figure 3).



1) Ligation of adaptors and first hybridization

2) Second hybridization

3) PCR amplification

Figure 3:

For each dissected embryos GFP cortical cells were sorted by flow cytometry (FACS). Analysis of GFP cells by FACS revealed the presence of two cell populations at E12.5, one expressing high level of GFP and one expressing low level of GFP (figure 1B and 1C). By analyzing GFP populations by flow cytometry at different stages (E11.5, E12.5, E13.5 and E15.5), Olivier Britz observed that the relative proportion of low and high level expressing GFP cells changes as development progress, with an increase of high level expressing cells at the expense of the other in late stages. Knowing that the pick of *Ngn2* expresion in the cortex is near E13.5 and that GFP accumulate in postmitotic cells, these data suggest that low GFP expressing cells correspond to progenitor cells in the VZ and high GFP population to postmitotic neurons. I thus sorted by flow cytometry only the low GFP expressing cells, yielding 30.000 cells for each embryo. Total RNA was extracted immediately after sorting.

b Subtraction

Total RNA from $Ngn1^{-/-}$;$Ngn2^{GFP/GFP}$ double mutants and $Ngn1^{+/+}$;$Ngn2^{GFP/+}$ (ie control) sorted cells were used for cDNA synthesis and amplified by PCR (see Materials and Methods, Construction of the subtracted library, p67). I then realized two rounds of subtraction in both directions to generate the libraries {control minus *Neurogenins* mutants} and {*Neurogenins* minus control cells}, enriched in wild-type or *Neurogenin* mutants specific genes respectively (figure 2 and figure 3).
In order to assess the quality of enrichment in each subtraction, cDNA from each subtraction were labelled with dCTP-P^{32} and used as probe for the detection of reference genes. The subtraction

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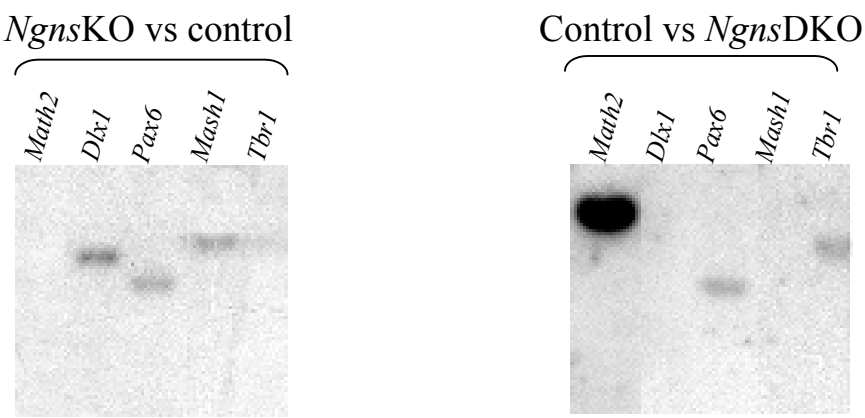
Figure3: Method of subtraction by Supression Substractive Hybridization (SSH). Two populations of cDNA (wild-type and *Ngns*KO for instance) are used alternatively as driver or tester to realize the subtraction in both directions (wild-type minus *Ngns*KO, and *Ngns*KO minus wild-type). Step 1) The cDNA in which specific transcripts are to be found is called “tester,” and the reference cDNA is called “driver.” The tester and driver cDNAs are digested with Rsa1 which yields blunt ends. The tester cDNA is then subdivided into two portions, each of which is ligated to a different ds cDNA adaptor. Each tester is then hybridized separately with an excess of driver (which is not ligated to adaptors). cDNA in common between the driver and the tester hybridize together (type c of molecules), while differentially expressed genes are single stranded (population a). Step 2) The two primary hybridization (which differ by the adaptor ligated to the cDNA) are mixed and hybridized with fresh denature driver. The type a population can now associate with the complementary tester to form a double stranded cDNA with different adaptor sequence on each end (population e). Step3) After filling the end of each adaptors, only the type e molecules can be exponentially amplified by PCR, allowing the selective enrichment of differentially expressed genes.

A



B

Function/Category	***Ngns*DKO *vs* Ctrl (%)**	**Ctrl *vs* *Ngns*DKO (%)**
Role in neurogenesis	2.4	4.9
Transcription factor	1.2	2.9
Cell cycle	2.4	0
Growth factor	3.6	0
Metabolism/structural	10.8	12.6
Signalling	1.2	1
Transport	3.6	0
Other	12	20.4
EST	22.9	32
Genomic DNA	39.9	27.2

Figure 4 : Validation of subtractive libraries. A) Subtracted cDNA pooled were radioactively labeled and hybridized to membranes on which equivalent quantity of control plasmids were blotted. Ventral markers (*Mash1* and *Dlx1*) are enriched in *NgnsD*KO vs Control (Ctrl) subtraction (left). Cortical markers (*Math2* and *Tbr1*) are enriched in Control vs *Ngns*DKO subtraction (right). B) 100 genes from each subtractive library were randomly sequenced. The table provide the % of genes in each categories.

{*Neurogenins* minus wild-type cells} is enriched in ventral specific genes (*Mash1*, *Dlx1*) and is depleted of cortical markers (*Math2*, *Tbr1*) (figure 4A). In contrast, the subtraction {control minus *Neurogenins* mutants} yielded an expected increased in hybridization to cortical markers like *Math2* and *Tbr1*, whereas ventral markers *Dlx1* and *Mash1* were downregulated (figure 4A). These data confirm that the subtraction lead to the enrichment of expected genes. The cortical regionalization gene *Pax6* is present at equivalent level in both libraries.

c Subtractive libraries

Subtracted cDNA from each {wild-type minus *Neurogenins* mutants} and {*Neurogenins* minus wild-type cells} subtractions were cloned in PCR2.1 (Invitrogen) to generate two libraries enriched in genes putatively regulated by *Neurogenins* or *Mash1* respectively. I performed a random sequencing of 100 clones from each library in order to assess the degree of redundancy in each library. Seven cDNA in {wild-type minus *Neurogenins* mutants} library and two in {*Neurogenins* minus wild-type cells} library were found more than once. A classification of sequenced clones based on their function is provided in figure 4B. Proportion of clones corresponding to genomic sequence is relatively high (27% and 40%) suggestive of a possible genomic DNA contamination in the two subtractive libraries.

B Screening by cDNA microarray

a Spotting of the Neural4k cDNA microarray

I amplified by PCR the cDNA inserts of 2000 clones from each library. After purification and quantification of the PCR products, the 4000 cDNA were spotted in triplicates on glass slide in collaboration with Bernard Jost and Phillipe Gerber (IGBMC, microarray facility, Strasbourg France) to produce a home made cDNA microrray. This "*Neural4k*" chip should be enriched in genes and EST regulated by proneural genes and potentially involved in neuronal specification and/or differentiation.

b Microarray hybridization

I used the *Neural4k* microarray to compare WT and *Ngn2*KO cortical tissue. Briefly, cortices of E13.5 embryos were carefully dissected to remove adjacent tissue, including pia, and processed individually for total RNA extraction directly after dissection. Three to five RNA samples of each tissue were pooled to reach the minimal requirement of 10µg of total RNA needed for cDNA synthesis and to average biological variability among samples. cDNA were labelled with Cy3 or Cy5 fluorochrome in collaboration with Christine Bole-Feysot **(**IGBMC, microarray facility,

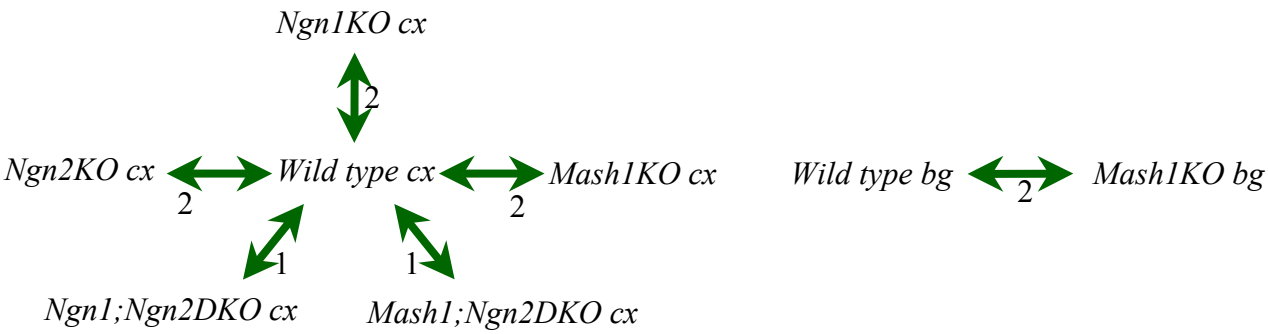
Strasbourg France). *Ngn2*KO samples were hybridized to microarray in duplicates by swapping the fluorochromes with the reference (flip-flop).
A total of 743 cDNA were significatively up or downregulated. I selected 200 genes (the 100 cDNA most up and downregulated) and sequenced them to identify the cDNA. Preliminary data show that among the 100 upregulated cDNA, 8 cDNA were found more than once. Among the genes represented only once, 44 were previously characterized in the mouse, 27 matched EST and 12 correspond to chromosomic DNA. Among the upregulated genes are found the LIM only transcription factor *Crip2*, expressed in the brain and possibly involved in the dynamics of actin cytoskeleton (van Ham et al., 2003), *Otx1*, involved in neuronal differentiation in the cortex (Panto et al., 2004; Acampora et al., 1998), the neuronal microtubule associated protein (Teng et al., 2001; Tucker, 1990), the cell cycle regulators *cyclinB1* and *cdc20* (Lin et al., 2003a) and miscellaneous genes like the maternally expressed genes *Meg3* or *transthyretin* (Herbert et al., 1986).

This subtractive screen allowed the detection few genes involved in neurological processes. Indeed, based on available annotations, 5% of sequenced genes are expressed in neuronal populations during development. In addition, transcription factors such as *Dlx1*, *Mash1*, *Ngn2*, *Nscl2* and *Math2*, misregulated in the cortex of *Ngn2*KO embryos (Fode et al., 2000), were not found among the 200 clones selected. It would be also important to hybridize on the *Neural4k* microarray the labeled cDNA obtained from each subtraction in order to detect genes expressed at low levels in the cortex. Further analyses are required to confirm the expression of candidates found in this screen. Characterization of ESTs and confirmation of regulation in *Ngn2*KO embryos by RNA *in situ* hybridization are under investigation.

2 Comparative analysis of proneural mutant mice with Affymetrix microarray

The data obtained with the subtractive library described in the precedent section (see, Identification of proneural genes effectors by screening a subtractive cDNA library, p36) are limited and need further analysis (for instance larger number of genes sequenced, hybridization with other probes). In order to have rapid insight in the pathways controlled by proneural genes, I used high density microarrays from Affymetrix. I compared the transcriptional program of wild-type and proneural knock out embryos at embryonic stage E13.5. At this stage *Mash1* and *Neurogenins* are expressed in the ventricular zone (VZ) to specify neuronal population in the cortex and in the basal ganglia respectively, and postmitotic neurons accumulate in the cortical and in the mantle zone. Genes either involved in neuronal determination or in neuronal differentiation are thus expected to be misregulated in proneural mutant embryos at E13.5.

A



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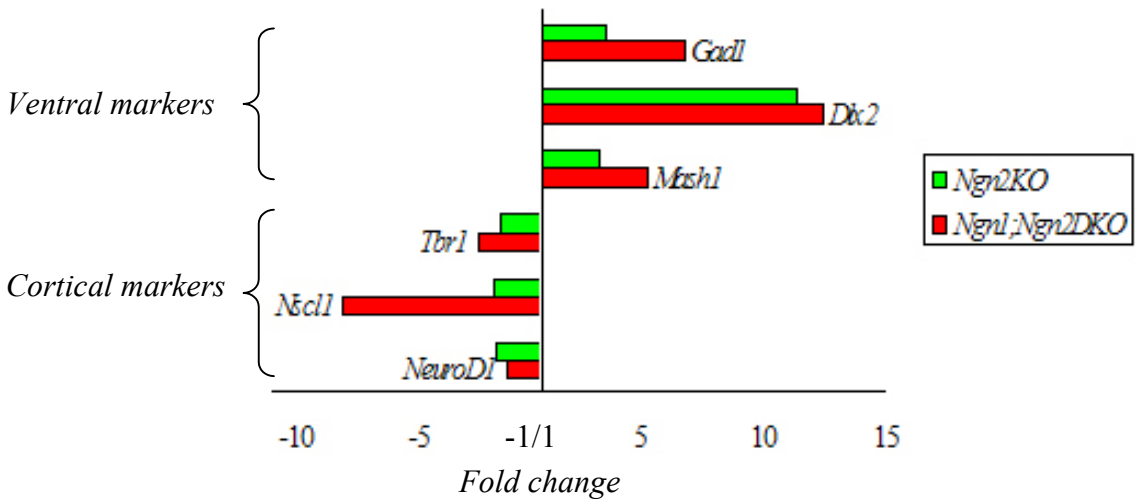


Figure 5: **Cortical markers are downregulated and ventral markers upregulated in the cortex of *Ngns*KO embryos.** A) Genotypes and tissues compared with Affymetrix microarrays. Number of replicates realized are indicated under each arrow. B) Regulation of ventral (*Gad1*, *Dlx2*, *Mash1*) and cortical (*Tbr1*, *Nscl1*, *NeuroD1*) markers in *Ngn2* single and *Ngn1;Ngn2* double mutant. KO: mutant, DKO: double mutant, cx: cortex, bg: basal ganglia.

Cortex and basal ganglia from E13.5 mice embryos were carefully dissected to remove adjacent tissues, including pia, and processed individually for total RNA extraction directly after dissection. Three to five RNA samples of each tissue were pooled to reach the minimal requirement (at the time) of 10µg of total RNA needed for cRNA synthesis and to average biological variability among samples (see Material and Methods, RNA extraction and hybridization to Affymetrix microarray). In total, five different genotypes (the single knock out *Mash1*KO, *Ngn1*KO, *Ngn2*KO, and the double knock out *Ngn1*;*Ngn2*DKO and *Ngn2*;*Mash1*DKO) and two different tissues (cortex and basal ganglia) were analyzed with Affymetrix U74v2A and U74v2B microarrays, to analyse the expression of 24833 genes or EST (Expressed sequence tag). Comparative analysis between wild-type and mutant tissue (cortex or basal ganglia) is described in figure 5A.

A Regulation of reference genes

To validate the experimental design of the microarray experiments, I first checked the expression of genes known to be misregulated in embryos lacking *Ngns* and/or *Mash1* expression. Previous studies showed, using RNA in situ hybridization, that expression of cortical markers like *NeuroD*, *Tbr1*, *Nscl* are downregulated in the cortex of *Ngn2*KO and *Ngn1;Ngn2*DKO (*Ngns*DKO) embryos, while genes normally expressed in the basal ganglia (*GAD*, *Mash1*, *Dlx genes*) are upregulated (Fode et al., 2000). The microarray data indicated that expression of *NeuroD*, *Tbr1* and *Nscl1* are downregulated significatively in *Ngn2*KOand *Ngns*DKO, whereas *Mash1, Dlx2* and *GAD1* are strongly upregulated in the cortex of *Ngns* knock out mice (figure 5B). These results validate the reliability of microarray data. Notably, increases of striatal markers and decreases of cortical genes are more drastic in *Ngns*DKOcompared to *Ngn2*KO, suggesting a more drastic phenotype in the double mutant. This observation confirms the previous finding that *Ngn1* can partially compensate for the loss of *Ngn2* in the cortex and indicates also that Affymetrix microarrays provide reliable quantitative data (Fode et al., 2000).

B Clusterization of microarray data

Microarray data obtained from six different genotypes (*Mash1*KO, *Mash1*;*Ngn2*DKO, *Ngn1*KO, *Ngn2*KO, *Ngn1*;*Ngn2*DKO and wild-type) are complex to analyze. To facilitate the analysis, I performed a hierarchical clustering of data obtained from mutant and wild-type cortical tissue together with data from basal ganglia of wild-type embryos, because many neurons in *Ngn* mutant cortices acquire a basal ganglia-like identity (Fode et al., 2000; Parras et al., 2002; Schuurmans et al., 2004). Genes analyzed by clustering were selected on the basis of four different criteria: i) genes must be detected significatively at least one time, ii) “absolute call” (see Material and Methods, Statistic analysis of Affymetix microarray data, p66) must be superior to 50 in at least one experiment, iii) “average difference” (see Material and Methods, Statistic analysis of Affymetix

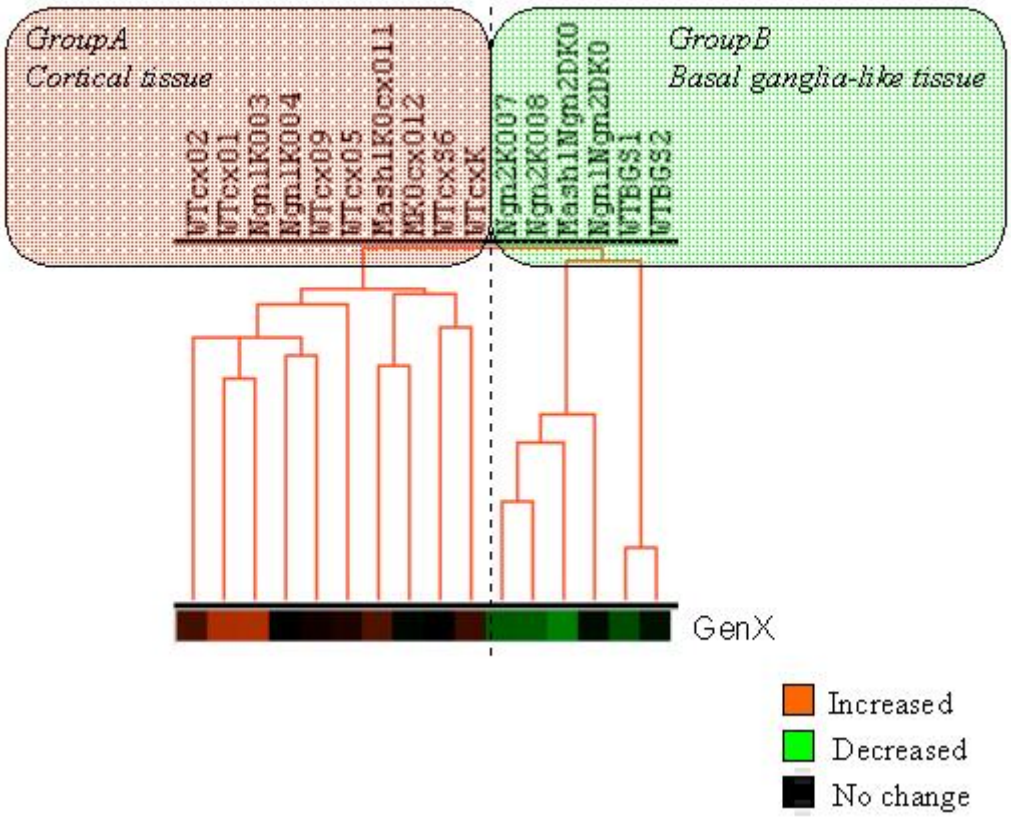


Figure 6: **Two groups of genotypes are distinguished by clustering cortical microarray data.** After hierarchical clustering of microarray data, cortical tissues from wild type, *Ngn1*KO and *Mash1*KO embryos are clustered together (groupA). Wild type basal ganglia and cortex of *Ngns* mutants form a distinct group (groupB). WT: wild-type, cx: cortex, BG: basal ganglia, KO: mutant.

microarray data, p66) must be significant in at least two out of the four comparative analysis possible for duplicate experiments, iv) fold change must be superior or equal to 1.5 times. A total of 1622 genes were selected based on those criteria and subjected to hierarchical clustering using the average linkage method (Cluster3.0, M. de Hoon and Eisen, (Eisen et al., 1998)).

a Comparison of expression profiles of the different genotypes

Data from clustering were first analyzed by assessing the degree of similarity between the different genotypes. In this type of analysis, expression profiles obtained from the different genotypes were compared to each other to determine which are more closely related (vertical phylogenetic tree). As described in figure 6, the genotypes segregate into two distinct groups. **Group A** is constituted by cortical samples obtained from wild-type, *Ngn1*KO and *Mash1*KO. In this group, the expression profile of *Ngn1*KO and *Mash1*KO cortical tissues are very similar to wild-type cortical tissue, suggesting that those two mutants have none or very subtle role in the cortex. Indeed, only 23 and 70 genes are misregulated in the cortex of *Ngn1*KO and *Mash1*KO embryos, respectively (annex1 and annex2). **Group B**, is constituted by samples from wild-type basal ganglia, *Ngn2*KO cortex, *Ngn1*;*Ngn2*DKO cortex and *Mash1*;*Ngn2*DKO cortex. The presence of *Ngns*KO in this group can most probably be explained by the loss of cortical markers in these mutants and by the respecification of cortical progenitors into a GABAergic fate, the main neuronal phenotype in the basal ganglia (Fode et al., 2000). In the case of the cortex of *Mash1;Ngn2*DKO embryos, cortical markers are also decreased and several basal ganglia determinants (such *Dlx1* and *Dlx2*) are still upregulated in the mutant cortex even in the absence of *Mash1* (Fode et al., 2000; Schuurmans et al., 2004), which explain the presence of this genotype in **Group B**.

b Characterization of Ngn2 and Mash1 specific cluster

Analysis of clustering results allowed the identification of two main clusters of genes. The first cluster (Cluster1) comprises 285 genes highly expressed in the basal ganglia compared to cortex in wild-type embryos, and strongly upregulated in the cortex of *Ngn2*KO and *Ngn1*;*Ngn2*DKO. Such pattern of expression is characteristic of striatal differentiation genes upregulated in the cortex of *Ngns* mutants. Indeed, the full GABAergic differentiation pathway regulated by *Mash1* is found in Cluster1, including *Mash1*, *Dlx1*, *Dlx2*, *Dlx6,* and the GABA synthesizing enzyme *GAD1* and *GAD2* (figure 7A) (Casarosa et al., 1999; Fode et al., 2000; Stenman et al., 2003; Stuhmer et al., 2002a; Stuhmer et al., 2002b; Anderson et al., 1997b; Alifragis et al., 2004; Cannizzaro et al., 2003). Other genes present in this cluster include transcription factors expressed in the basal ganglia such as *Lhx6*, *ER81*, *Pbx3* (Stenman et al., 2003; Alifragis et al., 2004; Toresson et al., 2000a), the potential target of *Dlx* genes, *Arx* (Cobos et al., 2005), the SRY box transcription factor *Sox6*, involved in neuronal differentiation of P19 cells (Hamada-Kanazawa et al., 2004), the brain specific

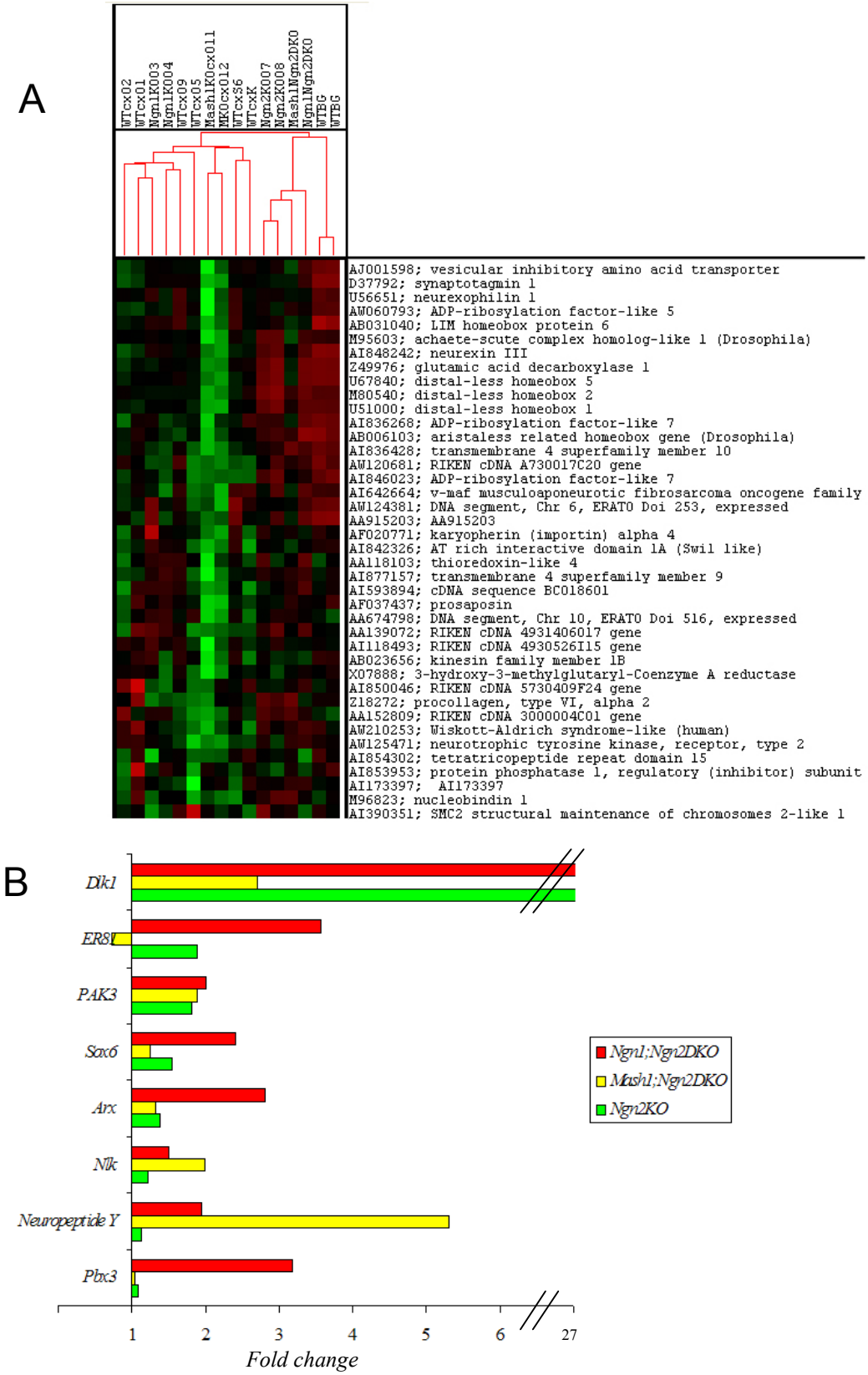


Figure 7: **Characterization of a *Mash1* specific cluster.** A) Part of cluster1 composed of 285 genes in total. Genes expressed at high level in the basal ganglia and upregulated in the cortex of *Neurogenines* mutant compared to wild type cortical tissue are present in this cluster. B) Fold change of some genes upregulated in the cortex of *Ngns* mutants embryos and present in cluster1.

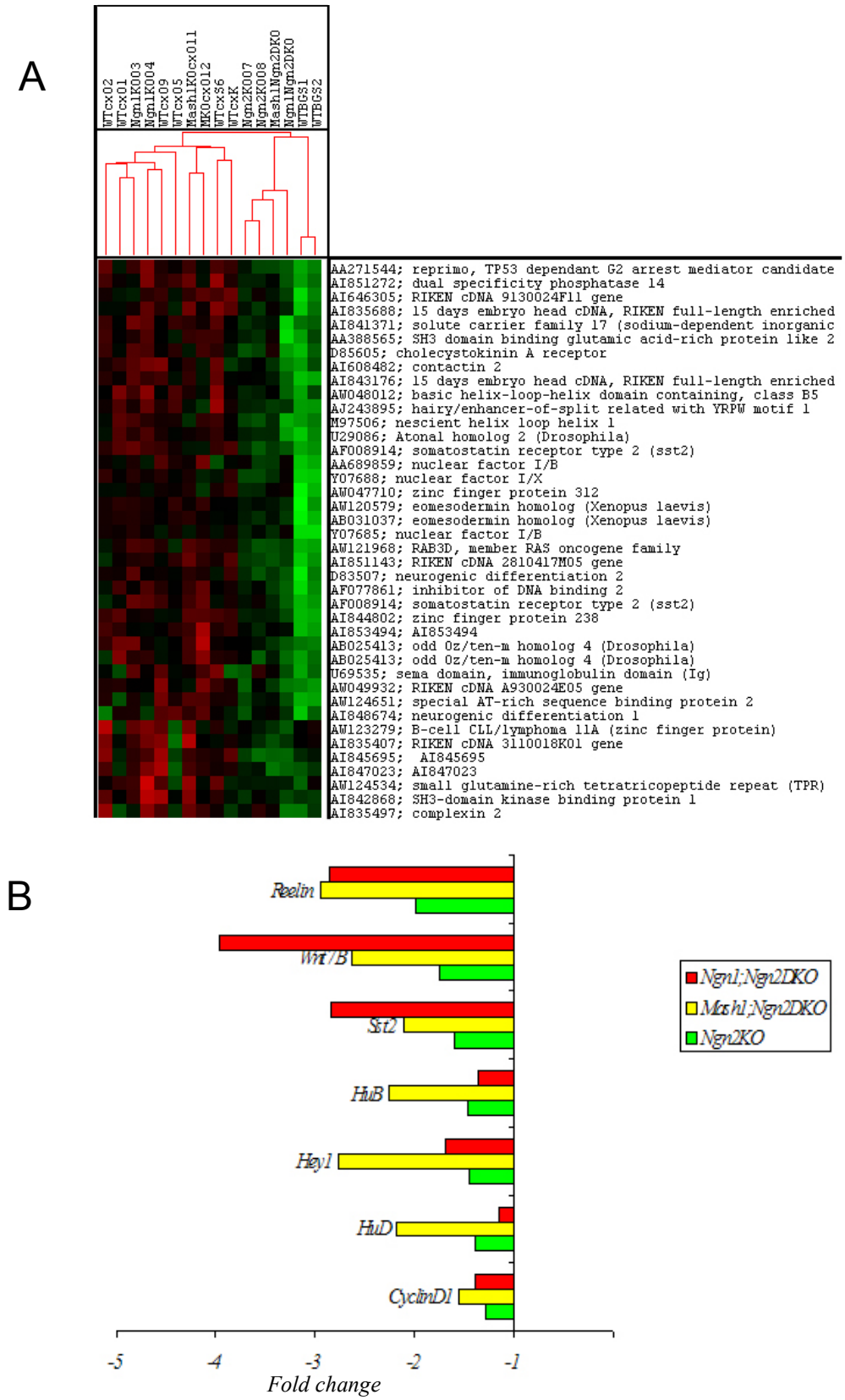


Figure 8: **Characterization of a *Ngn2* specific cluster.** A) Part of cluster2 composed of 277 genes in total. Genes expressed at high level in the cortex and downregulated in the cortex of *Neurogenins* mutants compared to wild type cortical tissue are present in this cluster. B) Fold change of genes upregulated in the cortex of *Ngns* mutants embryos and present in cluster2.

cell cycle inhibitor PAK3, neuropeptideY (Baraban and Tallent, 2004) and signalling genes like the modulator of *Wnt* signalling *Nlk* and the EGF like repeat-containing protein *Pref-1*/*Dlk1* (figure 7B) (Moon et al., 2002; Boda et al., 2004; Thorpe and Moon, 2004).

The second main cluster (Cluster2) is composed of 277 genes strongly expressed in the cortex compared to basal ganglia in wild-type mice, and downregulated in the cortex of *Ngn2*KO embryos. This profile of expression is characteristic of genes potentially regulated by *Ngn2* in the cortex. Cortical markers like the transcription factors *Math2*, *NSCL1*, *Tbr2* (*eomesodermin homolog*), *Tbr1*, *NeuroD1* are present in this cluster (figure 8A) (Kruger et al., 2004; Hevner et al., 2001; Englund et al., 2005). Also found in Cluster2 are the cell cycle regulators *p57* and cyclinD1, as well as *reelin* essential for proper cortical lamination (Ogawa et al., 1995), the RNA binding protein *HuB* and *HuD*, implicated in neuronal differentiation (Kasashima et al., 1999), the bHLH transcription factors *bhlhb5/Beta3* and the mediator of *Notch* signalling *Hey1* (figure 8B) (Belandia et al., 2005). The complete list of genes in Cluster1 and Cluster2 is presented in Annex3 and Annex4 respectively.

C In situ screen of genes regulated by proneural genes

Based on the microarray data and the subsequent hierarchical clustering, I generated a list of candidates to include in a RNA *in situ* hybridization screen for the characterization of downstream effectors of *Mash1* and *Ngn2* in the telencephalon. Indeed, confirmation of microarray data is necessary and study of expression pattern of candidates should allow the descrimination between generic and subtype specific determination or differentiation genes. A total of 36 genes were selected based on their function (transcription factors and signalling proteins mainly) and/or regulation in the various proneural mutant embryos (listed in table1). Expression patterns were examined by RNA *in situ* hybridization on wild-type E13.5 sections. At this stage the VZ and SVZ are formed in the basal ganglia as well as in the lateral region of the cortex, and postmitotic neurons start to differentiate in the CP of the cortex and in the MZ (mantle zone) of the ganglionic eminences. As *Ngn2* and *Mash1* expression is restricted in the VZ of the cortex and the basal ganglia respectively, downstream effectors of proneural activities are expected to have partial overlapping expression with proneural genes. This criterion is important as gene expression lost in postmitotic neurons in the CP or in the MZ can be a consequence of the loss of neurons in proneural mutant embryos.

a Pattern of expression of candidate genes in wild-type embryos

From the 36 selected genes, 6 were not detected on wild-type section at E13.5, although a present call was found by Affymetrix detection algorithm in at least one wild-type tissue. Among the 30 remaining genes, 12 were found to be regionally expressed in the wild-type cortex or in the basal

Acc#/Id	*Description*	*WT*	*Ngn2*KO	*Mash1*KO
MIBP	TF	cx CP	↓cx CP (-3X)	No change
NM029758	Unknown	cx CP	↓cx CP (-2X)	No change
L1CAM	Adhesion	cx CP, bg MZ	↓cx CP (-1,8X)	No change
BC052038	Unknown	cx CP	↓cx CP (-1,5X)	No change
AI840429	Unknown	cx CP, bg VZ/MZ	↓cx CP (3X)	Not checked
NM172710	Unknown	cx CP, bg SVZ	↑cx SVZ (1,7X)	No change
Gadd45g	Apoptose	cx VZ, bg VZ	↑cx VZ (1,5X)	Not checked
AI842002	Unknown	cx CP, bg /MZ	↑cx VZ (2X)	Not checked
Meg3	Unknown	cx CP, bg MZ	↑cx CP (5X)	No change
Arx	TF	cx VZ, ge VZ/MZ	↑cx CP (1,3X)	Not checked
NG2	Adhesion	cx CP, ge MZ	↑cx CP (1,4X)	Not checked
Sialyltransferase	Enzyme	cx VZ/CP, bg MZ	↑cx CP (2X)	Not checked
Pref-1/Dlk1	signal transduction	bg VZ/MZ	↑cx SVZ (27X)	↓bg VZ (-1,2X)
Nlk	kinase	bg SVZ/MZ	↑cx CP (4X)	↓bg MZ (-1,3X)
Sox6	TF	cx VZ, bg MZ	No change	↓bg MZ (1,5X)
Nrarp	TF	cx VZ, bg VZ	Not checked	↓bg VZ (2X)
Gp38	Adhesion	bg VZ	No change	↓bg VZ (-2,5X)
Supth16	Unknown	cx SVZ, bg SVZ	No change	↑bg SVZ (2X)
Peg1	Enzyme	bg MZ	No change	↑bg VZ (2,5X)
Asb4	Unknown	bg MZ	No change	↑bg VZ (2,4X)
Peg3	TF	bg SVZ	No change	↑bg VZ (3X)
Tssn1	Unknown	bg SVZ	No change	↑bg VZ (1,3X)
mEvf1	Unknown	bg MZ	cx CP (3X)	↑bg VZ (not available)
Lhx6	TF	bg MZ	No change	↑bg VZ (1,3X)
AA673799	Unkown	cx VZ, bg VZ	No change	Not checked
IA1	TF	cx VZ, ge VZ/MZ	No change	Not checked
Brn4	TF	cx VZ, ge VZ	No change	No change
Sox9	TF	cx VZ, ge VZ	No change	No change
Ayk1 (AuroraA)	Cell cycle	cx VZ, ge VZ	No change	No change
AW258842	Unkown	cx pp, bg MZ	No change	Not checked
AA691557	Unkown	not detected		
Zic1	TF	not detected		
Phdtf	TF	not detected		
BC059223	Unknown	not detected		
Ebfaz	TF	not detected		
AK081204	Unknown	not detected		

Table 1: **Summary of genes expressed in the E13.5 telencephalon.** Description of changes in the expression patterns of regionalized genes in *Ngn2* and *Mash1* mutants at E13.5. Fold change from Affymetrix data is indicated in brackets. VZ, ventricular zone; SVZ, subventricular zone; CP, cortical preplate; MZ, mantle zone; cx, cortex, bg, basal ganglia; TF, transcription factor.

ganglia (see table1). The transcription factor MIBP1 and two genes with unknown function (Gen Bank accession number NM_029758 and BC052038) are specifically expressed in the cortex in the preplate or in the SVZ (see figure 9). In contrast, the transcription factors *Lhx6*, *Peg3*, the modulator of Wnt signalling *Nlk*, and various genes with unknown function in the brain including *Asb4*, *Peg1*, *Tssn1, mEvf1* and *Dlk1* are expressed specifically in the SVZ and/or MZ of the basal ganglia (figure 9). Another category comprises 14 genes expressed in both cortex and ventral forebrain. Among them are the cell adhesion molecule L1CAM, the transcription factors *Sox6*, *Sox9*, *Brn4* and *Arx*, the *Notch* regulated gene *Nrarp*, the cell death progression factor *Gadd45g* and various ESTs (Gen Bank number NM172710, AI840429 and AI842002) (figure 9 and figure 10) (Vairapandi et al., 2002; Pirot et al., 2004; Haspel and Grumet, 2003). Finally, other genes display complex expression pattern in the embryonic telencephalon, including the glycoprotein *Gp38* (*Aggrus*) (Kotani et al., 2003), expressed in the VZ in the basal ganglia as well as in the lateral cortex and in the cortical hem, and the paternally expressed gene *Peg1* (figure 10).

b Regulation in the cortex of proneural mutant embryos

I next examined by RNA *in situ* hybridization the regulation of candidate genes in mutant embryos for *Mash1* or *Ngn2* at E13.5. As summarized in table 1, 24 genes out of 30 candidates genes were visibly either up or downregulated in the telencephalon of proneural mutant mice. Three genes specifically expressed in the cortex were downregulated in postmitotic neurons of the preplate of *Ngn2*KO cortices: the transcription factor MIBP1 and two proteins with unknown function, (Gen Bank BC052038 and NM029758) (figure 9). However, as these genes are expressed mainly in the preplate (where postmitotic neurons reside), they are unlikely to mediate directly the function of *Ngns* and probably reflect the loss of cortical identity observed in *Ngn2*KO. Among the pan-neuronal genes expressed in all neurons in the telencephalon, the brain specific adhesion molecule L1CAM is specifically lost in the SVZ and the preplate of *Ngn2*KO embryos (figure 9). The loss of this protein, essential for proper axonal guidance and neurite outgrowth in the brain, could account for precedent findings suggesting a defect in thalamocortical pathfinding in the cortex of *Ngn2* mutant embryos (Mattar et al., 2004; Seibt et al., 2003; Haspel and Grumet, 2003).

Importantly, in the cortex of *Ngn2KO* embryos, misregulation of genes can be attributed either directly to the lack of *Ngn2* activity or to the ectopic expression of *Mash1*-dependent or independent pathways in the cortex. To evaluate this, I examined microarray data obtained from the cortex of *Mash1*;*Ngn2*DKO. The analysis of expression data in figure 11B show decreased expression of cortical genes like MIBP1, L1CAM, and the EST NM029758, BC052038 and AI840429 in the cortex of *Mash1*;*Ngn2*KO, indicating that these genes are (even indirectly) regulated by *Ngn2*.

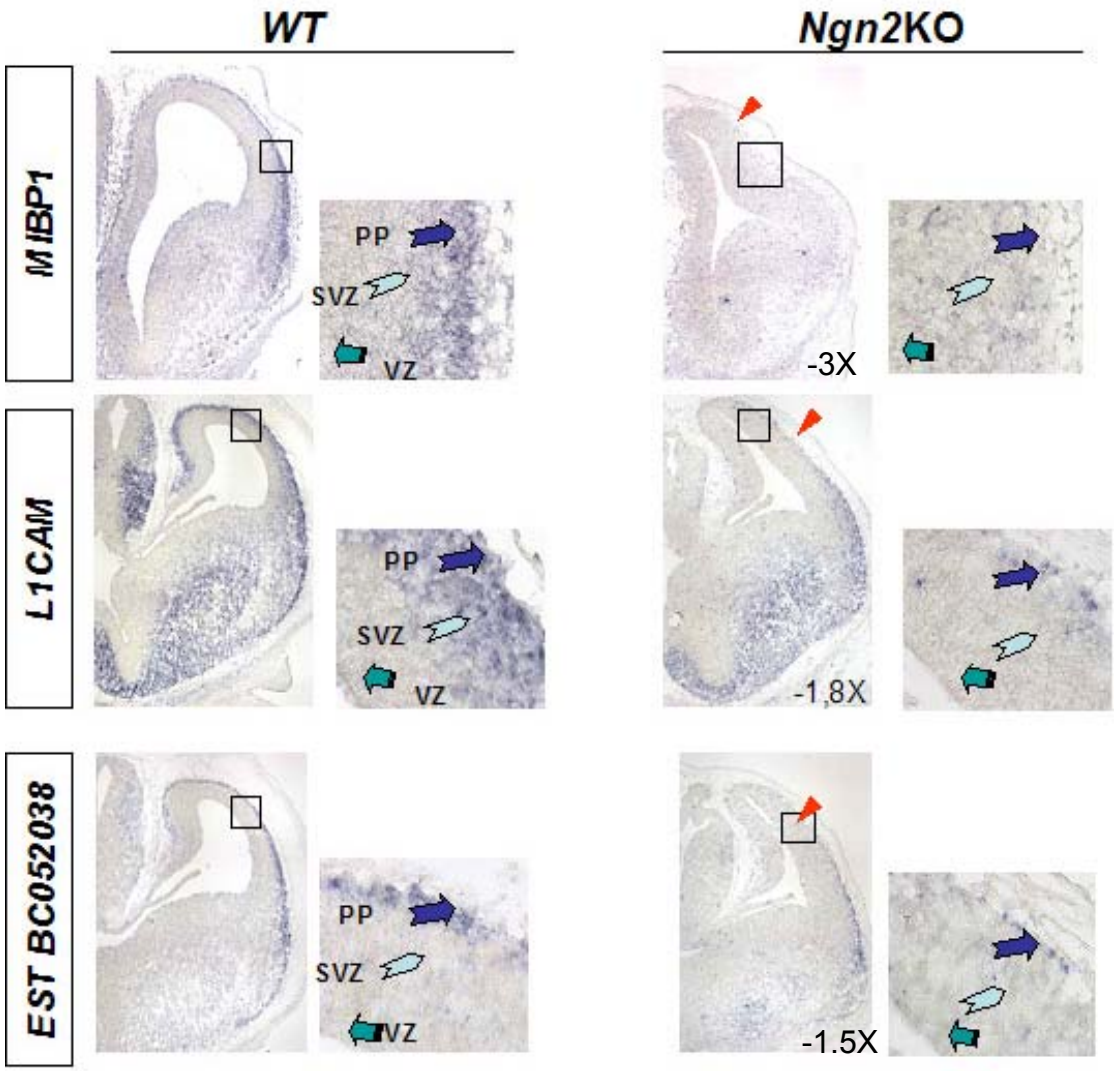


Figure 9: **Expression analysis of genes expressed in the cortex of wild type embryos and downregulated in the cortex of *Ngn2* mutant at stage E13.5.** Red arrowheads show the loss of expression of genes. Affymetrix fold change is indicated for each gene. PP, preplate, SVZ, subventricular zone, VZ, ventricular zone.

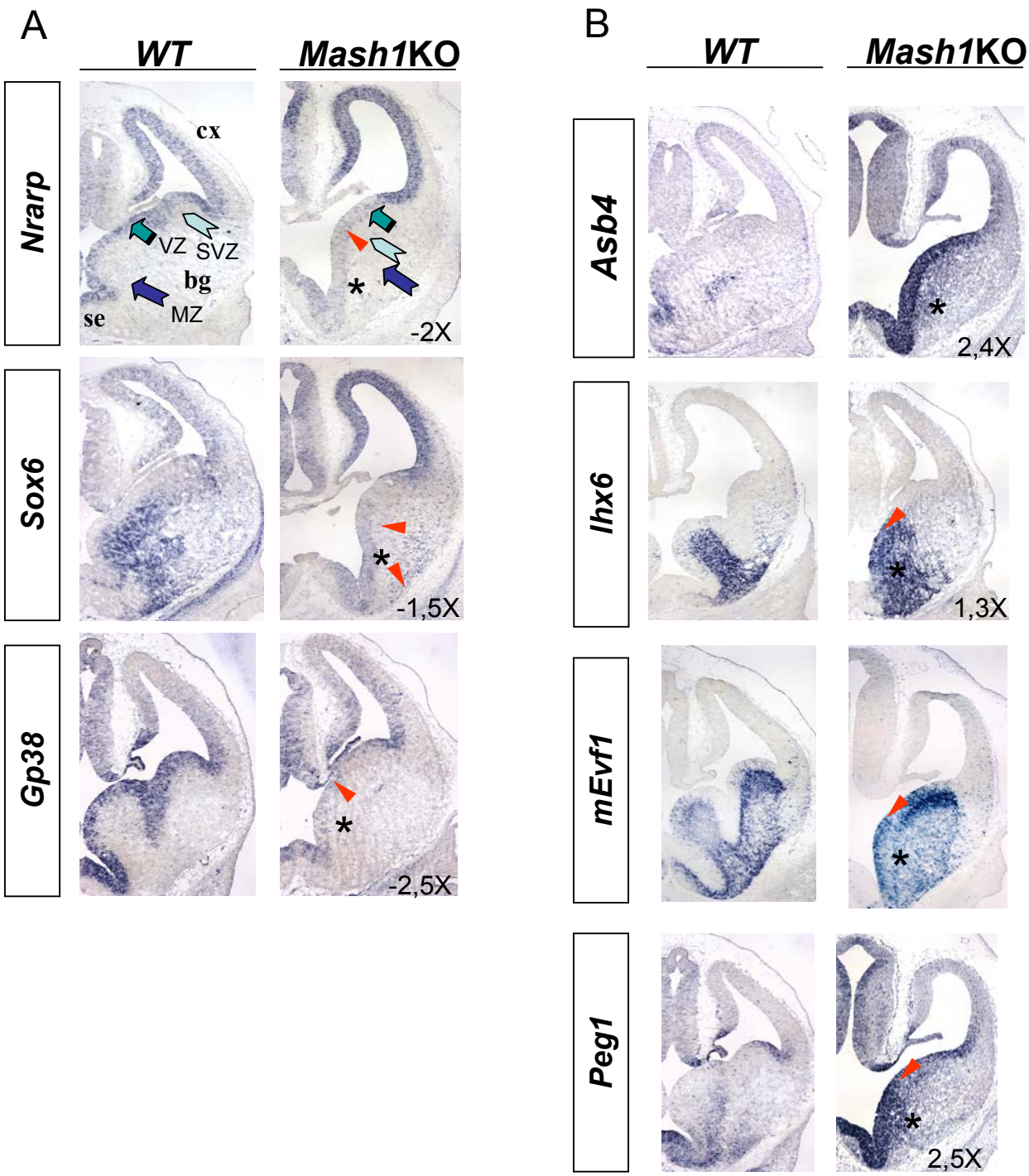


Figure 10: **Expression analysis of genes expressed in the basal ganglia of wild type embryos and *Mash1*KO at E13.5**. A) Genes downregulated in *Mash1* mutants basal ganglia. B) Genes upregulated in *Mash1* mutant basal ganglia. Red arrowheads show the change of expression. Asterisks (*) indicate the mutant MGE. Affymetrix fold change is noted for each gene. MZ, mantle zone, SVZ, subventricular zone, VZ, ventricular zone, cx: cortex, bg: basal ganglia, se: septum.

c Regulation in the basal ganglia of proneural mutant embryos

In wild-type embryos, *Mash1* is expressed in the VZ of both medial (MGE) and lateral ganglionic eminence (LGE). In *Mash1*KO, progenitors of the MGE are lost leading to an atrophied MGE, whereas progenitors of the LGE are still specified but express prematurely SVZ markers like *Dlx5* and *GAD67* (Casarosa et al., 1999). Several genes were found to be strongly misregulated in the basal ganglia of *Mash1*KO embryos (complete list of regulated genes in *Mash1*KO basal ganglia in Annex 5). The modulator of *Notch* signalling *Nrarp* and the glycoprotein *Gp38* (Pirot et al., 2004), are specifically lost in the VZ of the ganglionic eminence in *Mash1*KO embryos (figure 10). These expression profiles strongly suggest that *Nrarp* and *Gp38* are regulated by *Mash1* in the basal ganglia. On the other hand, several genes are strongly upregulated in progenitors of the basal ganglia in *Mash1*KO embryos. For instance *Asb4/Socs4*, a negative regulator of JAK/STAT and EGFR signalling (Kario et al., 2004), and the transcription factor *Peg3* are both strongly upregulated in the VZ of *Mash1*KO basal ganglia (figure 10). The same is observed for the homeobox gene *Lhx6*, implicated in the specification of neuronal population in the MGE (Alifragis et al., 2004), and *mEvf1* a potential target of *Dlx* genes in the basal ganglia (Faedo et al., 2004) (figure 10). The signification of such upregulation in the basal ganglia of *Mash1*KO embryos is currently not known and, to our knowledge, was not reported before. An attractive hypothesis would be that some of these genes could account for the compensatory mechanism of specification of GABAergic phenotype observed in the LGE of *Mash1*KO embryos (Casarosa et al., 1999).

Finally, several genes normally expressed in the basal ganglia are upregulated in the cortex of *Ngn2*KO, like the kinase *Nlk*, a modulator of *Wnt* signalling (Hyodo-Miura et al., 2002; Thorpe and Moon, 2004), the EGF repeat family member *Pref-1/Dlk1* (Moon et al., 2002; Smas and Sul, 1993) and *mEvf1* (figure 11A). Such upregulation can be the consequence of a derepression due to the loss of *Ngn2* in the cortex, or directly due to the ectopic expression of *Mash1*. To discriminate between these two possibilities, I verified the expression of *Nlk* and *Pref-1* in the cortex of *Mash1*;*Ngn2*DKO. In the cortex of these double mutants, *Pref-1* and *Nlk* are almost not changed (figure 11B): *Nlk* expression is not increased significatively, and the upregulation *Pref-1* is much less drastic compared to *Ngns*DKO data (3 times compared to 27 times). These data indicate that *Pref-1* and *Nlk* are upregulated (at least in part in the case of *Pref-1*) in response to the ectopic expression of *Mash1* in the cortex of *Ngn2* mutant embryos.

D Expression pattern and functional analysis of two candidates genes

Genes regulated in the telencephalon of proneural mutant embryos are potential downstream effectors of *Ngn2* or *Mash1*. To investigate the role of such candidate genes in the development of the central nervous system, I started with Lydia Teboul, a postdoctoral fellow in the laboratory, a

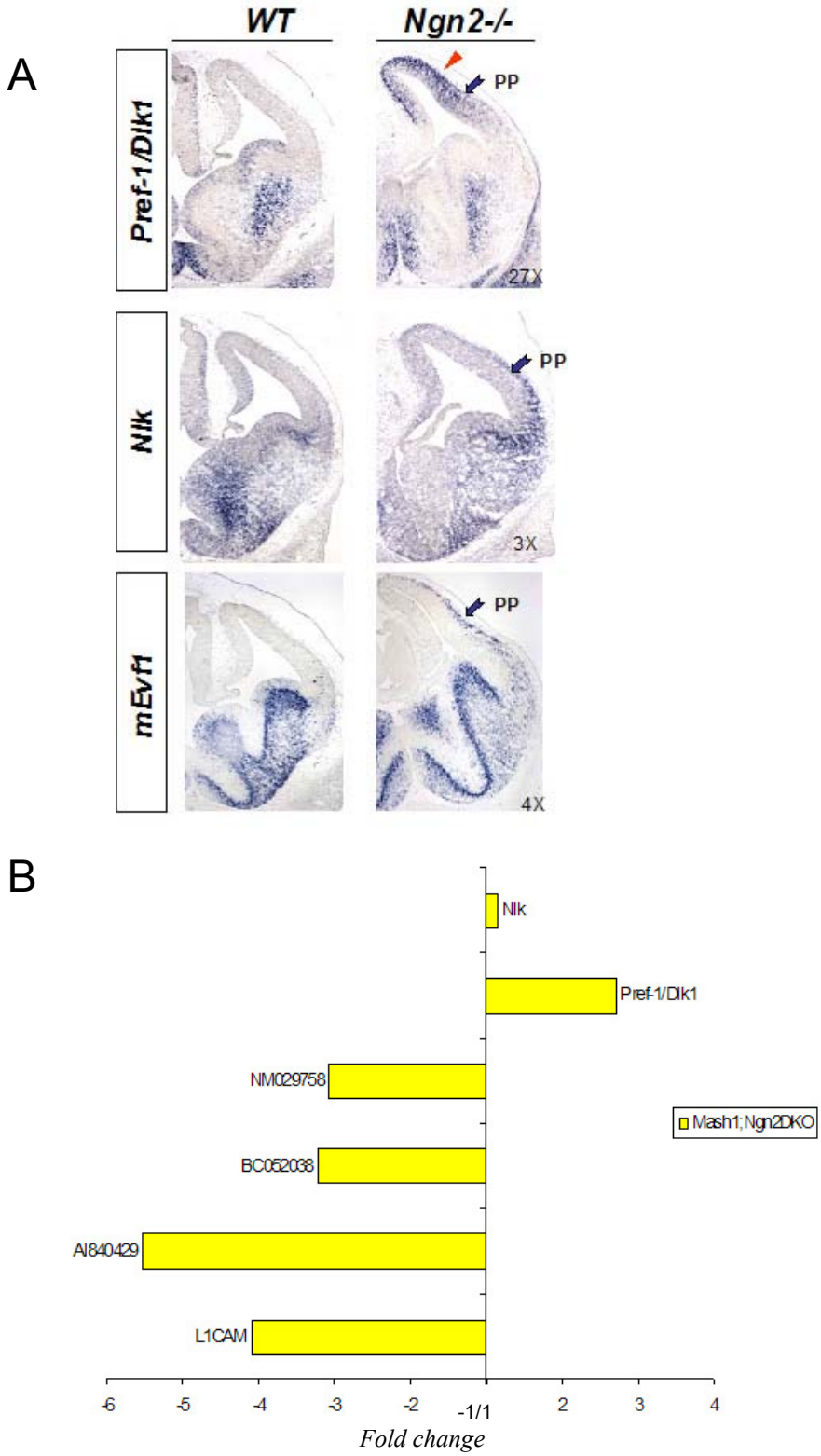


Figure 11: **Upregulation of ventral genes in the cortex of proneural mutant embryos.** A) *Nlk*, *mEvf1* and *Pref-1* are expressed specifically in the basal ganglia of wild-type embryos and are upregulated in the cortex of *Ngn2*KO at E13.5 (red arrowheads). Affymetrix fold change is indicated for each gene. PP, preplate. B) Regulation of *Nlk*, *Pref-1* and four cortical genes in the cortex of *Mash1;Ngn2* double mutants embryos. PP: preplate

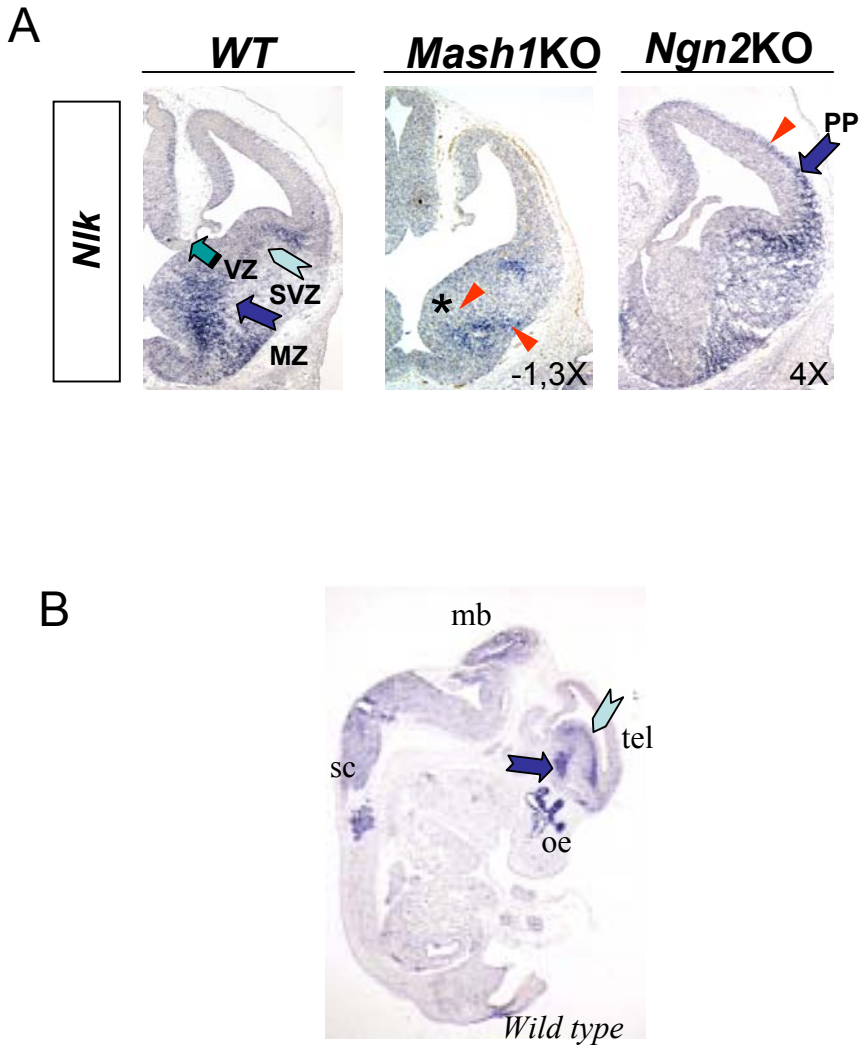


Figure 12: ***Nlk* expression at E13.5.** A) *Nlk* expression in the telencephalon in wild type, *Ngn2*KO and *Mash1*KO embryos coronal sections at E13.5. *Nlk* expression is lost in the SVZ of *Mash1*KO embryos and upregulated in the PP of *Ngn2*KO embryos (red arrowheads). B) Expression pattern of *Nlk* on sagital sections at E13.5. SVZ: subventricular zone, tel: telencephalon, mb: midbrain, sc: spinal cord, oe: olfactory epithelium, pp: preplate. Asterisks (*) indicate the mutant MGE.

functional analysis of two genes potentially regulated by *Mash1* in the basal ganglia: the kinase *Nlk*, which modulates *Wnt* signalling in the *Xenopus*, and *Pref-1*, an EGF repeats containing protein structurally related to *Delta*.

a Nemo like kinase

Nlk is a serine/threonine protein kinase similar to MAP (Mitogen-Activated Protein) kinase. Originally identified in *Drosophila*, *Nemo* was shown to be required for the establishment of epithelial planar polarity in developing ommatidia, a process mediated by the non-canonical Wnt-pathway involving Disheveled (Dsh), RhoA and JNK (Jun N-terminal kinase) (Mlodzik, 1999; Choi and Benzer, 1994), and was subsequently shown to act downstream of Frizzled signalling (Strutt et al., 1997). Homologs have been identified in humans (Dres16), mice (*N*emo-*l*ike *k*inase, Nlk), zebrafish, *Xenopus* and *C. elegans* (Lit-1) (Banfi et al., 1996; Brott et al., 1998; Meneghini et al., 1999; Thorpe and Moon, 2004; Hyodo-Miura et al., 2002). A role of *Nemo* homologues in the canonical Wnt pathway was also proposed in *C. elegans* and in vertebrates. In this pathway the binding of Wnt ligand to the receptor Frizzled leads to the stabilization of cytoplasmic β-catenin via the cytoplasmic Disheveled protein which antagonizes the destabilizing effect of glycogen synthetase kinase 3 (GSK3) and Axin protein, (Cadigan and Nusse, 1997; Gumbiner, 1998). This process allows the β-catenin to translocate into the nucleus where it interacts with a member of the TCF/LEF family of transcription factors to activate the transcription of target genes. During the early step of neural induction in the *Xenopus*, *Nlk* has been shown to antagonize *Wnt* signalling by phosphorylating TCF thereby inhibiting DNA-binding of β-catenin/TCF complex (Ishitani et al., 1999). Furthermore, *Nlk* was demonstrated to induce, in synergy with *Sox11*, the anterior neural differentiation marker *Otx2* and the pan-neural marker N-CAM (Hyodo-Miura et al., 2002). In the mouse, *Nlk* mutants are growth retarded, show aberrant differentiation of bone marrow stromal cells and seems to have neuronal defects, as sited by Kortenjann et al. (Kortenjann et al., 2001). However, a potential role of *Nlk* in the mouse CNS has not been described to date.

In the microarray analysis, *Nlk* is present in Cluster1 (see, Characterization of *Ngn2* and *Mash1* specific cluster, p39), which suggests a high level of expression of *Nlk* in the basal ganglia compared to cortex in wild-type embryos. I therefore examined more closely the expression of *Nlk* in the telecephalon of wild-type mouse embryos at stage E13.5. In the basal ganglia *Nlk* mRNA was detected in the SVZ and in the MZ of the MGE (figure 12). I also found expression of *Nlk* in the VZ of the spinal cord, midbrain and hindbrain and in the olfactory epithelium (figure 12B). I next analyzed the expression of *Nlk* in proneural mutant embryos. Microarray data suggests a significant increase of *Nlk* transcripts in the cortex of *Ngn2*KO embryos at E13.5. I*n situ* hybridization confirms that *Nlk* is ectopically expressed in the cortical plate of *Ngn2*KO embryos, as already showed in figure 11A. Such ectopic expression in postmitotic neurons in the cortex of *Ngn2*KO

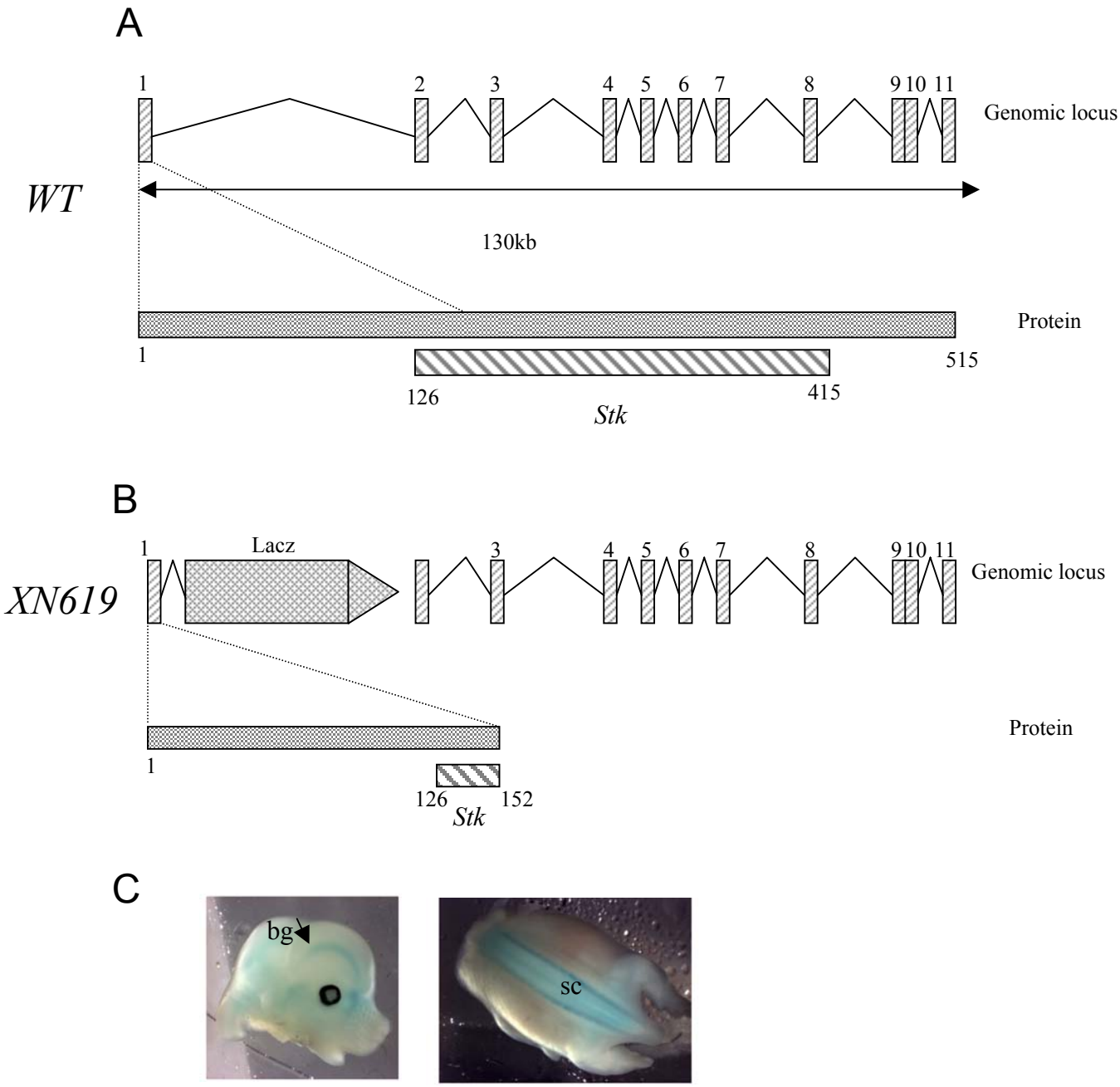


Figure 13: **Description of *Nlk* genomic locus.** A) *Nlk* genomic locus is organized into 11 exons, dashed gray rectangles. *Nlk* protein is composed of 515 amino acids. The serine/threonine protein kinase domain (*Stk*) is located between position 126 and 415. B) The line XN619, obtained from Bay Genomics (San Francisco Bay Area, USA), contains a *LacZ* insertion which disrupts *Nlk* coding sequence between the first and the second exons. The truncated *Nlk* protein of 144 resulting from the *LacZ* insertion lacks most of the serine/threonine protein kinase domain. C) X-Gal staining of *Nlk*+/LacZ E13.5 embryos in the brain (left) and in the spinal cord (right). Bg: basal ganglia, sc: spinal cord.

embryos is very similar to the expression of *Dlx* genes and suggests that *Nlk* could be expressed in GABAergic neurons. I thus investigated the expression of *Nlk* in the basal ganglia of *Mash1*KO embryos. On E13.5 *Mash1*KO sections, *Nlk* expression is lost in a subpopulation of cells in the SVZ and in the MZ of the MGE (figure 12A). Together these results show that *Nlk* is regulated by proneural genes and suggest a role of *Nlk* in the specification and/or differentiation of neuronal population in the basal ganglia.

In order to adress the function of *Nlk in vivo, Nlk* mutant mice were produced from a gene trap line (cell line XN619) obtained from Bay Genomics (San Francisco Bay Area, USA) in a 129/Sv genetic background. In these mice, the coding sequence of *Nlk* is disrupted between the first and the second exons by the insertion of the *LacZ* gene leading to a truncated *Nlk* protein of 144 amino acides lacking the protein kinase domain and expected to be not functional (figure 13). *Nlk* mutant embryos therefore express *LacZ* under the control of *Nlk* enhancer elements (figure 13C), and are currently under analysis. Mutation of *Nlk* in this line was assessed indirectly by RNA *in situ* hybridization with a probe located in the deleted region (ie after the second exon). No expression of the transcript was detected in homozygous mutants at E15 and E18 (data not shown). Wild-type, heterozygote and mutant P14 (14 days old) pups were obtained at Mendelian ratio but no adult mutant mice were detected yet. Previous description demonstrated that *Nlk* mutant mice died before 6 weeks of postnatal life and displayed abonormal bone marrow differentiation (Kortenjann et al., 2001). In the telencephalon *ER81*, *Mash1*, *GAD67* or *Ngn2* as well as *Hes5*, *Dll1* and the paneuronal marker *SCG10* seems to be normal expressed (data not shown). However, preliminary results suggest a loss of a subset of TH+ interneurons in the olfactory bulb of *Nlk* mutant embryos at stage E18.5 (ongoing analysis).

b Pref-1/Dlk1

Pref-1 (*Pre-adipocyte factor 1*), encoded by the paternally imprinted gene *Pref-1*/*Dlk1,* is a member of the EGF-like protein family structurally related to *Delta*. Pref-1 is a transmembrane protein that undergoes proteolytic cleavage (via a unknown protease) of its extracellular part to produce two soluble products of 50kDa and 25kDa (Mei et al., 2002). As previously indicated, *Pref-1* sequence is similar to *Delta-like* genes, the ligands of Notch. However, *Pref-1* lacks the DSL (Delta/Serrate/Lag-2) domain, suggesting that this protein cannot activate the *Notch* receptor as Delta related proteins. Interestingly, a recent study found Pref-1 in a yeast two-hybrid screen to interact with Notch1 in a specific manner (Baladron et al., 2005). The authors demonstrate that, *in vitro*, ectopic expression of *Pref-1* decrease the activity of a CSL/RBP-Jk/CBF-1-dependent promoter in luciferase assay, and that inhibition of *Pref-1* expression using antisense DNA is

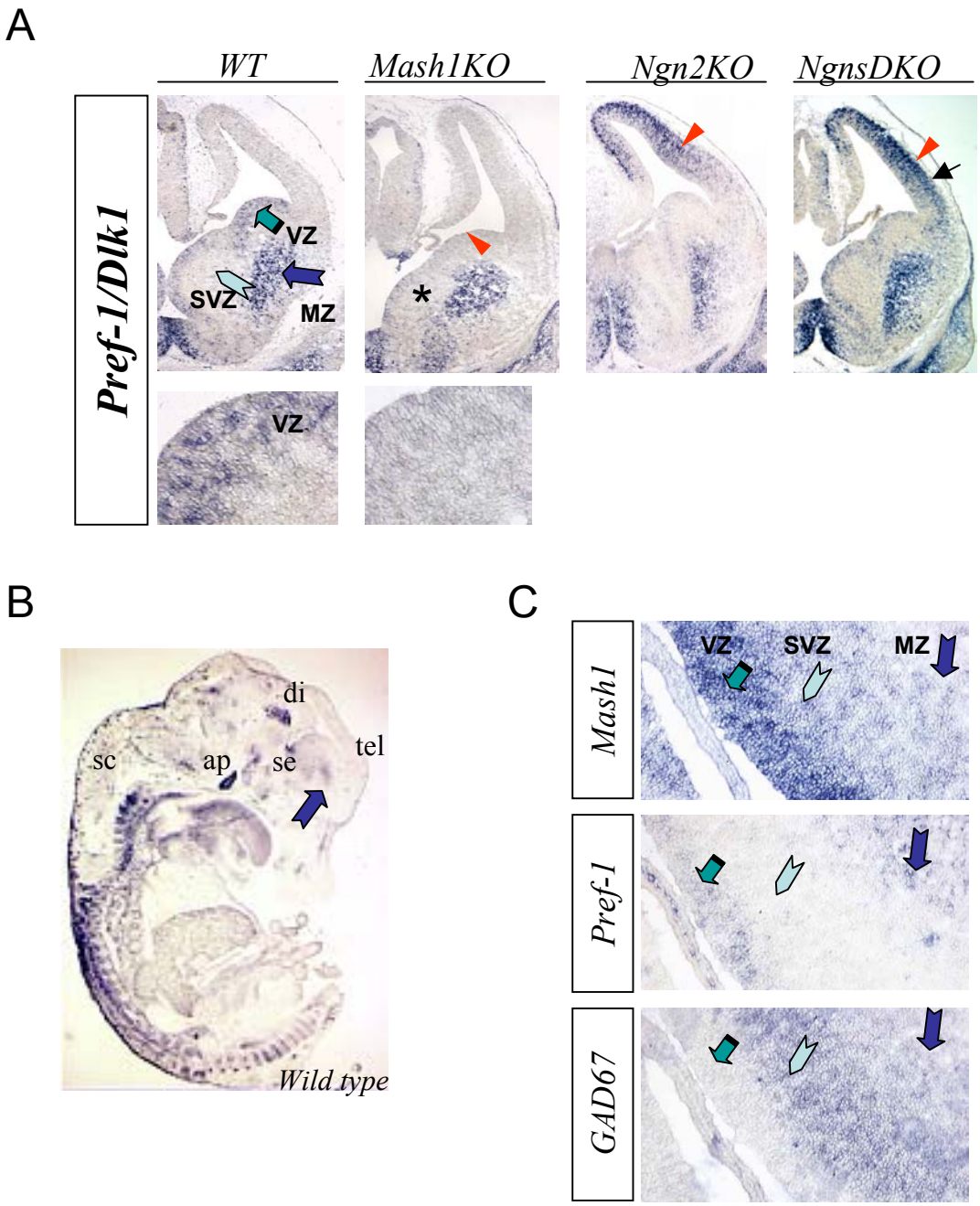


Figure 14: **Expression of Pref-1 in E13.5 embryos.** A) Pref-1 expression at E13.5 in wild type, *Mash1*KO, *Ngn2*KO and *Ngns*DKO embryos. Pref-1 is lost in the VZ of Mash1KO basal ganglia, and upregulated in the cortex of *Ngn2*KO (red arrowheads). In the *Ngns*DKO, Pref-1 is also ectopically expressed in the lateral cortex (black arrow). B) *Pref-1* expression on sagital E13.5 wild-type embryos. C) RNA *in situ* hybridization with *Mash1*, *Pref-1* and *GAD67* probes on wild-type E13.5 adjacent sections. SVZ: subventricular zone, VZ: ventricular zone, MZ: mantle zone, di: diencephalon, tel: telencephalon, sc: spinal cord, se: septum, ap: anterior pituitary. Asterisks (*) indicate the mutant MGE.

associated with an increase in *Hes-1* expression (Baladron et al., 2005). This data suggest a possible role of *Pref-1* in the modulation of the Notch signalling pathway, at least *in vitro*.

A role of *Pref-1* in regulation of cell differentiation was first caracterized during adipogenesis. During differentiation of adipocytes, *Pref-1* expression promotes progenitor stage and inhibits adipocyte differentiation. *In vitro*, constitutive expression of *Pref-1* in 3T3-L1 cells inhibits adipocyte differentiation, whereas forced downregulation of *Pref-1* enhances adipogenesis (Smas and Sul, 1993; Mei et al., 2002; Smas et al., 1999; Smas et al., 1998). In the mouse, null mutation of *Pref-1* induces perinatal death in 50% of the newborn. Surviving pups are growth retarded, obese and display skeletal malformations (Moon et al., 2002). In contrast, transgenic mice overexpressing the complete ectodomain of Pref-1 show both decreased adiposity and glucose tolerance (Lee et al., 2003b). *Pref-1* was shown to be expressed in the CNS in adult rat and human brain (neurons of the Edinger-Westphal's nucleus as well as in substantia nigra, ventral tegmental area (VTA), locus coeruleus and in certain parts of the raphe nuclei), but its function in this tissue is unkown (Jensen et al., 2001a).

To investigate *Pref-1* expression pattern in the CNS, RNA *in situ* hybridizations were performed. *Pref- 1* is expressed at low level in the VZ and at high level in the MZ of the striatum on wild-type E13.5 sections; no expression is found in the cortex (figure 14). These results are consistent with microarray data as *Pref-1* is specifically found in Cluster1 (see, Characterization of *Ngn2* and *Mash1* specific cluster, p39). *Pref-1* expression is also found in the septum, diencephalon and in the anterior pituitary, as reported before (figure 14B) (Jensen et al., 2001b). I next analyzed the expression of *Pref-1* in proneural mutant embryos. *Pref-1* is found ectopically in the VZ and PP in *Ngn2*KO embryos at stage E13.5 (figure 14A). The ectopic expression in the cortex follows a dorso-ventral gradient, with dorsal parts expressing high level of *Pref-1* and more lateral areas of the cortex containing no *Pref-1* positive cells in *Ngn2* single mutants (see figure 14A). This expression may be due to the expression of *Ngn1* in the lateral part of the cortex which would inhibit *Pref-1* in this area (Fode et al., 2000). In agreement with this hypothesis, such dorso-ventral gradient is not observed in the *Ngn1*;*Ngn2*DKO embryos, and *Pref-1* is detected ectopically in the lateral cortex (see figure 14A).

In the basal ganglia, *in situ* hybridization on adjacent sections shows that *Pref-1* is expressed in some *Mash1* positive cells in the VZ, and in postmitotic neurons in the MZ in cells expressing low levels of GAD67 (Figure 14C). In *Mash1*KO embryos at E13.5, *Pref-1* expression is completely lost in the VZ of the basal ganglia, whereas expression is still present in the MZ (figure 11A). In summary, *Pref-1* is expressed in VZ progenitors in the basal ganglia, as well as in a subpopulation of postmitotic neurons in the striatum and its regulation in proneural mutant indicates a potential role in GABAergic differentiation.

To address the function of *Pref-1*, I started to analyse a few *Pref-1* mutants embryos that I obtained from H.S. Sul's laboratory (Moon et al., 2002). As described before, these mice are growth retarded and obese. No morphological abnormalities were detected in *Pref-1* mutant telencephalon at E13, E15, E18 and P0. Expression of ventral markers like *Dlx1*, *Dlx5*, *EBF1*, *ER81*, *Sox1*, *GAD65*, *GAD67* as well as cortical markers such as *Ngn2*, *Pax6* and the paneuronal marker *SCG10* were tested by *in situ* hybridization on *Pref-1* mutants embryos at embryonic stages E13.5 and E15.5. I also checked expression of patch and matrix markers in the striatum at E18.5. These analyses revealed that ventral and cortical markers are normally expressed in the telencephalon of *Pref-1* mutant embryos. It would be still interesting to analyse proliferation markers like phospho-histone H3 and KI67, as this gene in expressed in proliferating precursors in the VZ.

In overall, this microarray analysis realized with embryos lacking proneural activities allowed the characterization of genes regulated in the telencephalon of proneural mutant embryos. Two groups of genes (Cluster1 and Cluster2) were shown to be either upregulated (Cluster1) or downregulated (Cluster2) in the cortex of *Ngns*KO embryos, making them good candidates to be regulated by *Ngns* or *Mash1* respectively. In addition, I characterized 9 genes specifically expressed in the basal ganglia (see table 1). I also started functional analysis of two genes expressed in basal ganglia: *Nlk* and *Pref-1*. These two genes are upregulated in the cortex of *Ngn2*KO embryos, and are lost in specific neuronal population in the basal ganglia of *Mash1*KO embryos. Functional studies are still ongoing to determine their role in the telencephalon.

3 Characterization of putative direct targets of proneural genes

The microarray analysis of proneural knock out mice provides insightful information on the genetic pathways regulated by *Ngn2* and *Mash1*, as demonstrated in the precedent section. However, such loss-of-function strategies are limited by potential functional redundancies that probably masks some of the proneural activities in knock out embryos. Furthermore, it is not possible to decipher between genes regulated either directly or indirectly by proneural genes. In order to overcome these limitations, I initiated with Laurent Nguyen and Diogo Castro (two postdoctoral fellows in the laboratory) gain-of-function studies of proneural genes in the mouse telencephalon at embryonic stage E10.5. At this stage, the telencephalon is formed by a single layer of multipotent neuroepithelial cells specified by regional determinant to form the prospective cortex and basal ganglia. The proneural genes *Ngns* and *Mash1* start to be expressed in the cortex and basal ganglia respectively, but neuronal differentiation has not started. At this stage the neuroepithelium is thus competent for proneural activity and gain-of-function studies should allow the detection of genes potentially directly regulated by *Mash1* or *Ngn2*.

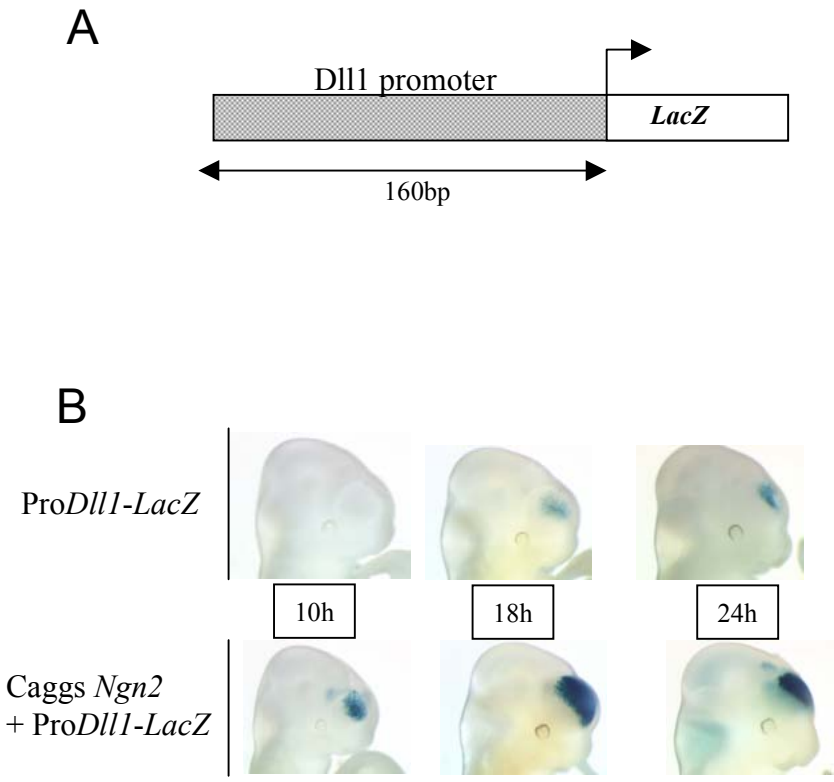


Figure 15: **Ngn2 ectopic expression in the cortex of E10.5 embryos.** A) The *LacZ* coding sequence was put under the control of a 160bp long *Dll1* promoter fragment (Pro*Dll1-LacZ*), also called HOM2 (See figure 22, from Beckers et al. 2000). B) β-Galactosidase staining of electroporated embryos with *ProDll1-LacZ* and control or *Ngn2* expressing vector.

A Mouse electroporation assay with proneural activity reporter

To test the feasibility of a gain-of-function strategy, I compared the activity of endogenous and exogenous proneural proteins using a reporter construct for proneural activity, which includes *LacZ* under the control of a 160bp long *Dll1* promoter (Pro*Dll1-LacZ*) (figure 15A, and see figure 22) (Beckers et al., 2000). Telencephalon of E10.5 mice embryos were electroporated with the Pro*Dll1-LacZ* reporter and control or CAGGS-*Ngn2* expression vector and grown in *ex-vivo* culture (Osumi and Inoue, 2001) (see, Material and Methods, Electroporation of mouse embryos). Activity of the *LacZ* reporter was assessed 10hrs, 18hrs and 24hrs after electroporation. Control embryos electroporated with Pro*Dll1-Lacz* reporter and empty CAGGS vector (control) exhibited low activity of the *LacZ* reporter starting from 18hrs and remaining low 24hrs after electroporation (figure 15B). In embryos electroporated with *Ngn2* expressing vectors, *LacZ* reporter activity is detected 10hrs after electroporation, as assessed by X-Gal staining. After 18hrs, activity of the reporter reached higher levels compared to endogenous proneural activity and remained high after 24hrs, as showed by X-Gal activity of the *LacZ* reporter (figure 15B).

These results suggested that 18hrs after electroporation, targets of proneural genes can be induced by electroporation of *Ngn2* or *Mash1* at sufficient levels to be distinguished with endogenous proneural activities. We thus choose to do our gain-of-function experiments on microarray by incubating the embryos 18hrs after electroporation. This experiment should allow the detection of direct and indirect targets of *Mash1* and *Ngn2*.

B Microarray analysis of mice overexpressing proneural genes

We made the hypothesis that Affymetrix analysis would provide quantitative data making possible to detect increased proneural activity in electroporated embryos compared to endogenous levels of *Ngn2* or *Mash1*. Briefly, E10.5 embryos were electroporated with vectors expressing either *Ngn2* or *Mash1* by injecting DNA solution into the telencephalic vesicles (see, Material and Methods, Electroporation of mouse embryos). For electroporation, embryos were oriented in the electroporation chamber to target either the cortex, for *Ngn2* expression, or the basal ganglia for *Mash1*. A *Green fluorescent protein* (GFP) expressing vector was co-electroporated in order to visualise the electroporated area and assess electroporation efficiency. After 18hrs of whole embryo culture (Osumi and Inoue, 2001), GFP positive (electroporated) regions were dissected, and total RNA extracted immediately. Five to six dissected fragments of basal ganglia or cortex were pooled to reach 1µg of total RNA and subjected to cRNA amplification following Affymetrix guidelines (see, Material and Methods, RNA extraction and hybridization to Affymetrix microarray). Hybridizations to Affymetrix microarrays MOE430_2 were performed in duplicates for each experimental condition. Given the precedent assay with the Pro*Dll1-Lacz* reporter, this

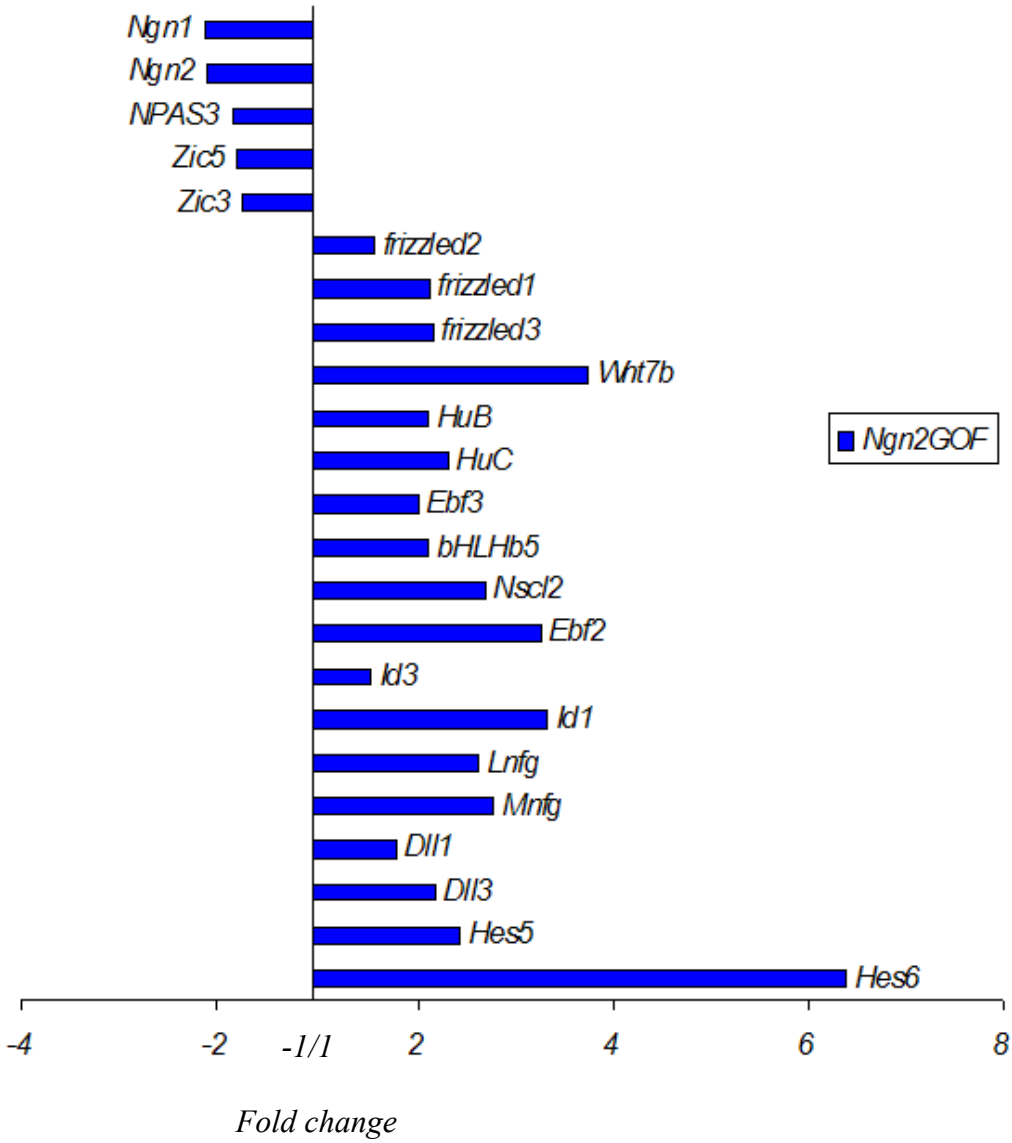


Figure 16: **Genes up- or downregulated in the cortex of E10.5 embryos electroporated with *Ngn2* expressing vector.** Example of genes misregulated after Ngn2 electroporation. Potential targets of Ngn2 such as *Nscl2*, *Nscl2*, *Dll1*, *Dll3* and *Ebf* genes are upregulated. GOF: gain of function

overexpression strategy should allow the caracterization of genes regulated either directly or indirectly by proneural genes.

a Ngn2 gain-of-function

A total of 2456 genes were either up or downregulated significatively in the cortex of embryos overexpressing *Ngn2*, based on the criteria indicated in Clusterization of microarray data (p.38). Notably, the number of genes upregulated in the cortex were much higher than the number of downregulated ones (1834 compared to 622 genes), which correlates with a function of *Ngn2* mainly as transcriptional activator. Among the upregulated genes, several mediators of *Notch* signalling were found such as *Dll1*, *Dll3* and *Hes5*, but also *Hes6*, a positive regulator of *Ngns* (Koyano-Nakagawa et al., 2000), and modulators of *Notch* signalling *Lnfg*, *Mfng* (Ishii et al., 2000; Donoviel et al., 1998) and *Sel1-homologue* (see figure 16). Also upregulated in the cortex were many components of *Wnt* signalling, including *Wnt7b*, several *Frizzled* receptors (*Fzd1, Fzd2* and *Fzd3*) and the signal transducer *Dishevelled2*, cortical differentiation genes like the bHLH transcription factors *Nscl2*, *Bhlhb5* (Mattar et al., 2004; Kruger and Braun, 2002) and other genes involved in neuronal differentiation like *EBF1* and *EBF2*, which stabilize neural commitment (Garcia-Dominguez et al., 2003) or the RNA binding protein *HuC*, *HuB* and *HuD* (Akamatsu et al., 1999). Inhibitors of proneural activity *Id1* and *Id2* were also found to be upregulated in *Ngn2* electroporated cortices (Lyden et al., 1999).

In contrast various transcription factors like *Zic3*, *Zic5* and the neuronal bHLH/PAS domains transcription factor *NPAS3* (Brunskill et al., 1999) were downregulated in the cortex *Ngn2* electroporated embryos (figure 16). *Ngn1* and *Ngn2* transcripts were also downregulated by *Ngn2* overexpression in the cortex. This could be attributed to regulatory loops between *Ngn1* and *Ngn2*. However, RNA *in situ* hybridization showed a decrease of *Ngn1* expression in *Ngn2* mutants embryos suggesting the requirement of *Ngn2* for *Ngn1* expression in dorso/medial cortex (Fode et al., 2000).

b Mash1 gain-of-function

Analysis of *Mash1* gain-of-function data, using the same filters as described in Clusterization of microarray data (p.38), showed that 538 genes were missregulated in electroporated basal ganglia. A total of 269 genes were upregulated, among them components of the *Delta/Notch* pathway such *Dll1*, *Dll3*, *Hes5*, *Hes6*, and *Mfng* (similarly to what is observed in *Ngn2* gain-of-function) (figure 17). The proneural inhibitors *Id1*, *Id3* and *Id4*, upregulated by *Ngn2* overexpression in the cortex, were also upregulated by *Mash1*. Other upregulated genes were the neuronal determinants of the basal ganglia *Isl1*, *Lhx6* and *Lhx8*, as well as *NeuropeptideY*, the glycoprotein *gp38* and the cell cycle inhibitor *PAK3*. Genes implicated in GABAergic determination such as *Dlx* and *GAD* genes

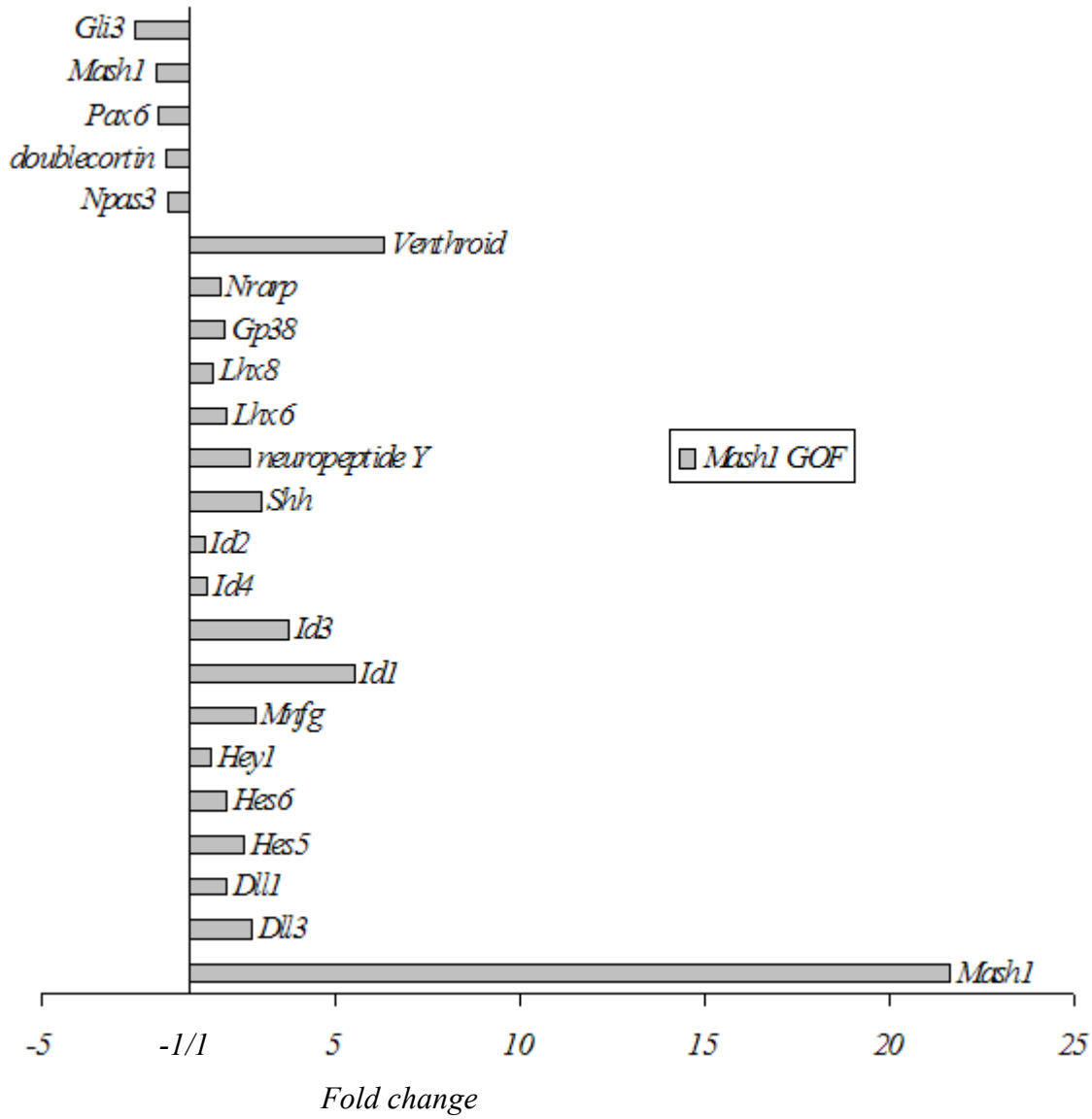


Figure 17: Genes up- or downregulated in the cortex of E10.5 embryos electroporated with *Mash1* expressing vector. Genes from the Notch signalling pathway are upregulated such as *Dll1*, *Dll3*, *Hes5*, and *Hes6*, as well as ventral markers like *Lhx6*, *Lhx8*, *NeuropeptideY* and *Shh*.

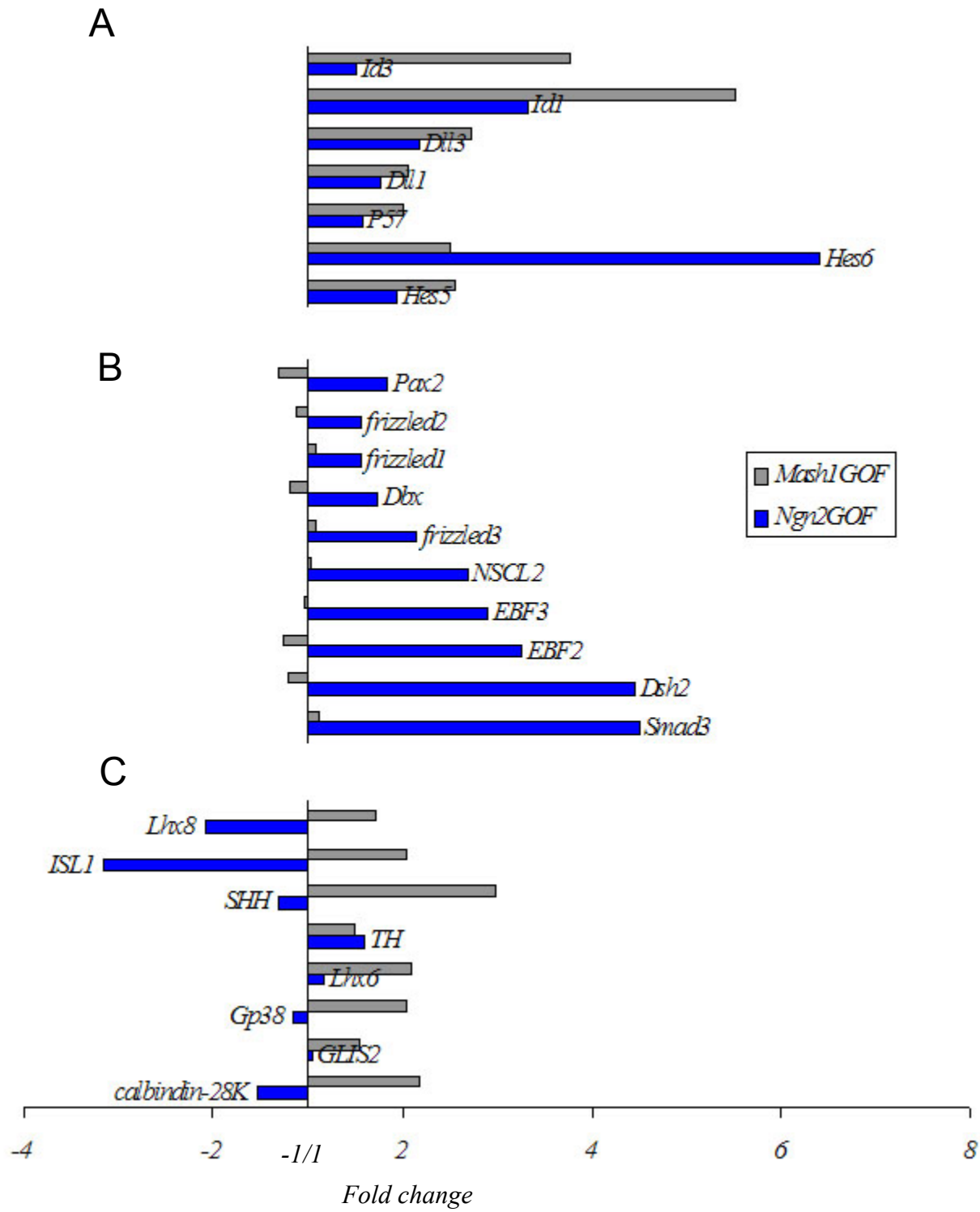


Figure 18: **Comparison between *Mash1*GOF and *Ngn2*GOF studies.** A) Genes upregulated by *Ngn2* and *Mash1* include genes implicated in Notch signalling pathway. B) Genes upregulated only by *Ngn2*. C) Genes upregulated only by *Mash1*.

were not significatively upregulated in our gain-of-function model. Among the 269 downregulated genes, we found the cortical determinants *Pax6*, as well as the microtubule associated protein *Dcx*, shown in a precedent screen to be downregulated in the cortex of *Ngn2*KO embryos (Mattar et al., 2004; LoTurco, 2004), the MADS box transcription factor *Mef2a* (Lyons et al., 1995), *NPAS3* and *Sel1-homologue*, both upregulated in *Ngn2* gain-of-function microarray data.

The total number of *Mash1* regulated genes (538 genes) is rather small compared to the 2469 genes identified in *Ngn2* gain-of-function studies. This surprising observation seems not to be the consequence of a low electroporation efficiency, since GFP was detected at similar levels in *Ngn2* and *Mash1* experiments. Furthermore, induction levels of genes upregulated by both *Mash1* and *Ngn2* such as *Dll1*, *Dll3*, *Hes5* and *Hes6* are comparable (compare figure 16 and figure 17). The numbers of upregulated and downregulated genes are almost identical, although *Mash1* is considered to work as a transcriptional activator. These observations could reflect a limitation in the number of target genes activated by *Mash1* in the context of our model system. Interestingly, the number of downregulated genes is also relatively small in the *Mash1*KO basal ganglia (see, Regulation in the basal ganglia of proneural mutant embryos, p41). It is thus possible that the small number of genes regulated by *Mash1* is a consequence of a genuine difference between *Mash1* and *Ngn2*.

c Common and distinct downstream effectors of proneural genes

Analysis of gain-of-function microarray data suggests that *Mash1* and *Ngn2* are able to activate both distinct and common target genes. An attractive hypothesis would be that genes upregulated by *Mash1* and *Ngn2* gain-of-function account for the panneural function of proneural genes, while genes specifically activated by *Mash1* or *Ngn2* are implicated in the specification of particular neuronal identities. To investigate this hypothesis, I compared data from *Ngn2* and *Mash1* gain-of-function. In total 42 genes were found to upregulated by *Ngn2* in the cortex and *Mash1* in the basal ganglia, including components of *Notch* signalling as described before, as well as *Id2*, *Id3*, the cyclin kinase inhibitor *p57*, the regulators of G-protein signalling *Rgs16* (figure 18A) (Hiol et al., 2003). *Ngn2* induces specifically the expression of 1266 genes, including *EBF1, EBF2*, *Nscl2*, *Dbx*, *smad1*/*3* and the *Wnt* receptors *Frizzled1*/*2*/*3*/*6* (figure 18B). In contrast only 94 genes are upregulated in the basal ganglia after Mash1 electroporation, including *Isl1*, *Lhx6*, *Shh*, the catecholamine synthesising enzyme tyrosine hydroxylase (TH) and the Gli-Kruppel zinc-finger transcription factor *Glis2* (or NKL, neuronal Kruppel-like protein) (figure 18C), a target of *X-Ngn1* in *Xenopus* (Lamar et al., 2001b). Complete list in Annex 6.

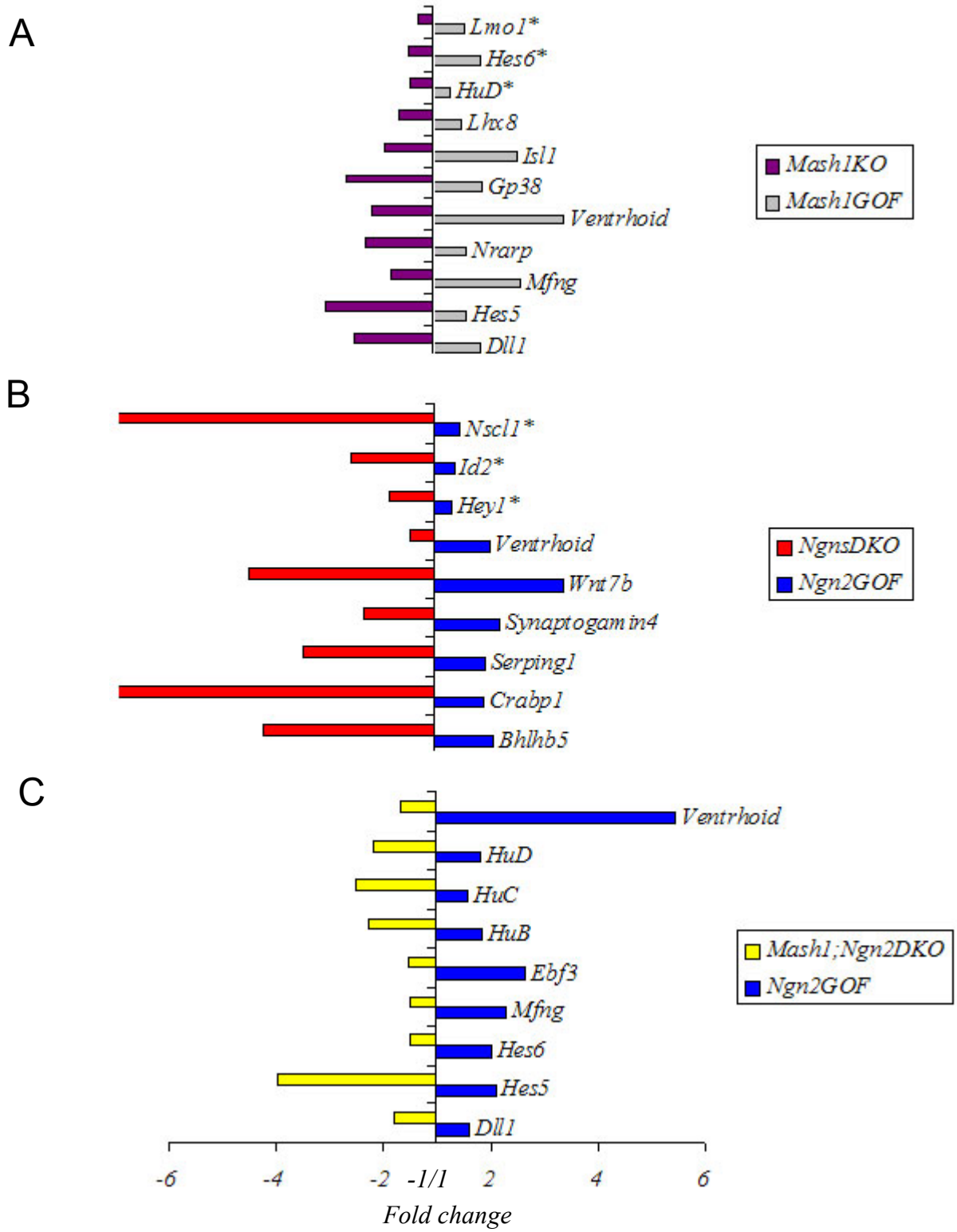


Figure 19: **Comparison between gain of function and loss of function studies.** A) Genes upregulated in the basal ganglia by *Mash1GOF* and downregulated in *Mash1*KO. B) Genes upregulated in the cortex by *Ngn2GOF* and downregulated in *NgnsD*KO. C) Genes upregulated in the cortex by *Ngn2GOF* and downregulated in *Mash1;Ngn2D*KO. (*) Genes misregulated more than 1.2 times.

d Comparison of proneural gain and loss-of-function microarray data

Comparison of microarray data from gain and loss-of-function analysis should provide an important source of information for the selection of genes regulated by proneural genes. Indeed, downstream effectors of proneural activity are expected to be upregulated in gain-of-function studies and downregulated in knock out embryos. However, we can expect a relative high number of false negatives due to functional redundancy encountered in proneural mutant embryos. In the case of *Mash1*, this strategy is in addition impaired by the low number of genes up or downregulated in gain and loss-of-function studies.

By using criteria described previously for the selection of candidate genes (see, Clusterization of microarray data, p.38), only 12 genes are upregulated in *Mash1* gain-of-function and downregulated in basal ganglia of *Mash1*KO embryos (figure 19A). Among these candidates we find *Dll1*, *Hes5*, *Mng*, the *Notch* regulated gene *Nrarp* (Pirot et al., 2004), the transcription factors *Lhx8*, *Isl1*, the glycoprotein *Gp38* and *Ventrhoid*, a modulator of EGF signalling specifically expressed in the ventral neural tube (Jaszai and Brand, 2002). By decreasing the fold change from 1.5 to 1.2 times, 13 additional genes were selected, among them *Hes6*, *Lmo1*, the histone desacetylase *Hdac5*, cyclin G2 shown to block cell cycle entry (Horne et al., 1997) and a transmembrane protein with EGF domain expressed in the neural tube, *tomoregulin-1* (Eib et al., 2000). Complete liste in Annex 7.

In the case of putative *Ngn2* targets, 30 genes are upregulated in *Ngn2* gain-of-function and decreased in *Ngns*KO mice (figure 19B). Among them are found *Wnt7b*, *synaptogamine4*, the transcription factor *bhlhb5* and the cellular retinoic acid receptor *Crabp1*. 43 additional genes are obtained by decreasing the fold change to 1.2, among them, *Nscl1*, *Id2* and *Hey1*. No neurogenic genes (but *Hey1*) are present, due probably to the functional compensation of *Mash1* in the cortex of *Ngns* mutant embryos. When data from cortex of *Mash1*;*Ngn2*DKO embryos are crossed with *Ngn2* gain-of-function data, 44 genes are selected based on criteria described as before (figure 19C). Genes involved in *Notch* signalling are then selected, such *Dll1*, *Hes5*, *Hes6*, *Mfng*, as well as the transcription factor related to *Drosophila Collier*, *Ebf3* (Dubois et al., 1998), the RNA binding protein *HuB*, *HuC* and *HuD*, and *Ventrhoid* (Kasashima et al., 1999; Jaszai and Brand, 2002). Complete list in Annex 8.

C Delta like 1, is a direct target of Mash1

The microarray data strongly suggest that *Ngn2* and *Mash1* are necessary and sufficient for the expression of several genes involved in *Notch* signalling such *Dll1*, *Hes5*, *Hes6* and *Mfng*. By RNA *in situ* hybridization, *Dll1* is coexpressed with *Mash1* in the VZ in the basal ganglia, and precedent study in *Mash1*KO embryos showed a loss of *Dll1* in mutant basal ganglia (Casarosa et al., 1999).

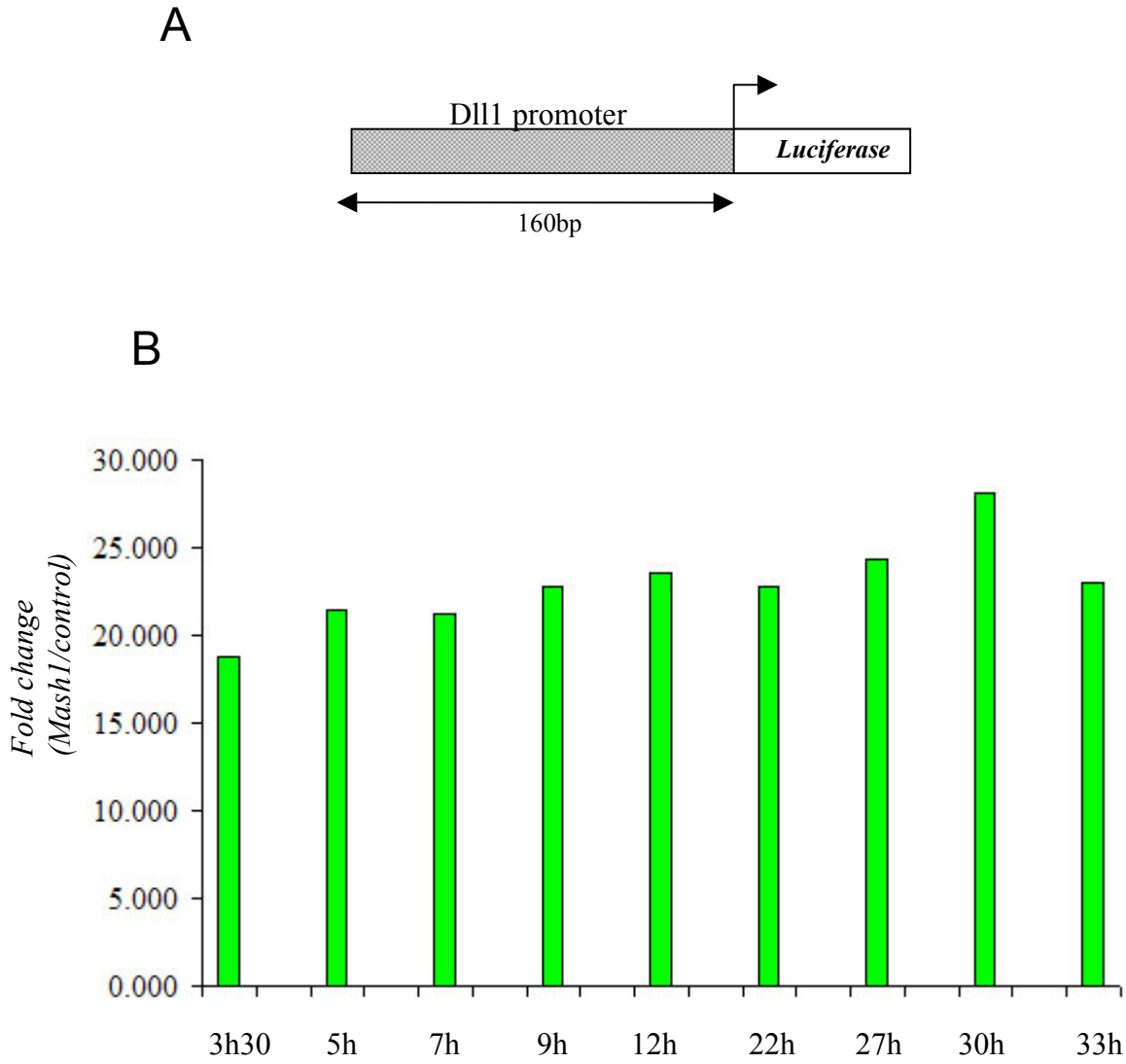


Figure 20: **Time course of Mash1 activity by luciferase assay in P19 cells.** A) The reporter Pro*Dll1-Luc* (comprising 160bp of the *Dll1* promoter, called HOM2. See figure 22, from Beckers et al. 2000) was transfected with *Mash1* or control expression vector. B) Luciferase was detected on cell lysat at different time after transfection, as indicated. Fold change between *Mash1* and control transfected cells is shown (mean of three independent experiment). The luciferase reporter is activated as soon as 3h30 after transfection and remains at constant levels in subsequent times.

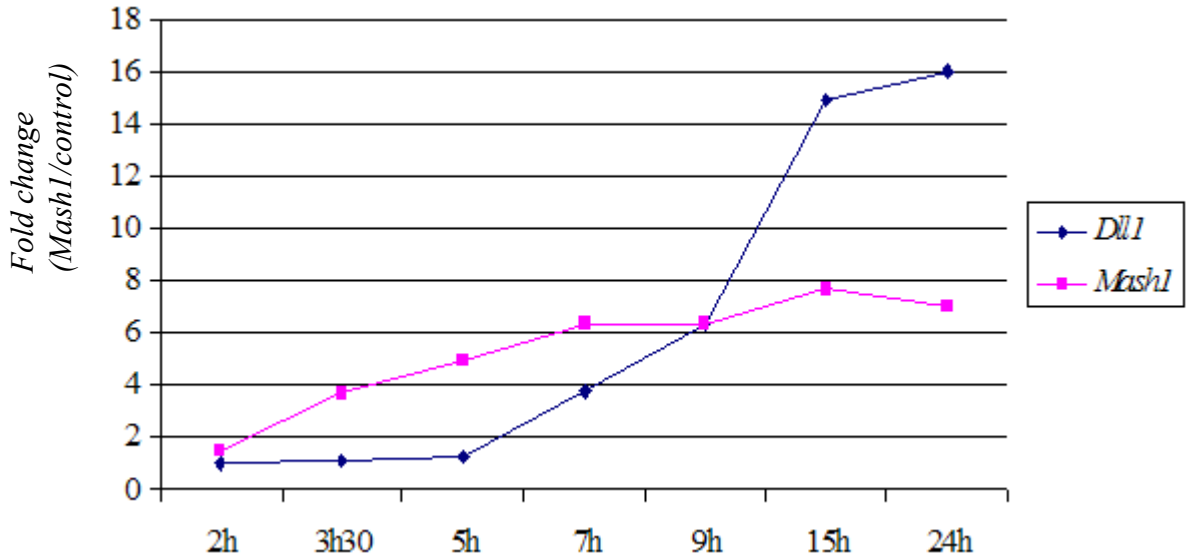


Figure 21: **Dynamic of expression of endogenous *Dll1* transcript and *Mash1* in P19 cells by quantitative RTPCR.** Induction of the endogenous *Dll1* transcript was determine at different times after electroporation with *Mash1* or control expression vector. Data were normalised with the housekeeping gene hydroxymethylbilane synthase. *Mash1* fold changes were calculated in log10 to be comparable with *Dll1* at the same scale.

In order to confirm microarray data obtained from electroporated embryos and investigate a possible direct regulation of *Dll1* by Mash1, I chose a simpler model based on transient transfection of P19 embryonic carcinoma cells. P19 cells are pluripotent cells and were shown to specifically differentiate into neurons when induced by retinoic acid or when transfected with *Mash1*, *Ngn1* or *NeuroD* expressing vectors (Farah et al., 2000; Johnson et al., 1990).

a Dll1 upregulation in P19 transfected cells

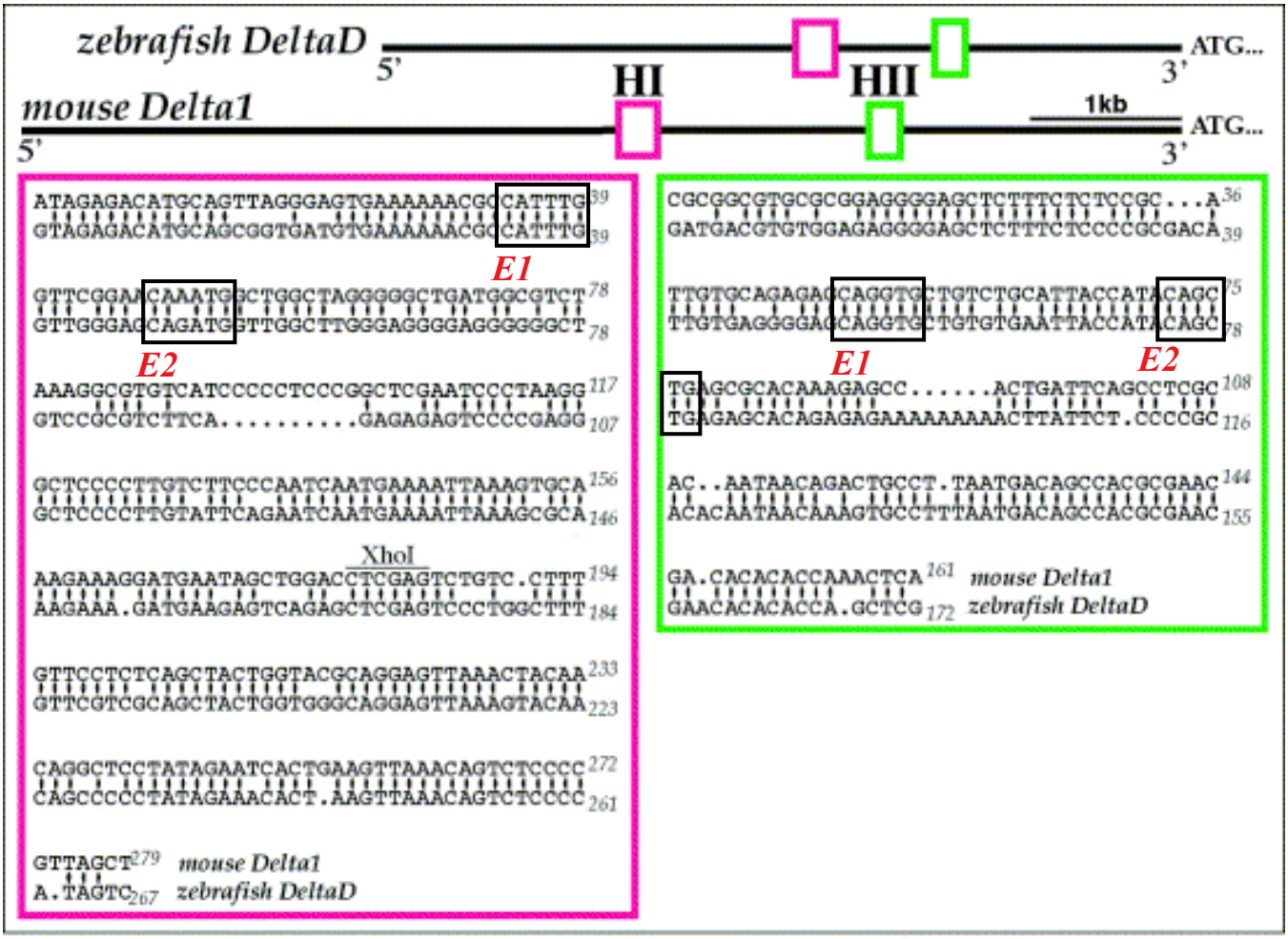
To test the activity of *Mash1* after transfection in P19 cells, Diogo Castro in the laboratory used a luciferase reporter under the control of a part of the *Dll1* promoter (Pro*Dll1-Luc*) (160bp of Dll1 promoter called HOM2, see figure 22, from Beckers et al. 2000). Luciferase assay showed a clear activation of the reporter (near 20 times) in *Mash1* transfected cells compared to control experiment, already 3h30 after transfection (figure 20). This result suggests that Mash1 protein is produced very rapidly after transfection and activates the *Dll1* promoter in a luciferase assay.

I then investigated the regulation of endogenous *Dll1* by *Mash1*. P19 cells were transiently transfected with *Mash1* and *E47* expression vectors and grown for a maximum of 24hrs. Transfection efficiency was followed by cotransfection of a GFP expressing vector. Total RNA from transfected cells was extracted 2hrs, 3h30, 5hrs, 7hrs, 9hrs, 15hrs and 24hrs after transfection and cDNA synthesised for each sample. Quantitative PCR (qPCR) were then realized to measure expression of target genes between control and *Mash1* transfected cells (see, Material and Methods, P19 cells transfection and quantitative RT PCR).

I analysed by qPCR the sequential activation of *Mash1* and its putative target *Dll1*. As shown in figure 21, level of *Mash1* transcript was upregulated 3h30 after transfection and increased regularly to reach a maximal level at 15hrs. In the case of *Dll1*, its mRNA was upreguated 4 times, 7hrs after *Mash1* transfection. Fold change increased then regularly in subsequent time to reach a maximal at 24hrs (figure 21). These results demonstrate that transient overexpression of *Mash1* can induce expression of endogenous *Dll1* in P19 cells, confirming the microarray data (see, *Mash1* gain of function, p48). It is also interesting to note that four additional hours (3hrs versus 7hrs) are required for the activation of endogenous *Dll1* promoter compared to the Luciferase assay. This difference is most than probably the consequence of chromatin modifications required for induction of endogenous *Dll1* promoter.

b Dll1 promoter contain functional EBoxes

It seems thus that *Mash1* activate *Dll1* in P19 cells, possibly through a direct regulation of the *Dll1* promoter. Diogo Castro studied the *Dll1* promoter and found EBoxes consensus binding sites in two previously identified regions of homology (Beckers et al., 2000), HOM1 and HOM2, conserved between the mouse and zebrafish (see figure 22). Diogo demonstrated that, these two enhancers



From Beckers et al. , 2000

Figure 22: **Characterization of two enhancers of *Dll1*.** Beckers et al. characterized two conserved regions, H1 and H2 (HOM1 and HOM2), in the *Dll1* promoter between the mouse and the zebrafish genome (Beckers et al. 2000). Two conserved Eboxes (E1 and E2) are present in HOM1 and HOM2 enhancers.

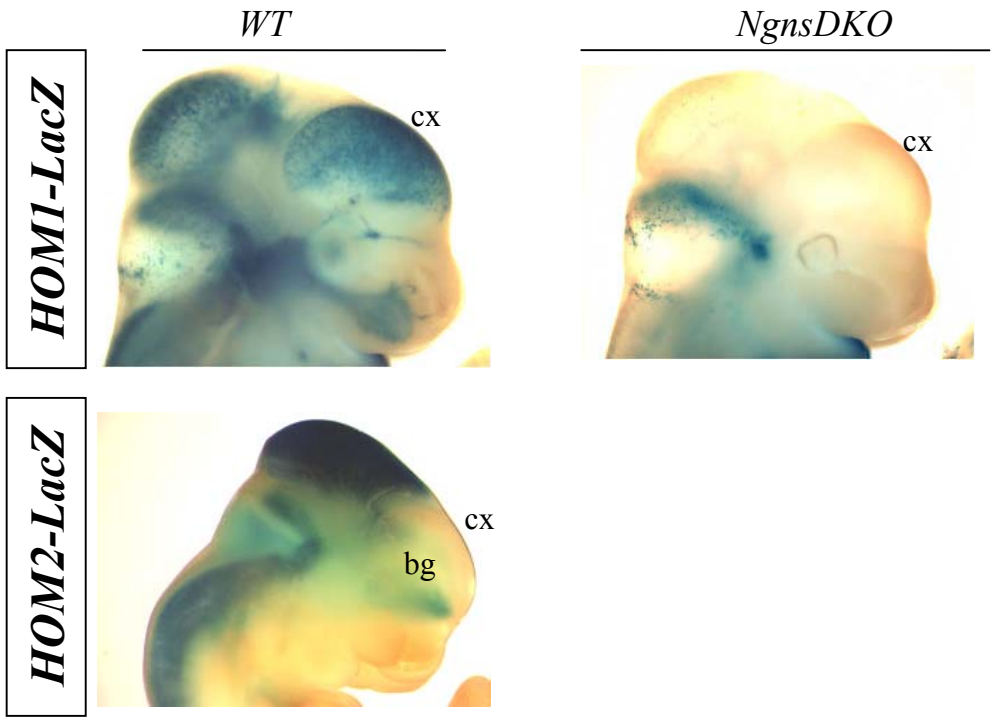


Figure 23: **HOM1 and HOM2 enhancers drive LacZ expression in *Ngns* and *Mash1* expression domain respectively.** X-Gal staining of HOM1-*LacZ* and HOM2-*LacZ* transgenic embryos at E11.5. In wild-type embryos, HOM1-*LacZ* is activated in the cortex (cx) and HOM2-*LacZ* in the basal ganglia (bg). In a *Ngns*DKO background HOM1-*LacZ* expression is totally lost.

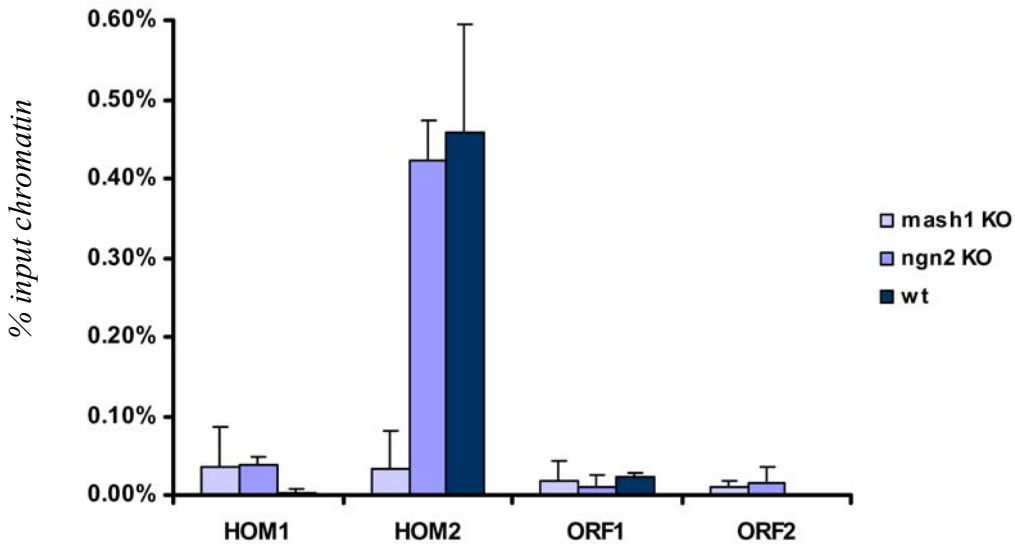


Figure 24: **Mash1 interacts physically with HOM2 enhancer.** Chromatin immunoprecipitation in E12.5 telencephalon. HOM2 genomic sequence is immunoprecipitated with Mash1 antibody from wild–type (dark blue bars) and *Ngn2*KO (midle blue bars) embryos. In absence of Mash1, HOM2 is not immunoprecipitated (light blue bars). ORF1 and ORF2 correspond to control with set of primers located in the *Dll1* coding sequence. The y axis correspond to the quantification of the immunoprecipitated sequence by quantitative PCR.

were sufficient to drive specific expression of *Dll1* in the cortex or in the basal ganglia, in overlapping regions of *Ngn2* and *Mash1* respectively. Indeed, in transgenic embryos with enhancer HOM2 or HOM1 controlling *LacZ* expression, X-Gal staining is detected in the cortex for HOM1-*LacZ* and in the basal ganglia for HOM2-*LacZ* (figure 23), indicating that HOM1 and HOM2 are specific element for *Ngn2* and *Mash1* respectively. In addition, no X-Gal staining is detected in the HOM1-*LacZ* transgenic embryos in a *Ngns*KO background (figure 23), showing that *Ngns* expression is necessary and sufficient to drive HOM1 expression *in vivo*. By doing similar experiment with HOM2-*LacZ* transgenic embryos bread in a *Mash1*KO background, no X-Gal staining was detected in the basal ganglia, showing that *Mash1* is required for the activation of HOM2 enhancer *in vivo* (data not shown).

In order to prove direct physical interaction between the Mash1 proteins and the *Dll1* promoter *in vivo*, chromatin immunoprecipitation specific to Mash1 were realized, using genomic DNA from E12.5 telencephalon. As described in figure 24, the enhancers HOM2 is pulled down with the Mash1 antibody in wild-type telencephalon. This experiment demonstrates that Mash1 directly binds to the *Dll1* promoter and, more specifically, recognizes EBoxes present in the HOM2 enhancer.

D Characterization of new potential targets of Mash1

Study of *Dll1* demonstrates that microarray data can be used in conjunction with promoter studies for the characterization of direct targets of proneural genes in the telencephalon. I have selected a total of 14 genes, mainly transcription factors and signal transduction proteins, upregulated in *Mash1* gain-of-function studies (liste in table 2). Expression pattern analysis by RNA *in situ* hybridization on E12.5 wild-type and *Mash1*KO sections, as well as, analysis of expression dynamic in *Mash1* transfected P19 cells, are under investigation. These results should provide the basis for further functional studies involving: i) promoter study to find conserved element containing putative EBoxes, and ii) chromatin immunoprecipitation to confirm binding to the predicted EBoxes.

My preliminary data with *Nrarp* and *Mfng*, demonstrate that these two candidates are expressed in the VZ of the cortex and basal ganglia and specifically lost in the *Mash1*KO ventral telencephalon (figure 25). *Mash1* seems thus necessary for the expression of *Mfng* and *Nrarp* in the basal ganglia at 12.5 days of development. RNA *in situ* hybridization screen for the remaining 12 potential Mash1 targets is ongoing. I also started to characterize the promoter of *Nrarp* and *Mfng* to find conserved EBox consensus binding sites. *Mfng* and *Nrarp* genomic sequences within the first intron and up to 10kb upstream of the transcriptional start site were analyzed with the multiple alignment tool from the University of California Santa Cruz (UCSC) (http://genome.ucsc.edu), to search for evolutionary conserved elements. I found two regions of about 200bp conserved between mouse,

GB	Name	Description	Ratio *Mash1*GOF
NM_021459	*Isl1*	transcription factor LIM homeodomain	2.5
NM_013915	*Zfp238*	transcription factor zinc finger	1.5
NM_007897	*Ebf1*	transcription factor collier	1.1*
AV306664	*Sox3*	transcription factor Sox	1.2*
NM_057173	*Lmo1*	transcription factor LIM	1.6
D49658	*Lhx8*	transcription factor LIM homeobox	1.5
NM_011546	*Zfhx1a*	transcription factor zinc finger homeobox	1.1*
NM_007570	*Btg2*	BMP/Smad modulator	1.6
NM_023117	*Cdc25b*	cell cycle tyrosine phosphatase	1.9
BM230524	*Rapgef5*	guanine nucleotide exchange factor	1.6
NM_008595	*Mfng*	glycosyltransferase	2.5
NM_010329	*Gp38*	glycoprotein	1.9
NM_025980	*Nrarp*	ankyrin repeat protein	1.6
NM_010488	*HuD*	RNA binding	1.5

Table 2: **List of genes upregulated in *Mash1*GOF.** (*) average value <1.5. GB: Gen bank accession number.

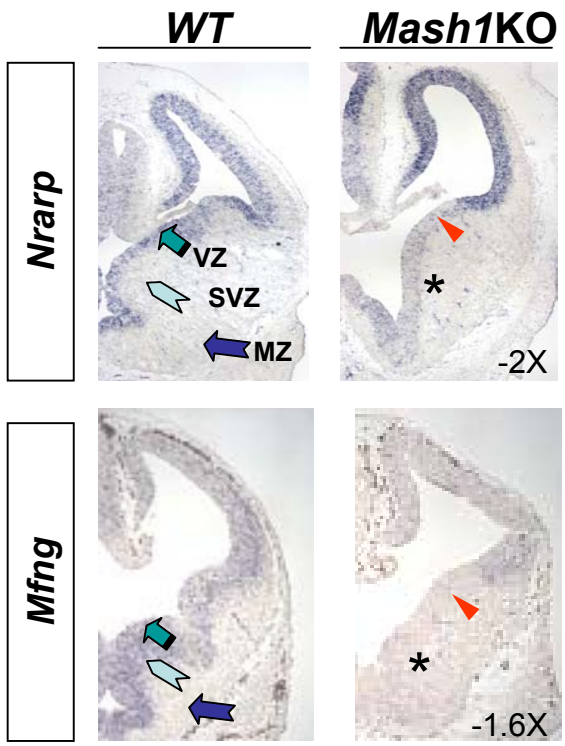


Figure 25: ***Mfng* and *Nrarp* expression in *Mash1*KO embryos.** *In situ* hybridization on wild-type and *Mash1*KO E12.5 embryos. Fold change obtained with Affymetrix is indicated. (*) atrophied MGE.

Nrarp homology region 1 (H1) (-100bp)

```
mm5.chr2      acgaccccgcctccgccccgcccccacgtgggcaccacccaatcgaaagagccctggtgtgcgcgtcaag
rn3.chr3      acgaccccgcctccgccccgcccccacgtgggcaccacccaatcgaaagtgccctggtgtgtgcgtcaag
hg17.chr9     acggcaccgcccacgccccgcccccatgtgggcgcggcccaatcccggggccgccggcgcgcgcgtcacg
canFam1.chr9  acg-ccccgccca-gccccgcccccacgtgggcgccgcccaatcgtagggccgccggcgtgcgcgtcacg
              *** * *****   **********.** ****** *  *******    *  * * ** * * ****** *
mm5.chr2      agcgtct-attgttatgcaaatataagaacc----gtaaaagacggcgagaagcggcgggcgaattagaa
rn3.chr3      agcatcc-attgttatgcaaatataagaaccaaccgtaaaa-actccgggaagctgcgggcgaattagaa
hg17.chr9     ggctgcc-attgttatgcaaatataaagaag----gtaaaaggcgccccggcgcggcgggcgagctagtg
canFam1.chr9  ggcgccctattgttatgcaaatataaaggggg----gtaaaaggcgccccggcgcggcgggcgggcgagtg
              **   *  ******************         ****** *   *  *  ** *******    **
```

Nrarp homology region 2 (H2) (-1600bp)

```
mm5.chr2      -tctaccaggtgtctgggctgctgggaagaaacactctcctctttcccacgctccccaacacattttaat
rn3.chr3      ttctaccaggtgtctgggctgctgggaagaaacactctcctctttcccacgctccccaacacattttaat
hg17.chr9     ttctaccaggtgtctgggctgctgggaagaaacactctcctctttcccacgct-cccgacacattttaat
canFam1.chr9  ttctaccaggtgtctgggctgctgggaagaaacactctcctctttcccacgctccccgacacattttaat
               ********************************************** *** ************
mm5.chr2      gaattagctggaagccccgccccccactgcccac-----tggcctccctcccctgctcagcgcctttaag
rn3.chr3      gaattagctggaagccccgccccccactgcccac-----tggcctccctcccctgctcagcgcctttaag
hg17.chr9     gaattagctggaagccccg-cccccactgcccgc-----caccctccct-ccctgcttagcgcctttaag
canFam1.chr9  gaattagctggaagccccgccccgcccgtccgcccgcgcgccctccctcccccgcctagtgc--ttaag
              ****************** ****  * * ** *       ******* *** **  ** **  *****
```

Figure 26: **Part of the multiple alignment of Nrarp genomic sequence.** Two regions (H1 and H2) conserved in the mouse, the human, the rat and the dog genome are found in the enhancer region of Nrarp. H1 is located 100bp and H2 1600bp upsteam from Nrarp transcriptional start site. One conserved EBox (black box) is found in each homology region.

human, rat and dog genomes located 100bp (region H1) and 1600bp (region H2) respectively upstream of *Nrarp* transcriptional start site. A search of transcription factor binding sites among these two homology regions using MatInspector (http://www.genomatix.de), revealed the presence of one conserved EBox in H1 and one EBox in H2 (figure 26). Analysis of *Mfng* genomic sequence using the same strategy revealed the presence of one Ebox in a region conserved between mouse, human, rat and dog located 150bp upstream the transcriptional start site and two conserved Eboxes within the first intron. These promoter analyses should provide the basis for the design of chromatin immunoprecipitation experiments in order to demonstrate that *Nrarp* and *Mfng* could be directly regulated by *Mash1* in the telencephalon. Notably, this strategy of promoter analysis successfully found the two homology regions found in the *Dll1* promoter (data not shown, figure 22).

This approach combining microarray data and promoter analysis should provide insightful information on genes probably directly regulated by proneural genes in the telencephalon. In addition the analysis of expression dynamic of candidates by quantitative PCR on *Mash1* transfected P19 cells constitute an interesting model to decipher rapidly between potential direct or indirect targets of *Mash1*.

DISCUSSION

Proneural transcriptions factors are essential for the proper acquisition of neural fate both in vertebrates and invertebrates. However very little is known about the downstream effectors of proneural proteins and how this program directs cells to adopt a neural fate. To begin to elucidate the genetic network regulated by proneural genes in the embryoninc mouse telencephalon, I developped a microarray strategy combining both gain and loss-of-function studies. Two complementary methods were used: i) screening of a subtractive library produced from *Neurogenins* mutants versus wild-type embryos, and ii) gene profiling using Affymetrix microarray. The present work is the first study comparing the regulatory cascades regulated by *Mash1* and *Ngns* in the mouse telencephalon. By combining both, loss and gain-of-function approach as well as promoter studies, we show that *Dll1* is a direct target of *Mash1* in the telencephalon. I also characterized 14 new genes potentially directly regulated *Mash1* in the basal ganglia. Further studies are ongoing to confim this possibility.

A precedent screen by Mattar et al. investigated *Neurogenins* downstream effectors using a strategy very similar to the subtractive library described here (see, Construction of a subtractive library, p34) (Mattar et al., 2004). However, the present study provide a more comprehensive analysis of proneural downstream effectors, since: i) genes analyzed by Affymetrix microarray spans a large part of the mouse genome, ii) *Mash1* and *Ngn2* downstream transcriptional programs were analyzed, allowing to study common and specific regulatory cascades of proneural genes, iii) gain-of-function analysis are not limited by compensatory mechanisms and indirect effects that occur in loss-of-function studies (ie mutants embryos). The subtractive screen I designed should also have the advantage to screen for genes regulated in progenitors rather than in postmitotic neurons. Indeed, to build the subtractive library, I specifically sorted low expressing GFP cells by flow cytometry and exclude the high expressing GFP cells fraction which are likey to correspond to postmitotic neurons where GFP is accumulating. This assumption has however to be confirmed, as data obtained from this subtractive screen are very preliminary.

1 Characterization of downstream effectors of proneural genes

Mash1, *Neurogenins1* and *Neurogeneins2* are the three known proneural transcription factors expressed in the mouse telencephalon during neurogenesis. *Neurogenines* are absolutely required to induce a glutamatergic fate in early cortical neurons, and *Mash1* is necessary to induce a GABAergic phenotype in ventral neurons. Very few components of the downstream pathway induced by proneural genes are known. It includes *NeuroD*, *Nscl1*, *Tbr1*, *Math2*, *VGlut1* and *VGlut2* for *Neurogenins*, and *Dlx*, *GAD1* and *GABA* transporters for *Mash1* (Schuurmans et al.,

2004). New potential targets of proneural proteins were characterized using a microarray approach on mouse embryos lacking proneural genes expression.

I first validate my Affymetrix microarray approach by analysing the expression data of genes known to be up- or down-regulated in the cortex of *Ngns*KO embryos and characterized 12 genes misregulated in the basal ganglia of *Mash1* mutant embryos and 12 genes in *Ngn2* mutant cortices. These genes were selected based on microarray data (fold change, absolute signal) and genes annotations (focusing primarily on transcription factors and signalling molecules). However, if I succeeded to find many genes expressed in the ventricular zone (VZ) and subventricular zone (SVZ) (ie dividing progenitors) in the basal ganglia, genes in the cortex were mainly expressed in the cortical plate (CP). It is probable that genes misregulated in the postmitotic neurons in the CP or in the MZ are not directly regulated by *Ngn2* or *Mash1*, but rather were downregulated in proneural mutant embryos as a consequence of the loss of neurons or cortical identity. In this case comparison with gain-of-function studies is important, and should allow the identification of genes more directly regulated by proneural genes.

In the cortex, I confirmed the microarray data by *in situ* hybridization for 5 candidate genes: the adhesion molecule L1CAM, the transcription factor MIBP and 3 genes of unknown function (NM029758, BC052038 and AI840429), expressed mainly in the CP and downregulated in *Ngn2*KO embryos cortex. The transcription factor MIBP was shown to induce the expression of the somatostatin receptor II, the main receptor for somatostatin in the cortex (Dorflinger et al., 1999), which is also downregulated in the cortex of *Ngn1;Ngn2*DKO, based on microarray data (-2X compared to wild-type cortex). As somatostatin is involved in the process of cortical lamination (Imaki et al., 1994) and was shown to be misregulated in the cortex of *Ngn2* mutant embryos (Mattar et al., 2004), it is possible that the downregulation of MIBP1 in *Ngn2*KO embryos could account for the defect observed in the preplate of these mutants (Schuurmans et al., 2004). However this process can be quite indirect, since loss of MIBP1 could be due to the change of overall phenotype of CP neurons (as glutamateric neurons acquire a GABAergic fate in *Ngns*KO). The other gene with known function downregulated in the cortex of *Ngn2*KO embryos is L1CAM. This gene is expressed at high levels in the CP and at lower levels in the SVZ in the cortex of wild-type embryos. As L1CAM is an adhesion molecule important for axonal guidance (Haspel and Grumet, 2003), the loss of L1CAM expression could account for the thalamocortical pathfinding defects observed in *Ngn2* mutant cortices (Mattar et al., 2004; Seibt et al., 2003). However, this gene is not induced in *Ngn2* gain-of-function studies (as for the other genes MIBP, NM029758, BC052038 and AI840429), which indicates that even if L1CAM downregulation in the SVZ is a consequence of the loss of *Ngn2*, it is probably far downstream of *Ngn2* activation program.

In the basal ganglia, 5 genes expressed in the VZ or SVZ were found to be downregulated in *Mash1*KO: *Pref-1/Dlk1, Nlk, Sox6, Nrarp,* and *Gp38*. Expression pattern of these genes make them attractive candidates to be regulated by *Mash1* in the basal ganglia. *Pref-1, Nrarp,* and *Gp38* are in addition strongly upregulated in *Mash1* gain-of-function. The case of *Nrarp* is particularly interesting as this protein was demonstrated to be involved in the regulation of Notch signalling. Studies in *Xenopus* showed that Notch signalling activates directly *Nrarp*, which, in association with Notch intracellular domain ($Notch^{ICD}$) and XSu(H), result in the decreased level of $Notch^{ICD}$ (Lamar et al., 2001a; Pirot et al., 2004). Analysis of the Nrarp promoter showed in addition the presence of an evolutionary conserved EBox motif (Pirot et al., 2004) similar to the consensus binding site for *scute*-related transcription factors (CCACGTGC). An attractive hypothesis would involve the activation of *Nrarp* by proneural genes in the process of inhibition ofNotch signalling in neuronal precursors. In this case neuronal progenitors expressing proneural genes would decrease Notch signalling by the activation of *Nrarp* in a negative feedback loop, allowing neuronal differentiation. In other words, *Mash1* could induce directly *Nrarp* in order to promote the switch off Notch signalling in neuronal progenitors. The transcription factor *Sox6* is also an interesting candidate. *Sox6* is required for proper P19 cells differentiation into neurons (Hamada-Kanazawa et al., 2004). In the mouse, *Sox6* null mutation suggests that this transcription factor is involved in maintaining the normal physiological function of muscle tissues, such as of the heart (Hagiwara et al., 2000). However, the exact role of *Sox6* during development of the central nervous system remains to be elucidated.

Finally my loss-of-function studies showed that a small fraction of genes are misregulated in the cortex of *Mash1* and *Ngn1* single mutants. Since the role of *Mash1* in the cortex is still unknown, the analysis of misregulated genes in this mutant should provide precious information of Mash1 function in this tissue. Transcription factors such *v-Maf*, *Mef2c* as well as the ubiquitin-ligase *Ring1-b*, *Znrf2* and *Ariadne* are downregulated in the cortex of *Mash1*KO embryos. It would be interesting to confirm their expression in the cortex by *in situ* hybridization.

2 Genes upregulated in the basal ganglia of Mash1 mutant embryos, a potential compensatory mechanism?

Mash1 is the unique proneural genes known to be expressed in the basal ganglia to date. Precedent studies showed that *Mash1* is essential for the specification of GABAergic neurons in the medial ganglionic eminence (MGE) (Casarosa et al., 1999). However, Progenitors in the lateral ganglionic eminence (LGE) are still specified indicating that the proneural function of *Mash1* can be compensated by a still unidentified mechanism. In addition, beside in the specification of the GABAergic fate, downstream effectors of *Mash1* are unknown. This screen allowed the

identification of 6 genes upregulated in the VZ of *Mash1*KO basal ganglia: *Peg1, Peg3, Asb4, Tssn1, Lhx6* and *mEvf1*. Premature expression of *Dlx1* and *GAD67* was already shown in the VZ of *Mash1*KO embryos and ascribed to a loss of Delta-Notch signalling in the basal ganglia (Casarosa et al., 1999). Recently, Faedo *et al.* showed that *mEvf1* expression is lost in *Dlx1/2* mutant and ectopically expressed in the cortex of *Pax6* embryos, suggesting that *mEvf1* is regulated by *Dlx* genes in the embryonic telencephalon (Faedo et al., 2004). I show here that *mEvf1* is expressed prematurely in the VZ of *Mash1*KO embryos and in the CP of *Ngn2*KO embryos, probably as a consequence of premature *Dlx* expression. These results suggest that *mEvf1* could be involved downstream of *Dlx1* and *Dlx2* in GABAergic specification. The LIM homeobox genes *Lhx6* is also upregulated in the VZ of the basal ganglia, but only in the presumptive mutant MGE (MGE*). This gene was suggested to be involved in the specification of striatal interneuron expressing NeuropeptideY and Somatostatin (Marin et al., 2000). It was shown that GABAergic precursors are not specified in the MGE* of *Mash1*KO embryos, but other type of interneuron were not analyzed in *Mash1*KO embryos (Casarosa et al., 1999). It is thus possible that the premature expression of *Lhx6* in the VZ corresponds to a precocious differentiation of NeuropeptideY and somatostatin positive interneuron, in a way similar to what is observed in the mutant LGE (LGE*) with *Dlx* genes.

Overall, the role of upregulated genes in the *Mash1*KO basal ganglia is not known. However, it is tempting to raise the hypothesis that prematurely genes expressed such *mEvf1*, *Lhx6* and *Dlx*, are involved in compensatory mechanisms leading to GABAergic specification in a *Mash1*-independant pathway. Indeed, GABAergic neurons are still specified in the LGE in the absence of *Mash1* proneural activity, which indicates that others still unidentified factors can drive GABAergic specification in *Mash1*KO basal ganglia (Casarosa et al., 1999; Schuurmans et al., 2004). Genes upregulated in the VZ of *Mash1*KO and identified in this screen could be involved in this *Mash1* compensatory mechanism.

Another possibility is that *Mash1* represses particular genetic pathways or cell fate beside its role in GABAergic specification, or that in absence of *Mash1* some neural progenitors endow an alternative fate. It was shown for instance, that progenitors in the MGE has the potential to produce either GABAergic interneuron or oligodendrocyte (He et al., 2001). Interestingly, beside *mEvf1* and *Lhx6* which are prematurely expressed in the VZ, the four remaining genes upregulated in the VZ in the basal ganglia, *Tssn1*, *Peg1*, *Peg3* and *Asb4*, are also strongly ectopically expressed in the VZ of the septum and entopeduncular area (located more medially compared to the MGE, see figure 10), a region which produce GABAergic and cholinergic interneuron as well as oligodendrocyte (Soria et al., 2004; Taglialatela et al., 2004; Spassky et al., 2001; Marin et al., 2000). *Mash1* is expressed in the septum and in the entopeduncular area (Fode et al., 2000). Based on microarray data, I found

that several markers of interneuron subpopulations are upregulated in the basal ganglia of *Mash1*KO embryos, such as *Calb1*, *Calb2* and *NeuropeptideY* (Marin et al., 2000; Xu et al., 2004), suggesting that generation of these interneurons could be affected in *Mash1*KO embryos.I*n situ* hybridization can be envisaged to determine if other interneuron population (different from GABAergic interneuron) are misregulated in *Mash1*KO basal ganglia.

3 Nlk and Pref-1 two genes potentially implicated in neurogenesis in the mouse telencephalon

Pref-1 and *Nlk* were identified in my microarray screen to be strongly upregulated in the cortex of *Ngns*KO embryos and to a lesser extend in *Mash1;Ngn2*DKO cortices. In addition, *Nlk* and *Pref-1* expression are lost in the SVZ and VZ respectively in *Mash1*KO basal ganglia. Together these results suggest that *Pref-1* and *Nlk* are regulated by *Mash1* in the basal ganglia and in the cortex of *Ngn2*KO embryos. However, as described in figure 11B, *Pref-1* is still upregulated in the cortex of *Mash1;Ngn2* embryos (3 fold increase compared to 27 fold increase in the *Ngns*DKO), indicating that its upregulation in the cortex of *Ngns*KO embryos is also in part a consequence of the loss of *Ngns* inhibition. *Nlk* is not upregulated in gain-of-function studies, suggesting either that its regulation in proneural mutant embryos is an indirect consequence of the loss of proneural activity or that its regulation is not visible in the condition of our model system. The *Mash1*-dependant upregulation of *Nlk* and *Pref-1* in the cortex of *Ngn2*KO embryos and their expression in the VZ or SVZ in wild-type basal ganglia, suggest a potential role of these two genes in the specification of the GABAergic phenotype. Further experiments are required to investigate the possible involvement of *Nlk* and *Pref-1* in the specification of GABAergic neurons. Gain-of-function in the cortex by electroporation could provide a rapid answer.

Mutation of *Nlk* in the mice didn't show any defect in the telencephalon neither in dorsoventral patterning nor in specification of neuronal subpopulation in the ventral forebrain. However, a subset of interneuron expressing tyrosine hydroxylase (TH+) are probably lacking in the olfactory bulb of E18.5 embryos (preliminary data from Lydia Teboul, data not shown). Interestingly, interneurons in the olfactory bulb are produced in the SVZ of LGE and migrate rostrally along the rostral migratory stream to invade the granular layer of the olfactory bulb, from embryonic stage E13.5 and throughout adult life (Wichterle et al., 2001; Lois and Alvarez-Buylla, 1994; Stenman et al., 2003). Defect in the generation or in the migration of subsets of neuronal progenitors in the SVZ could thus account for the phenotype observed in the *Nlk* mutants.

It is also important to note that the genetic background modulates the phenotype of *Nlk* mutant mice. Indeed, *Nlk* mutant mice breed to 129/Sv genetic background survive up to 4-6 weeks after birth, while in the C57BL/6 genetic background embryos die before birth (Kortenjann et al., 2001). Our *Nlk* line was breed in a 129/Sv genetic background. We now plan to produce *Nlk* mutant mice

in a C57BL/6 genetic background to determine if these mice exhibit a stronger phenotype in the CNS.

In the case of *Pref-1* mutant embryos, no defect was detected in the telencephalon. *Pref-1* is a paternally imprinted gene. Imprinted genes are organized in clusters, where cis-acting regulatory elements are believed to control multiple genes. The mouse *Pref-1* and *Gene-trap locus2* (*Gtl2*) genes form a pair of linked reciprocally imprinted genes located on distal mouse chromosome 12 and human 14q32 (Wylie et al., 2000; Lin et al., 2003b). *Pref-1* is expressed only from the paternal allele, while *Gtl2*, which encodes a non-coding RNA, is expressed only from the maternal allele. Characterization of *Gtl2* and *Pref-1* expression has shown that these two genes are coexpressed in many tissues (Schmidt et al., 2000), and, interestingly, *Gtl2* is upregulated in the cortex of *Ngn2*KO embryos (2.6X, annex3), as *Pref-1*. Co-regulation of *Pref-1* and *Gtl2* is perhaps fortuitous, but *Gtl2* loss-of-function leads to growth retardation, as *Pref-1* mutants mice (Schuster-Gossler et al., 1996). It would then be interesting to characterize the phenotype of embryos miss-expressing both *Pref-1* and *Gtl2*. In addition, as *Pref-1* levels are important for proper embryonic development, we have started to characterize gain *Pref-1* of function in embryonic mice. We started to analyze the phenotype of embryos overexpressing *Pref-1* using BAC-Pref-1 transgenic embryos obtained from Anne Ferguson-Smith (Cambridge, UK). In these mice, BAC (Bacterial Artificial Chromosome) containing the *Pref-1* locus (coding sequence and part of its regulatory elements) were inserted randomly in the genome, allowing the overexpression of *Pref-1* in its normal domain of expression (Lee et al., 2001). Such study should allow to characterize the role of *Pref-1* during development and avoids possible functional redundancy of loss-of-function experiments.

4 Characterization of targets of proneural genes by gain-of-function studies

Gain-of-function of proneural genes allowed the characterization of genes regulated by *Ngn2* and/or *Mash1*. In the electroporation experiments, embryos were cultivated for 18hrs after electroporation. We choose this timing based on results with the Pro*Dll1-LacZ* reporter which was strongly activated 18hrs after *Mash1* electroporation (see figure 15), providing a good indication of the time needed to observe activation of a putative proneural direct target. However, it is not possible to conclude directly from our gain-of-function studies that upregulated genes are directly regulated by proneural genes as second order targets can also be activated (ie genes regulated by direct targets of proneural transcription factors).

Genes involved in Delta-Notch signalling, *Dll1*, *Dll3*, *Hes5* and *Hes6*, were found to be regulated by both *Ngn2* and *Mash1* in gain-of-function studies. This observation confirms precedent studies showing activation of *Delta* homologues by proneural genes, and suggests also that *Mash1* acts with *Hes6* in a positive feedback loop to promote neuronal differentiation, as it is the case for *Ngns*

(Koyano-Nakagawa et al., 2000). The finding of *Hes5* upregulation is more surprising as this transcription factor was shown to repress proneural activity (Ohtsuka et al., 2001). A possibility could be that this upregulation is the consequence of a strong Notch activation in cells adjacent to proneural transfected cells. Another explanation is that *Mash1* does activate *Hes5* transcription. In *Drosophila*, it has been shown that achaete directly activates the promoter of *E(spl)* (Kramatschek and Campos-Ortega, 1994; Singson et al., 1994), in order to maintain the origin steady state within the proneural cluster before singling-out of the neural progenitors (Jafar-Nejad et al., 2003; Modolell, 1997). Microarray data indicates that same find of mechanisms could occur within the mouse neuroepithelium in order to kepp an equilibium between differentiation and progenitor stage. Other genes were specifically induced by *Mash1*, including several ventral determinants like *Lhx6*, *Lhx8*, *TH* and *Isl1*, suggesting that *Mash1* would have a much broader panel of activation that GABAergic differentiation (*Lhx8* and *TH* are involved in the specification of cholinergic interneuron for instance (Zhao et al., 2003)). In this context, it is surprising that GABAergic differentiation markers like *Dlx1*, *Dlx2*, and *GAD67* are not upregulated in our *Mash1* electroporated embryos at a stage which is permissive for their expression (Eisenstat et al., 1999). This suggests a limitation in our *Mash1* gain-of-function model and would explain the relative low number of misregulated genes in this experiment. For instance the *Mash1* activity could be impaired by an essential cofactor expressed at limited levels in the basal ganglia.

Ngn2 specific targets were also identified with our gain-of-function model. I found that four *Frizzled* receptors (*Frizzled1*/*2*/*3* and *6*) are upregulated by *Ngn2* in the cortex. A recent paper from Backman et al. showed that the canonical Wnt signalling pathway is necessary for proper cortical identity (Backman et al., 2005). Indeed, by conditional inactivation of β-catenin in the telencephalon (using a *Nestin*-Cre line), the authors observed ectopic activation of *Mash1*, *Dlx2*, *Dlx5* and *Gsh2* in the cortex together with the loss of cortical marker like *Ngn2*, *Emx1*, *Emx2* and *Pax6*. Interestingly, this effect was observed only when Wnt signalling was impaired before E11.5. When β-catenin was inactivated at later stages (after E11.5), no dorso-ventral patterning defect were observed (Backman et al., 2005). In the *Ngns* loss-of-function data (realized at E13.5), no decreased of *Frizzled* receptors were observed. The gain-of-function data indicates that *Ngn2* could have a role in *Wnt*-mediated dorso-ventral patterning, for instance by giving the competence of dorsal cells to *Wnt* signalling. It would be interesting to see if *Frizzled* receptors expression is lost at E10.5 in the cortex of *Ngns;Mash1*DKO embryos, when *Wnt* signalling has a crucial role in dorso-ventral patterning.

The expression of *NeuroD* is not increased in the *Ngn2* gain-of-function data. This finding is in contradiction with precedent work in *Xenopus* and P19 cells showing an upregulation of *NeuroD* following *Neurogenin* overexpression (Farah et al., 2000; Perron et al., 1999). However, studies in

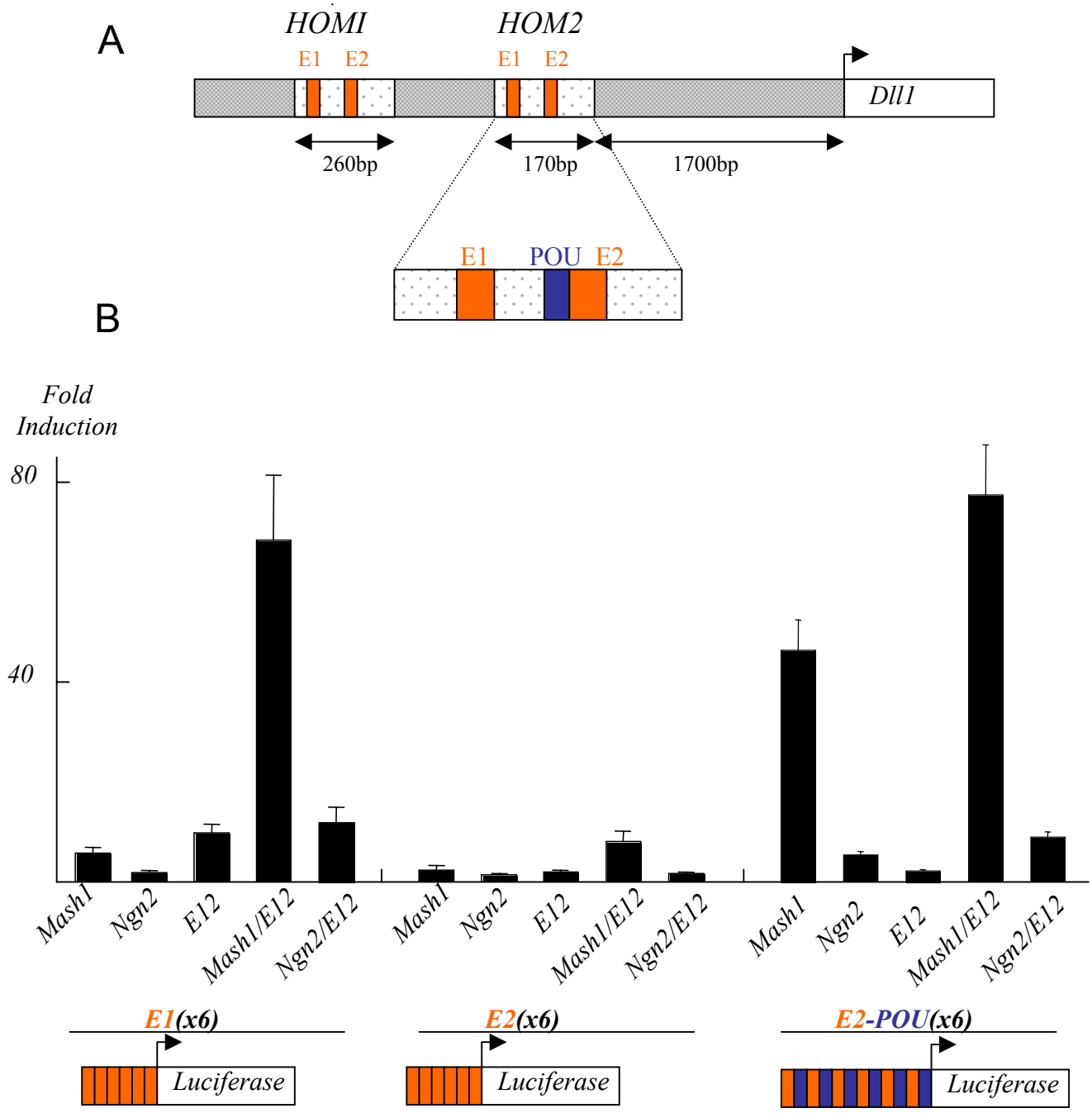


Figure 27: **E1 but not E2 is sufficient to drive Mash1 activity in P19 cells.** A) The enhancer HOM2 contains two conserved EBoxes (E1 and E2 in orange). B) Concatemers of E1 respond to the heterodimer Mash1/E12 but not to Ngn2/E12. E2 needs the presence of 8bp just adjacent to the Ebox which correspond to the consensus binding site for POU transcription factors (in blue, see figure 28).

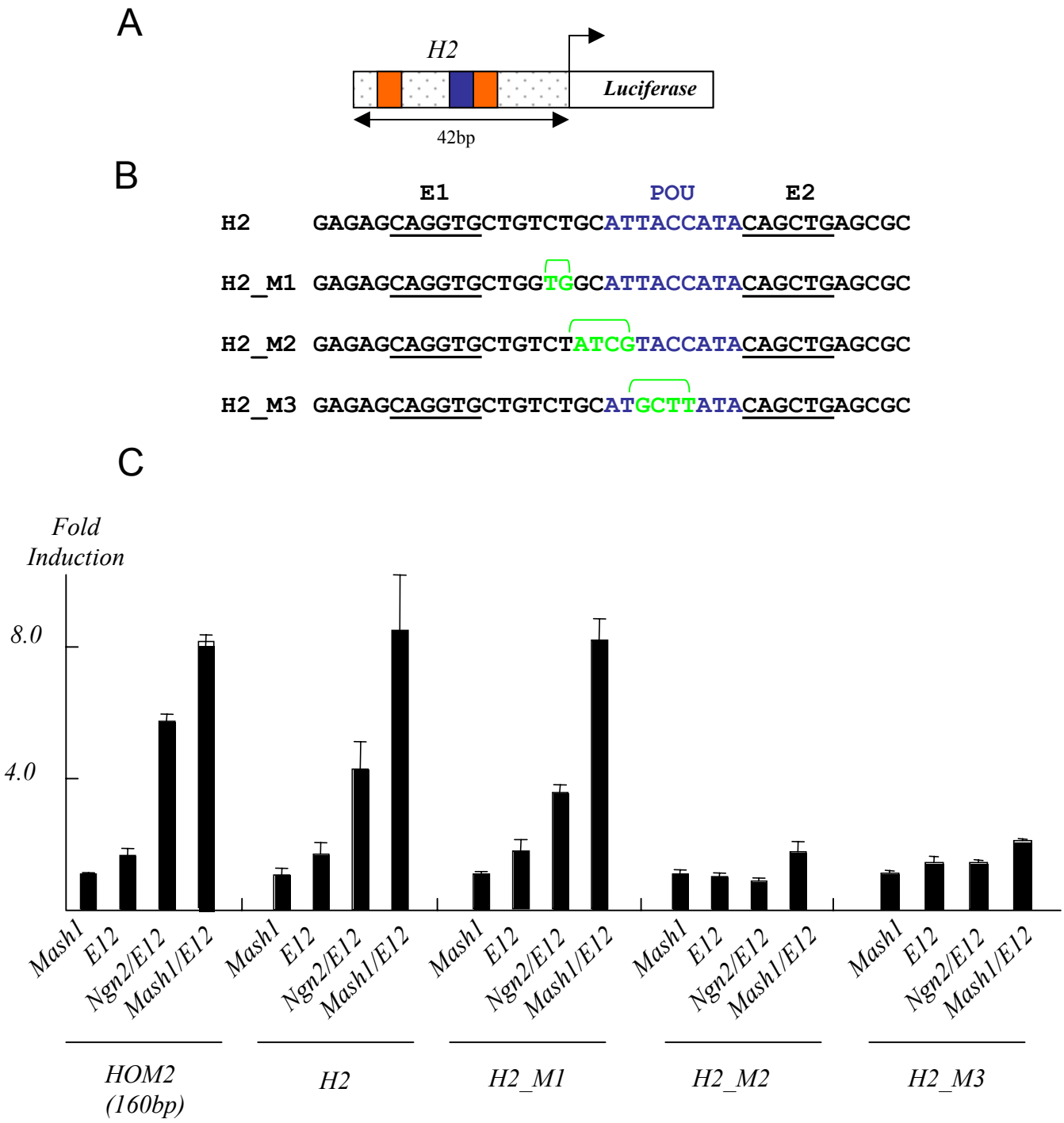


Figure 28: **The POU domain adjacent to EBox E2 is essential to drive Mash1 activity in P19 cells using luciferase assay**. A) H2 promoter is a part of the HOM2 enhancer of 42 bp comprising the two EBoxes and the adjacent POU domain (in orange). B) Detailed sequence of the H2 promoter and its mutated variants (H2_M1, H2_M2 and H2_M3) used to drive luciferase expression. Mutated positions are indicated in green. C) Luciferase assay with the different H2 constructs. The POU domain is required for Mash1 activity (mutated in H2_M2 and H2_M3).

Xenopus suggest that *NeuroD* is activated only when proneural expression reach a high level of expression in neural progenitors, a process showed to involve positive feedback loops between *XNgn1* and *XCoe2* (othologuous gene of the the mouse *Ebf2*) (Dubois et al., 1998). In addition, *in vitro* studies with *Neurogenins* transfected cell line showed that *NeuroD* is detected only 36hrs after transfection (Farah et al., 2000; Gasa et al., 2004). It is thus possible that in our model system *NeuroD* is not found 18hrs after electroporated but would have been detected after 24 or 36 hours.
In contrast genes involved in GABAergic differentiation in the striatum like *Mash1*, *Dlx1*, *Dlx2* and *GAD1* were found to be downregulated in *Ngn2* electroporated cortices. This finding is surprising as GABAergic determinants are already restricted in the basal ganglia at E10.5 and are not detected in the cortex at this stage by immunofluorescence (Yun et al., 2002). Nevertheless, *Mash1*, *Dlx1*, *Dlx2* and *GAD1* are expressed in control cortex and are decreased in *Ngn2*GOF, based on Affymetrix microarray data. This result could indicate that fragments of the LGE were taken with cortical transfected tissue during the dissection. If this is the case, several downregulated genes in the *Ngn2* gain-of-function study would be a consequence of forced *Ngn2* expression in the basal ganglia. However, this hypothesis contradict the observation that *Ngn2* can not affect the specification of striatal neurons as observed in the *Mash1*KI*Ngn2* mice (Parras et al., 2002).

5 Dll1 is a direct target of Mash1

Expression studies by *in situ* hybridization showed that, compared to wild-type situation, Dll1 was lost in the VZ of *Mash1* mutant embryos. In contrast, *Dll1* expression increased after *Mash1* or *Ngn2* overexpression, suggesting a regulation of *Dll1* by proneural genes. We show by chromatin immunoprecipitation that *Mash1* interacts physically with two Eboxes present in the *Dll1* enhancer. In addition, *Ngn2* and *Mash1* recognize distinct region of the *Dll1* enhancer, as showed by luciferase assay and transgenic mouse embryos. Remarkably, Eboxes present in *Ngn2* and *Mash1* enhancers (HOM1 and HOM2 respectively) are similar to consensus binding site expected for *atonal* and *scute* related specific EBox. Indeed, the two Eboxes present in HOM1 enhancer, CAGATG and CATTTG, are close to the consensus CA(G/T)GTG proposed for *ato*-related binding site (Powell et al., 2004). In HOM2, the two Eboxes found in this enhancer, CAGGTG (EBox1) and CAGCTG (EBox2), fit exactly the consensus binding site proposed for *scute*-related protein CAG(C/G)TG (Powell et al., 2004). In order to characterize further *Mash1* specific EBoxes in HOM2 enhancer, Diogo Castro in the laboratory realised luciferase assay in P19 cells showing that both Eboxes are not equivalent. Indeed, if concatemers of EBox1 are sufficient to drive *Mash1* activity, the Ebox2 requires the presence of 8bp flanking sequence (figure 27). Analysis transcription binding site shows that this flanking region contains a putative domain for the binding of POU (*Oct* and *Brn*) transcription factors which is required for Mash1 activation (figure 28). An attractive hypothesis is that, to be functional, Ebox2 requires the presence of Mash1 together with

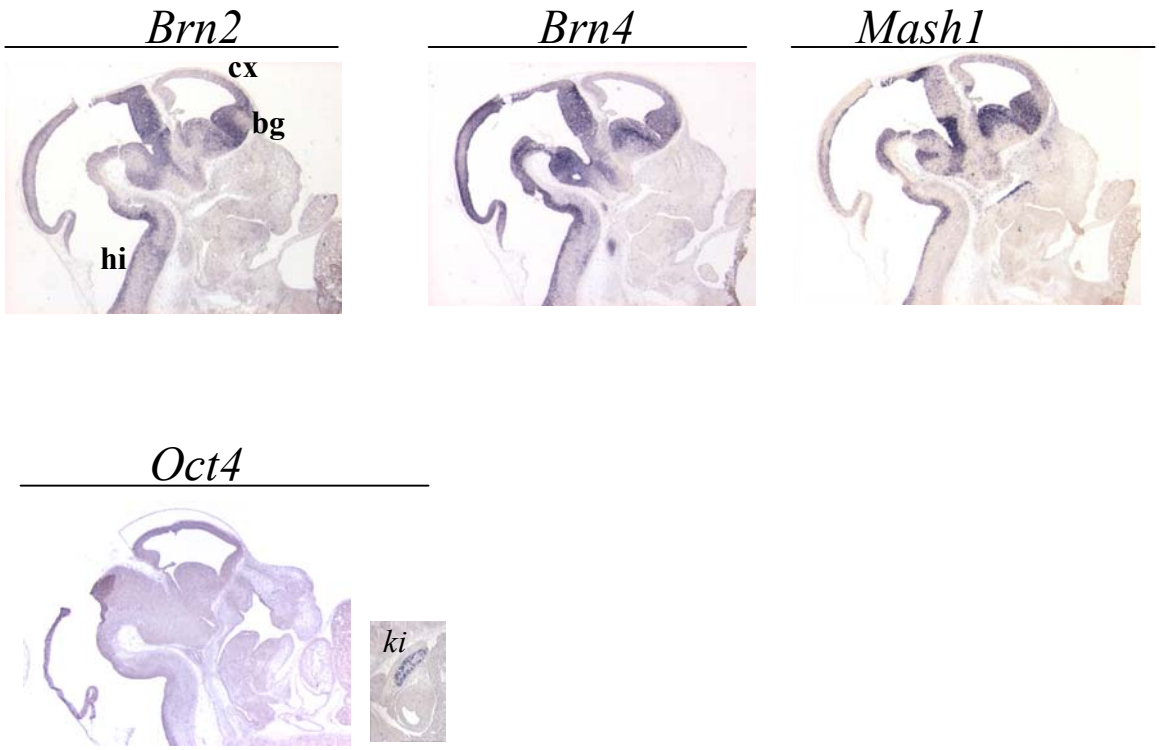


Figure 29: **Expression pattern of POU transcription factors in the embryonic CNS**. RNA *in situ* hybridization on E12.5 wild-type adjacent sections. *Brn2* and *Brn4* are localized in overlapping regions of the telencephalon and hindbrain with *Mash1*. *Oct4* is expressed in the kidney (ki) but not in the CNS. Cx: cortex, bg: basal ganglia, hi: hindbrain.

its putative POU cofactor. In other words, Mash1 and its cofactor must be coexpressed to activate EBox2 on the HOM2 enhancer. In order to characterize potential *Mash1* cofactor, I started RNA *in situ* hybridization with several members of the POU *Oct* and *Brn* family. Expressions of *Brn2* and *Brn4* overlap *Mash1* expression in several regions of the CNS including the basal ganglia as shown by RNA *in situ* hybridization on wild type E12.5 serial sections (see figure29). In contrast *Oct4* is not expressed in the CNS. I am currently testing expression of *Oct1*, *Oct2* and *Brn1*. These data should provide indication on *Mash1* putative cofactor for further studies. Luciferase essays are ongoing to test if POU proteins can synergise with Mash1 for the activation of the HOM2 Dll1 enhancer.

Taken together, these results suggest that *Dll1*, a target of both *Ngn2* and *Mash1*, is regulated through two distinct enhancers specific to *Mash1* (HOM2 enhancer) or *Ngn2* (HOM1 enhancer). In addition, *Mash1* HOM2 enhancer contains an EBox (E2) which require the presence of an adjacent POU domain. Interestingly, this EBox E2 is suboptimal in the flanking region of the EBox: consensus sequence GCAG(C/G)TG(G/T) compared to ACAGCTGA. If we consider that EBox2 is a suboptimal binding site for Mash1, a potential role of the cofactor would be to work in synergy with Mash1. Such mechanism was already proposed in vertebrates. For instance, in the spinal cord, Isl1 and Lhx3 synergize with NeuroM to activate the Hb9 promoter during motoneuron differentiation (Lee and Pfaff, 2003). An interesting possibility is that the EBox-Oct motif found in the *Dll1* promoter could be more generally involved in the regulation of genes implicated in neurogenesis. To investigate this possibility, we searched for EBox-Oct motif at the level of the mouse genome. This very preliminary analysis was done in collaboration with Ian Donaldson (Cambridge Institute for Medical Research, Cambridge, UK). We found, the EBox-Octamer motif in the promoter of several genes regulated by *Mash1* in the gain and loss-of-function studies, such as *Dll3*, *HuB*, *Ebf1* and *Isl1*, suggesting that this motif could be used for the regulation of other *Mash1* targets during neurogenesis. However, we still do not know if these motifs are functional and if they can bind Mash1. Chromatin immunoprecipitations are currently under investigation to answer this question.

6 Characterization of new potential targets of proneural genes

Microarray data from gain-of-function studies of proneural genes provides insights into the potential direct targets of *Ngn2* and/or *Mash1* during neurogenesis in the mouse telencephalon. In order to confirm microarray data and try to decipher between direct and secondary order targets of proneural genes, I designed an assay in P19 cells to analyze the dynamic of expression of potential targets after *Mash1* transfection. The expression of *Dll1* in this assay should provide a good reference to determine if one given gene is potentially directly regulated by Mash1.

GB	Name	Motif (POU-Ebox)*	POU-EBox position
NM_021459	*Isl1*	yes	126bp 5’
NM_013915	*Zfp238*	yes	95bp 5’
NM_007897	*Ebf1*	yes	within intron1
AV306664	*Sox3*	yes	525bp 3’
NM_057173	*Lmo1*	no	na
D49658	*Lhx8*	no	na
NM_011546	*Zfhx1a*	no	na
NM_007570	*Btg2*	yes	2kb 5’
NM_023117	*Cdc25b*	yes	within intron1
BM230524	*Rapgef5*	yes	57kb 5’
NM_008595	*Mfng*	no	na
NM_010329	*Gp38*	no	na
NM_025980	*Nrarp*	no	na
NM_010488	*HuD*	no	na

Table 3: **List of 14 genes selected for screening of potential direct targets of Mash1.** Several genes upregulated in *Mash1*GOF were found to have in their promoter an Ebox with the adjacent POU domain (Ebox-POU motif)*. These genes are very good candidates to be directly regulated by Mash1 in the telencephalon (*Isl1*, *Zfp238, Ebf1, Sox3, Btg2, Cdc25b, Rapgef5*). Position of the motif compared to the potential start of transcription is indicated (na: not applicable). Other genes do not possess the E-POU motif but are upregulated in *Mash1*GOF and were selected in this screen based on their function and/or expression (*Lmo1, Lhx8, Zfhx1a, Mfng, Gp38, Nrarp, HuD*).

POU Ebox

***** Sequence of the E-POU motif: TGCATTACCATACAGCTGA

We defined a strategy for the characterization of new *Mash1* targets as follows: i) upregulation in gain-of-function studies, ii) dynamic of expression similar to *Dll1* in the P19 essay, iii) promoter analysis to find putative EBoxes in evolutionary conserved regions (alignement tool from the University of California Santa Cruz (UCSC) (http://genome.ucsc.edu)), iv) confirmation of binding to EBoxes using chromatin immunoprecipitation. Similar strategy has been succesfully used recently in *Drosophila* for the characterization of proneural targets in the wing imaginal disc (Reeves and Posakony, 2005).

The list of genes we selected for the strategy described above is provided in table 3 (see also table 2). It comprises genes upregulated in *Mash1* gain-of-function study which possess the EBox-Octamer motif in their promoter, such as *HuB*, *Ebf1* and *Isl1,* and other presumably not regulated through this motif (*Nrarp*, *Mfng*, *Gp38*). It will be interesting to study the promoter of genes upregulated in *Mash1* gain-of-function studies but that lack the EBox-POU motif. Indeed, analysis of conserved element in these genes may allow the detection of new enhancers and perhaps new potential transcription factor binding site specific to *Mash1* cofactors. This possibility is currently under investigation.

Analysis of *Dll1* enhancer specific to *Ngn2* (HOM1) did not yield to the characterization of a conserved motif next the Eboxes. A possibility is that *Ngn2* does not require cofactors for the activation of *Dll1* promoter. However as *Ngn2* can not respecify GABAergic interneurons into glutamatergic excitatory neurons, it was proposed that *Ngn2* does require cofactors for its activity (Parras et al., 2002). In this context, we started to analyze conserved element in other genes upregulated in *Ngn2*GOF such as *Ebf1, Ebf2* and *Nscl2*, and potentially directly regulated by Ngn2 (Dubois et al., 1998; Talikka et al., 2002). Alternatively, proneural cofactors do not necessarily bind just next to EBoxes, making the search of functional motif more difficult.

Our data suggest that *Dll1*, a common target of proneural activity, could be regulated by separated enhancer specific to Mash1 or Ngn2 (HOM2 and HOM1 in this case respectively). Further characterization of the HOM2 enhancer show that concatemers of E1 is specific of Mash1 and is not activated by Ngn2 in Luciferase assay, indicating that specificity of proneural genes can be provide solely by EBox sequence. In contrast, the other Ebox (E2) in HOM2 requires the presence of a cofactor just next to it, which could provide an additional level a modulation of Dll1 expression in different tissue. However, we still do not know if EBox1 and EBox2-POU motif in the HOM2 enhancer are both required *in vivo*. Transgenic analyses are ongoing with mutated EBoxes to investigate this question. More generally, we do not know if this strategy of specific enhancer (HOM1 specific to *Ngn2* and HOM2 specific to *Mash1*) could represent a common mechanism for the activation of other proneural targets. A simple model would be that specific target of Mash1 and Ngn2 would possess only one specific enhancer, while common proneural target possess both

enhancers. The characterization of new proneural targets with the strategy described above should provide elements to understand the transcriptional mechanisms regulated by *Mash1* and *Ngn2* during neurogenesis.

CONCLU SION

In the past few years considerable progress has been made in our understanding of the function of proneural transcription factors during neurogenesis. Studies from our laboratory and other international groups revealed that proneural genes were essential for determination and specification of neuronal fate in many regions of the nervous system (reviewed in (Bertrand et al., 2002; Ross et al., 2003). During the course of my PhD, I realized a microarray screen to find the molecular pathways regulated by proneural genes during the development of the mouse telencephalon. This study revealed a role of *Neurogenins* in the specification of cortical identity. In particular, we showed that in the developing cortex, *Neurogenine1* and *Neurogenin2* are required for the acquisition of the glutamatergic phenotype and laminar character of early-born neurons, while simultaneously repressing an alternative GABAergic subcortical fate (Schuurmans et al., 2004). I also found several downstream effectors of proneural genes. In particular, I characterized two genes regulated by *Mash1* in the basal ganglia, *Nemo like kinase* and *Pref-1*, and potentially involved in the specification and/or differentiation of neuronal population in the basal ganglia. Functional studies are currently ongoing to determine the function of *Nlk* and *Pref-1 in vivo*. In order to analyze the genes directly regulated by proneural genes, I also developed a gain-of-function approach by electroporation in the mouse telencephalon. This strategy allowed me to characterize in the mouse, the transcriptional programs regulated by *Mash1* and *Ngn2* and to decipher between potential common and distinct targets. Ongoing studies combining my microarray data, promoter analysis and chromatin immunoprecipitation should lead to the characterization of new direct targets of proneural genes. In addition, we showed that the *Dll1* promoter is directly regulated by Mash1 and Ngn2 through two different enhancers. This finding should provide an important tool to understand how proneural genes regulate common and subtype specific target genes. In addition, our preliminary result indicates that transcription factors of the Oct and POU family could interact with Mash1 for the activation of Dll1 promoter. Further studies are required to confirm this hypothesis.

The role of *Mash1* in the basal ganglia was mainly studied in the context of GABAergic interneurons specification. My present work and other studies suggest that still unknown factors can compensate for the specification of GABAergic neurons in the basal ganglia of *Mash1* mutant embryos (Casarosa et al., 1999; Schuurmans et al., 2004). My microarray data in addition suggest that *Mash1* could have an additional role in the specification of others neuronal subtypes, such NeuroptideY or cholinergic interneurons. This data are however preliminary and have to be confirmed. More generally, the development of the basal ganglia is barely understood. This structure of the telencephalon is involved in many neurological disorders such as Parkinson,

Alzheimer and Huntington diseases. Understanding the mechanisms of neuronal specification in this tissue is thus essential to comprehend and treat these neurodegenerative diseases.

This study allowed the characterization of many genes regulated either directly or indirectly by proneural genes and I showed that the microarray approach provide an effective mean for the characterization of genes regulated by proneural transcription factors. In this work, I focused mainly on genes with known function, but many EST (Expressed Sequence Tag) and genes with unknown function were identified in my screen. It will be important in future studies to confirm that these new genes are regulated by proneural genes. The characterization of these candidates will provide important information on the understanding of proneural function and how proneural proteins direct the specification of neural fate.

MATERIAL AND METHODS

1 RNA extraction and hybridization to Affymetrix micorarray

Total RNA was extracted with Trizol Reagent (Invitrogen) following manufactor recommendations. After precipitation total RNA was resuspended in 100µl of DEPC water (Ambion) and subjected to an additional phenol and chloroform extraction. RNA precipitations were carried-out overnight at -20°C with 250µl of 95% ethanol and 10µl of 1M sodium acetate, and RNA was resuspended in 12µl of DEPC water (Ambion). Total RNA from three cortices were pooled to average genetic variations and 10 mg RNA used to generate probes, which were hybridized to Affymetrix U74v2 or MG430 2.0 chips following standard Affymetrix guidelines. The experiment was performed twice for single mutants and once for double mutants. Briefly, double-stranded cDNA was synthesized from 15 µg of total RNA with SuperScript Choice System (Invitrogen) by using oligo dT 24 primers, with a T7RNA polymerase promoter site added to its 3'end (Genset Corp, La Jolla, CA). The isolated cDNA was then used to generate biotinylated cRNA in vitro using the BioArray T7 RNA polymerase labeling kit (Enzo, Farmingdale, NY). After purification quality of ARNc was determined by capillar electrophoresis (Agilent), fragmented and hybridized onto the array. Each array was washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and amplified with biotinylated anti-strepavidin antibody (Vector Lab, Burlingame, CA). Each array was scanned to obtain image and signal intensities.

2 Statistic analysis of Affymetrix microarray data

Scanned output files were analyzed with the Affymetrix Microarray Suite 5.0 and global normalization was performed prior to comparison. The selection of differentially expressed gene was based on the "detection P-value" and the "absolute call", proportional to the level of mRNA present in the initial samples. To identify differentially expressed transcripts, pairwise comparison analysis was carried out with Data Mining Tool 3.0 (Affymetrix, Santa Clara, CA). The analysis compares the differences in values of perfect match to mismatch of each probe pair in the baseline array to its matching probe on the experimental array (the "average difference"). P-values were determined by the Wilcoxon's signed rank test and denoted as increase, decrease or no change. Analysis using Data Mining Tool also provides the signal log ratio, which estimates the magnitude and direction of change of a transcript when two arrays are compared (induced versus uninduced). Genes present in the different comparative analysis were selected based on the following criteria: i)

significant absolute call in at least one experiment (intensity signal detected superior to the background, as define by Affymetrix algorithm), ii) absolute call superior to 50 in at least one experiment (the number was determine empirically in order to have a chance to detect a signal by RNA *in situ* hybridization), iii) significant average difference in at least one experiment (as define by Affymetrix algorithm), iv) fold change superior or equal to 1.5.

3 Construction of the subtracted library

30.000 GFP+ cells were collected from each dissected cortex and sorted by flow cytometry and sorted directly into 200µl 4M guanidium isothiocyanate (EM Science). 1µg of glycogen (Invitrogen) were added in each sample to facilitate RNA precipitation. Between 170pg and 400pg of total RNA was extracted from each sample by adding 20µl of 3M sodium acetate pH4, 200µl of phenol water saturated and 40µl of chloroform. RNA was precipitated by adding 100µl of isopropanol into the aqueous phase and incubation at -80°C for 20 min. Pellet was washed with 200µl of 70% ethanol and resuspended into 10µl of DEPC water. RNA concentration and quality was assessed by spectrophotometry. About 0.5µg of total RNA was used for reverse transcription using the SMART system (Clontech) and two rounds of subtraction for wild-type minus mutant and mutant minus wild-type were carried out with the PCR-Select cDNA subtraction kit (Clontech). Resultant cDNA were cloned into TOPO-PCR2.1 vector (Invitrogen).

4 Electroporation of mouse embryos

Embryos obtained from CBA/CA X C57Bl/10 crosses were dissected without removing placenta at embryonic day E10.5 into Tyrodes solution. Embryos were precultured for 2hrs into a “Precision Incubator" (B.T.C. Engineering, Milton, Cambridge, UK) at 37°C with 65% oxygen in a solution of Rat serum supplemented with glucose (75% v/v Rat Serum (MRC, Mill Hill, UK) + 25% v/v Tyrodes Solution, 2mg/ml of glucose). Both telencephalic vesicles were injected with about 2µl of DNA solution, containing 3µg/µl expression vector and 2µg/µl GFP control vector, with a FemtoJet Microinjector (Eppendorf). We used expression vectors in which proneural genes are placed under the control of the CMV enhancer fused with the β-actin promoter (CAGGS expression vector) (Niwa et al., 1991). Electroporations were done in Tyrodes solution in a CUY520P20 chamber (Nepagene, Japan) using the BTX Electro Square Porator (Eppendorf), with the following settings: 70V, 5 pulses, 50 ms, 1s of interval. Embryos were grown in Rat serum supplemented with glucose (as above) for 18hrs. Electroporation efficiency was assessed for each embryo by detection of GFP uner the binocular. Dissected tissues were homogenized immediately into 300µl of Trizol

(Invitrogen). Total RNA was extracted as described in Materials and Method, RNA extraction and hybridization to Affymetrix micorarray.

5 P19 cells transfection and quantitative RT PCR

400.000 cells were seeded into 21cm² cell culture dish in DMEM media (Gibco) supplemented with 5% of normal goat serum and incubated overnight at 37°C. Cells were transfected with 3µg of DNA (1.4µg of *Mash1* expressing vector, 1.4µg of E12 expressing vector and 0.2µg of GFP control vector) and 6µl of JetPEI (Polyplus Transfection) following manufactor recommendations. Transfections were stopped after 3hrs by adding 2ml of fresh DMEM media supplemented with 5% of normal goat serum and Gentamycin 40µg/ml. Medium was removed and cells washed one time with Versen solution (Gibco). 6ml of Trizol were used to extract total RNA from a pool of 3 transfected P19 cells in 21cm² dishes. RNA extraction was realized as recommended by the manufactor and subjected to an additional phenol and chloroform extraction as described in Materials and Method, RNA extraction and hybridization to Affymetrix micorarray. Total RNA was precipitated overnight at -20°C with 250µl of 95% ethanol and 10µl of 1M sodium acetate. Pellet was resuspended in 50µl of DEPC water (Ambion) and RNA concentration determined by spectrophotometry. 6 µg of total RNA obtained from P19 transfected cells (see Material and Method, P19 cells transfection), was treated with 5Units of DNase I (Invitrogen) 15min at room temperature, following manufactor recommendations. DNase treated RNA was then reverse transcribed with Superscript III (Invitrogen). Quantitative PCR were realized with SYBR Green (Roche) in Light cycler (Roche). Quantification was realized with the hydroxymethylbilane synthase cDNA as internal control.

6 RNA in situ hybridization

RNA *in situ* hybridization was carried out on 10 µm cryostat sections as follows: digoxygenin (dig)-labeled RNA probes were generated using T3, T7, or SP6 RNA polymerases and a dig-RNA-labelling mix (Roche). Probes were hybridized to slide overnight at 65°C in a buffer composed of 50% formamide, 200 mM sodium, chloride, 20 mM sodium acetate, and 10% dextran sulfate, 1mg/ml tRNA, washed 2 times at 65°C in 50% formamide, 150 mM sodium chloride, 15 mM sodium citrate, 0.1% Tween 20. Sections were then incubated with an alkaline phosphatase (AP)-coupled anti-digoxygenin antibody (Roche), washed, and processed for alkalin phosphatase activity with NBT/BCIP (4-Nitro blue tetra- zolium chloride/5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine) (Roche).

Templates for Dig probes were obtained as follows (accession numbers in brackets): *MIBP* (NM_010437), *Pref-1* (Z12171), *Nlk* (AF036332), *L1CAM (*NM_008478), *IA1* (AF044669), *Meg3* (Y13832), *NG2* (NM_019389), *Gp38* (NM_010329), *Supth16* (NM_033618), *Peg1* (AF017994), *Asb4* (BC054745), *Peg3* (AF038939), *Tssn1* (NM_133236), *Phdtf* (NM_013629), *Ebfaz* (NM_033327), *mEvf1* (AK038694), *Sox9* (BC034264), *Ayk1* (U80932), *NM_029758, BC052038*, *AK081204*, *BC059223*, *AA691557, NM_172710*, *Gadd45g* (IMAGE:3493618), *AI842002* (IMAGE:5686394), *Sialyltransferase8* (IMAGE:6405078), *Sox6* (IMAGE:1068053), *Nrarp* (IMAGE:3993751), *AW258842* (IMAGE:3591395), *AI840429* (IMAGE:5369051), *Arx* (obtained from Vania Broccoli, San Raphaela Institute, Milan, Italy), *Lhx6* and *Lhx7* (obtained from Wassilis Pachnis, NIMR, London, UK).

Annex 1: list of genes regulated in the cortex of *Ngn1* mutants

Liste of genes regulated significatively in the cortex of *Ngn1* mutants embryos at E13.5. Fold change is calculated as the mean of *Ngn1* mutant data divided by the mean of wild-type cortical data. Acc: Gen bank accession number.

Acc	***Name***	***Ratio Ngn1/WT***
AA655308	Polyadenylate binding protein-interacting protein 1	4.9
AI507519	AI507519	2.3
AW125346	Transmembrane protein 14C	1.9
X00686	Mouse gene for 18S rRNA	1.8
AI841987	Diacylglycerol kinase, delta	1.8
AI180489	Par-6 partitioning defective 6 homolog gamma (C. elegans)	1.7
AF020771	Karyopherin (importin) alpha 4	1.6
AA874620	AA874620	-1.5
X15986	Lectin, galactose binding, soluble 1	-1.6
AI591449	Transcribed locus	-1.6
AW121563	Zinc finger protein 207	-1.7
AW209924	AW209924	-1.7
AW050335	Lipoma HMGIC fusion partner-like 2	-1.7
AI506490	Formin binding protein 3	-1.8
M13125	Hemoglobin X, alpha-like embryonic chain in Hba complex	-1.8
Y13832	GTL2, imprinted maternally expressed untranslated mRNA	-1.8
AI647599	Solute carrier family 15 (H+/peptide transporter), member 2	-1.9
U41341	S100 calcium binding protein A11 (calizzarin)	-2.0
U03419	Procollagen, type I, alpha 1	-2.2
X52046	X52046	-2.3
AI323358	Transcribed locus	-4.7
U63841	U63841	-8.9
AW214353	Expressed sequence AL117927	-13.3

Annex 2: list of genes regulated in the cortex of *Mash1* mutants

Liste of genes regulated significatively in the cortex of *Mash1* mutants embryos at E13.5. Fold change is calculated as the mean of *Mash1* mutant data divided by the mean of wild-type cortical data. Acc: Gen bank accession number.

Acc	*Name*	*Fold change Mash1/WT*
M95603	achaete-scute complex homolog-like 1 (Drosophila)	-5.2
AI853550	RIKEN cDNA 3110035E14 gene	-3.9
AW012608	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	-2.7
AV301161	RIKEN cDNA 1700022N24 gene	-2.5
AW214136	AW214136	-2.5
AW122924	Similar to hypothetical protein FLJ39873 (LOC207787), mRNA	-2.3
AA693044	abhydrolase domain containing 6	-2.1
M21041	microtubule-associated protein 2	-2.0
C76063	RIKEN cDNA 2900097C17 gene	-2.0
X05546	RIKEN cDNA 3110007P09 gene	-2.0
AA517145	AA517145	-1.9
AA184423	RIKEN cDNA E430003D02 gene	-1.9
AI848242	neurexin III	-1.9
AA755396	RIKEN cDNA 2010011I20 gene	-1.9
AI132554	myocyte enhancer factor 2C	-1.8
AW048257	phosphodiesterase 2A, cGMP-stimulated	-1.8
AW050143	RIKEN cDNA 1500005N04 gene	-1.8
D25540	transforming growth factor, beta receptor I	-1.8
AW121030	asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)	-1.8
AV145574	small nuclear ribonucleoprotein B	-1.7
X07888	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.7
AW060793	ADP-ribosylation factor-like 5	-1.7
AI841987	diacylglycerol kinase, delta	-1.7
AA739359	RIKEN cDNA C330002I19 gene	-1.7
M57647	kit ligand	-1.7
U62673	histone 2, H2bb	-1.7
AA432497	RIKEN cDNA 1110033M05 gene	-1.6
AF067180	kinesin family member 5C	-1.6
AF058799	3-monooxgenase/tryptophan 5-monooxgenase activation protein, gamma polypeptide	-1.6
AJ130977	ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (Drosophila)	-1.6
AI842625	thyroid hormone receptor associated protein 3	-1.6
AW125412	SAC1 (supressor of actin mutations 1, homolog)-like (S. cerevisiae)	-1.6
AA219946	mitogen-activated protein kinase 11	-1.5
AI877157	transmembrane 4 superfamily member 9	-1.5
AW124717	synaptotagmin 1	-1.5
AI845584	dual specificity phosphatase 6	-1.5
AA764001	transformation related protein 53 inducible nuclear protein 1	-1.5
U42384	fibroblast growth factor inducible 15	-1.5
AF037437	prosaposin	-1.5
AV358770	lysophospholipase 1	-1.5
AW047038	zinc finger/RING finger 2	-1.5
AI846682	solute carrier family 15 (H+/peptide transporter), member 2	-1.5
D90174	NFI-B protein	-1.5
AW123162	SUMO/sentrin specific protease 6	-1.5
X76850	MAP kinase-activated protein kinase 2	-1.5
X97720	Friend virus susceptibility 1	-1.5
Y12783	ring finger protein 2	-1.5

AW121194	expressed sequence AI504432	1.5
AW214621	AW214621	1.5
AA183037	AA183037	1.5
AV003378	hemoglobin alpha, adult chain 1	1.5
AA874329	RIKEN cDNA 1110008H02 gene	1.5
AW124295	RNA terminal phosphate cyclase domain 1	1.5
AW125076	RIKEN cDNA 1700054N08 gene	1.6
V00722	hemoglobin, beta adult major chain	1.6
AW045576	Kruppel-like factor 7 (ubiquitous)	1.6
AI854154	spastic paraplegia 21 homolog (human)	1.6
AI850484	AI850484	1.6
X14061	hemoglobin Y, beta-like embryonic chain	1.6
AW125272	RIKEN cDNA 5930418K15 gene	1.7
AI854488	RIKEN cDNA 2300004C15 gene	1.8
AA682038	AA682038	1.8
AI846274	AI846274	1.9
AV245229	runt related transcription factor 2	1.9
AJ223733	cyclin-dependent kinase 2	2.2
AA960124	retinoic acid induced 14	2.2
AI173309	RIKEN cDNA 6230416J20 gene	2.5
AI156452	AI156452	3.0
AI158278	ring finger protein 125	3.8
AI874856	RIKEN cDNA 4933439C20 gene	4.7

Annex 3: list of genes in cluster1

Part of the list of the 285 genes present in cluster1. Genes are highly expressed in the basal ganglia compared to cortex in wild-type embryos, and strongly upregulated in the cortex of *Ngn2* and *Ngn1*;*Ngn2DKO*.

INDEX	*NAME*	*Ngn2KO/WT*	*Mash1Ngn2DKO/WT*	*Ngn1Ngn2DKO/WT*
M95603	achaete-scute complex homolog-like 1 (Drosophila)	3.3	-10.7	5.3
AI852838	GTL2, imprinted maternally expressed untranslated mRNA	3.3	1.6	5.9
AI842002	RIKEN cDNA 6530413N01 gene	3.0	-1.5	4.3
D42051	glutamic acid decarboxylase 2	2.9	2.8	5.1
AA611885	AA611885	2.7	2.0	1.3
AF011543	transient receptor potential cation channel, subfamily C, member 4	2.5	1.2	3.3
AV332839	RIKEN cDNA 2210016L21 gene	2.4	1.1	1.5
AI838057	AI838057	2.3	-1.7	10.9
AI173397	AI173397	2.3	3.4	1.1
M16465	S100 calcium binding protein A10 (calpactin)	2.1	1.2	1.0
AV117844	ubiquitin-conjugating enzyme E2C	2.1	-1.1	1.3
AV293396	S100 calcium binding protein A14	2.1	-1.1	-1.0
X86000	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D	2.0	2.8	1.7
AV351758	tubulin, beta 2	2.0	1.2	1.5
AV245229	runt related transcription factor 2	2.0	-2.7	1.6
AV207739	glutathione S-transferase, mu 2	2.0	1.1	1.2
M83344	Carcinoembryonic antigen 2	1.9	-1.1	1.2
X84896	purinergic receptor P2X, ligand-gated ion channel, 1	1.9	-1.3	1.0
L10426	ets-related protein 81 (ER81)	1.9	-1.4	3.6
U81603	eyes absent 2 homolog (Drosophila)	1.8	3.5	2.3
AI154580	neurobeachin	1.8	1.1	2.2
AI317205	mitogen activated protein kinase kinase kinase 1	1.8	1.4	1.8
AV153952	DiGeorge syndrome critical region gene 6	1.8	-1.3	-1.2
AA152809	RIKEN cDNA 3000004C01 gene	1.8	1.6	1.5
U39738	p21 (CDKN1A)-activated kinase 3	1.8	1.9	2.0
M92378	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	1.8	1.2	3.1
AV367375	protein kinase C, zeta	1.8	1.0	-2.1
AW123143	AW123143	1.8	1.5	2.2
AA466892	RIKEN cDNA 5730406M06 gene	1.8	1.0	1.5
AV266785	AV266785	1.8	-1.0	1.2
AA153229	AA153229	1.8	-1.3	1.1
AV257523	DNA segment, Chr 4, Brigham & Women's Genetics 1540 expressed	1.8	1.0	1.5
M88301	POU domain, class 3, transcription factor 4	1.7	3.2	6.7
AW124265	RIKEN cDNA 6430519N07 gene	1.7	2.6	2.5
AI158957	dual specificity phosphatase 10	1.7	1.3	1.4
AV216468	expressed in non-metastatic cells 1, protein	1.7	-1.3	1.8
U67841	distal-less homeobox 6	1.7	2.1	9.9

AV367240	potassium voltage-gated channel, subfamily Q, member 1	1.7	-1.3	1.5
AW125471	neurotrophic tyrosine kinase, receptor, type 2	1.7	2.1	2.8
AF065145	G-protein-coupled receptor 50	1.6	-1.2	-1.1
Z18272	procollagen, type VI, alpha 2	1.6	1.8	-1.2
AA815831	CDNA clone IMAGE:30033444, partial cds	1.6	-2.0	1.4
D32167	Zic family member 1 (odd-paired homolog, Drosophila)	1.6	2.6	2.0
AI747057	ollistatin-like 5	1.6	1.1	1.9
AF047714	transient receptor potential cation channel, subfamily M, member 1	1.6	1.1	1.0
AA716925	ras homolog gene family, member E	1.6	-1.5	1.2
AW210072	ras homolog gene family, member E	1.6	-1.8	1.8
M12130	polymerase (RNA) II (DNA directed) polypeptide A	1.6	-1.4	1.3
AI848242	neurexin III	1.6	-1.1	2.3
AW049234	centaurin, gamma 2	1.6	1.2	1.3
AV253142	cDNA sequence BC003324	1.6	1.2	-1.8
AV149007	PDZ and LIM domain 1 (elfin)	1.6	1.0	-1.1
AJ010605	SRY-box containing gene 6	1.6	1.3	2.4
AV375661	glycolipid transfer protein	1.5	-1.3	1.2
AI847631	calcium regulated heat stable protein 1	1.5	-1.1	1.4
AV239949	GTP binding protein 1	1.5	-1.4	1.2
AV367141	UPF3 regulator of nonsense transcripts homolog B (yeast)	1.5	1.1	-1.4
AA008996	calcium channel, voltage-dependent, alpha 2/delta subunit 2	1.5	-1.2	1.3
AW061228	ATPase, Ca++-sequestering	1.5	-1.2	1.1
AI846023	ADP-ribosylation factor-like 7	1.5	1.2	1.8
X75959	poly A binding protein, cytoplasmic 2	1.5	-1.2	1.0
D17292	adrenomedullin receptor	1.5	-1.9	-1.1
AI850878	discs, large (Drosophila) homolog-associated protein 3	1.5	1.5	2.6
AA110885	AA110885	1.5	4.2	3.0
AF020199	pre B-cell leukemia transcription factor 3	1.5	1.3	2.4
X73985	calbindin 2	1.5	-1.3	2.7
X13586	2,3-bisphosphoglycerate mutase	1.5	-1.1	1.6
AI853896	RIKEN cDNA 2310045A20 gene	1.4	1.4	2.4
Y11245	forkhead box M1	1.4	1.2	-1.6
M63695	CD1d1 antigen	1.4	1.1	1.7
AW122202	parvin, alpha	1.4	3.7	1.4
AA189811	RIKEN cDNA A430106J12 gene	1.4	1.0	1.8
AA189811	RIKEN cDNA A430106J12 gene	1.4	1.0	1.8
AA711773	RIKEN cDNA 6330500D04 gene	1.4	1.0	2.0
AB006103	aristaless related homeobox gene (Drosophila)	1.4	1.3	2.8
AV172042	AV172042	1.4	-1.6	-1.8
AI447780	fibronectin leucine rich transmembrane protein 3	1.4	2.0	1.1
AI327488	eyes absent 1 homolog (Drosophila)	1.4	1.0	1.9
X68363	POU domain, class 2, transcription factor 1	1.4	1.4	1.6
AI430099	Musashi homolog 2 (Drosophila)	1.4	1.4	1.7
U86783	nuclear receptor subfamily 4, group A, member 2	1.3	1.8	1.9
AV325375	GTL2, imprinted maternally expressed untranslated mRNA	1.3	-1.5	2.6
AA866668	SRY-box containing gene 3	1.3	1.7	1.6

AJ131869	multiple PDZ domain protein	1.3	1.0	1.6
X94127	SRY-box containing gene 2	1.3	1.3	1.6
AW050310	RIKEN cDNA A230057G18 gene	1.3	-2.1	1.2
AA203872	RIKEN cDNA 6330414G21 gene	1.3	-3.1	2.2
AA739008	RIKEN cDNA 1110001A07 gene	1.3	1.1	1.7
X68363	POU domain, class 2, transcription factor 1	1.3	1.8	1.8
AI846076	coatomer protein complex, subunit gamma 2, antisense 2	1.3	-1.3	2.2
D45889	chondroitin sulfate proteoglycan 2	1.3	1.8	1.9
AI844617	dual specificity phosphatase 4	1.3	-3.2	-1.9
AI647612	RIKEN cDNA 5031439A09 gene	1.3	2.6	1.1
AA616757	RIKEN cDNA A430106J12 gene	1.3	-1.5	1.3
AJ005350	zinc finger protein 125	1.3	-1.6	1.2
AI839553	AI839553	1.3	-2.2	1.3
AA792015	glucocorticoid induced transcript 1	1.3	-1.5	2.3
AI836428	transmembrane 4 superfamily member 10	1.3	4.0	2.1
U01063	polo-like kinase 1 (Drosophila)	1.3	-1.2	1.6
AA189811	RIKEN cDNA A430106J12 gene	1.3	1.0	1.6
AF043939	transcription factor Dp 1	1.2	1.2	2.4
AF079366	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	1.2	-1.4	1.7
X83934	ryanodine receptor 3	1.2	-1.7	-1.2
AW125218	histone aminotransferase 1	1.2	2.4	-1.4
AI836268	ADP-ribosylation factor-like 7	1.2	-1.5	1.3
AW125891	AW125891	1.2	-1.2	1.5
M63697	CD1d1 antigen /// CD1d2 antigen	1.2	1.2	1.5
AW120868	TCDD-inducible poly(ADP-ribose) polymerase	1.2	1.4	1.8
AV330397	nemo like kinase	1.2	2.0	1.5
AA797843	DNA segment, Chr 11, ERATO Doi 530, expressed	1.2	1.8	1.8
Y11245	forkhead box M1	1.2	1.7	1.5
AW047093	slingshot homolog 2 (Drosophila)	1.2	1.4	1.8
AA739067	dual specificity phosphatase 4	1.2	-4.2	-1.2
X97581	sal-like 3 (Drosophila)	1.2	-1.4	2.0
D70849	zinc finger protein of the cerebellum 3	1.2	2.3	-1.3
AA647252	protein regulator of cytokinesis 1	1.2	1.2	1.5
AW210253	Wiskott-Aldrich syndrome-like (human)	1.2	1.0	1.9
D83203	expressed sequence AW125753 /// protein tyrosine phosphatase, receptor type, J	1.2	1.1	-1.9
AC002397	cell division cycle associated 3	1.2	1.6	-1.1
AW125480	Nur77 downstream gene 2	1.2	2.0	1.6
AV358817	2,3-bisphosphoglycerate mutase	1.2	1.1	1.5
AF020200	pre B-cell leukemia transcription factor 3	1.2	1.3	3.2
AF015768	lunatic fringe gene homolog (Drosophila)	1.2	-1.7	1.2
AA139072	RIKEN cDNA 4931406O17 gene	1.2	-1.1	1.5
AJ223293	kinesin family member 11	1.2	1.7	1.3
AJ001598	vesicular inhibitory amino acid transporter	1.2	-12.0	2.9
AW125218	histone aminotransferase 1	1.2	1.7	1.2
M96823	nucleobindin 1	1.1	1.5	-1.1
AI180489	par-6 partitioning defective 6 homolog gamma (C. elegans)	1.1	1.1	1.5
AI848386	neuropeptide Y	1.1	5.3	2.0

Annex 4: list of genes in cluster2

Part of the list of 277 genes present in cluster2. Genes are strongly expressed in the cortex compared to basal ganglia in wild-type mice, and downregulated in the cortex of *Ngn2*KO embryos.

INDEX	*NAME*	*Ngn2KO/WT*	*Mash1Ngn2DKO/WT*	*Ngn1Ngn2DKO/WT*
AB022100	cadherin 13	-3.9	-22.8	-8.0
AI851121	AI851121	-2.7	-13.2	-13.4
AF035400	neurotrophic tyrosine kinase, receptor, type 3	-1.6	-6.9	1.0
AI845695	AI845695	-3.2	-5.3	-8.6
M18775	microtubule-associated protein tau	-1.7	-5.3	-1.7
AI853813	cannabinoid receptor 1 (brain)	-1.9	-5.2	-4.1
L27220	internexin neuronal intermediate filament protein, alpha	-1.5	-5.0	-1.3
AI836561	AI836561	-2.9	-4.7	-3.0
AF028009	protein kinase C, epsilon	-1.8	-4.7	-1.6
AA822744	nuclear receptor subfamily 4, group A, member 3	-2.1	-4.6	-2.8
AI842602	monocyte to macrophage differentiation-associated 2	-1.4	-4.5	-3.5
AI839544	neuritin 1	-2.4	-4.5	-3.1
U85714	phospholipase C, beta 1	-1.9	-4.2	-4.9
AI837006	coactosin-like 1 (Dictyostelium)	-2.8	-4.2	-4.2
AW011738	expressed sequence AW011738	-1.2	-3.9	-1.1
AJ001700	euroserpin	-2.3	-3.9	-4.3
AW121968	RAB3D, member RAS oncogene family	-1.7	-3.7	-2.8
M73748	glycoprotein 38	-1.4	-3.6	-1.1
AF016482	ras homolog gene family, member N	-2.7	-3.6	-7.7
U49251	T-box brain gene 1	-1.7	-3.5	-2.6
AF026489	spectrin beta 3	-1.5	-3.4	-1.2
M89777	RAB3D, member RAS oncogene family	-1.9	-3.2	-2.6
U73487	calcium channel, voltage-dependent, alpha2/delta subunit 1	-1.9	-3.1	-1.7
AI131739	phospholipase C, beta 1	-1.8	-3.0	-2.6
AI608482	contactin 2	-1.6	-3.0	-2.6
U24703	reelin	-2.0	-3.0	-2.9
AW049675	contactin 2	-2.2	-2.9	-8.8
D29763	seizure related gene 6	-1.8	-2.9	-3.8
AB006141	insulin-like growth factor binding protein-like 1	-2.1	-2.9	-4.6
U29086	Atonal homolog 2 (Drosophila)	-1.5	-2.8	-8.5
AW123298	EGF-like repeats and discoidin I-like domains 3	-1.8	-2.8	-1.7
AI845815	RIKEN cDNA 5430432M24 gene	-1.7	-2.8	-1.9
AJ243895	hairy/enhancer-of-split related with YRPW motif 1	-1.4	-2.8	-1.7
AV354117	reprimo, TP53 dependant G2 arrest mediator candidate	-1.3	-2.7	-2.4
AI504506	0 day neonate thymus cDNA, RIKEN full-length enriched library, clone:A430017F18 product:unknown EST, full insert sequence	-1.6	-2.7	-3.6
M97506	nescient helix loop helix 1	-2.0	-2.7	-8.1
AA710215	TRAF2 and NCK interacting kinase	-1.9	-2.7	-2.8
AI851143	RIKEN cDNA 2810417M05 gene	-1.5	-2.6	-2.8

AF019385	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	-3.2	-2.6	-4.1
M89802	wingless-related MMTV integration site 7B	-1.8	-2.6	-4.0
AW125276	TRAF2 and NCK interacting kinase	-2.0	-2.6	-2.4
AI844322	RIKEN cDNA B230104P22 gene	-1.7	-2.6	-3.0
AI841945	AI841945	-1.9	-2.6	-5.8
AI838622	latrophilin 2	-1.3	-2.6	-1.5
Y00864	kit oncogene	-1.7	-2.6	-4.4
AI843150	RUN and SH3 domain containing 1	-1.7	-2.6	-1.3
AI851384	RIKEN cDNA 9130203F04 gene	-1.3	-2.6	-2.7
U29148	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C)	-1.4	-2.5	1.1
AI846211	DIX domain containing 1	-1.6	-2.5	-1.4
AF010254	serine (or cysteine) proteinase inhibitor, clade G, member 1	-2.4	-2.5	-3.1
AI843176	15 days embryo head cDNA, RIKEN full-length enriched library, clone:D930035P11 product:unknown EST, full insert sequence	-1.3	-2.5	-3.1
AV371846	Ena-vasodilator stimulated phosphoprotein	-1.4	-2.5	1.3
AW121058	cadherin 13	-1.6	-2.5	-2.3
AW125006	SLIT and NTRK-like family, member 1	-1.8	-2.5	-2.9
AI851180	RIKEN cDNA 6430704M03 gene	-2.1	-2.5	-2.3
AI152789	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	-1.6	-2.5	-1.7
U28068	neurogenic differentiation 1	-1.9	-2.4	-4.2
AI848490	SH3-domain GRB2-like 2	-1.3	-2.4	-1.9
AW121637	RIKEN cDNA C630028C02 gene	-1.3	-2.4	-1.6
AI847023	AI847023	-1.4	-2.4	-2.0
AI851600	RIKEN cDNA A130038L21 gene	-1.5	-2.4	-4.9
AW049175	RIKEN cDNA C630028C02 gene	-1.5	-2.3	-2.2
AI839232	AI839232	-1.5	-2.3	-1.6
AI840625	cortactin binding protein 2	-1.4	-2.3	-2.2
AA726364	lipoprotein lipase	-1.9	-2.3	-2.3
U22399	cyclin-dependent kinase inhibitor 1C (P57)	-2.6	-2.3	-3.2
AA739203	RIKEN cDNA 9130430L19 gene	-1.9	-2.3	-2.5
U29088	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	-1.5	-2.3	-1.4
AW120970	RIKEN cDNA 2300002D11 gene	-2.5	-2.3	-2.4
AI850678	RIKEN cDNA 2410066E13 gene	-2.3	-2.2	-2.0
AW215585	RIKEN cDNA 9130422G05 gene	-2.2	-2.2	-5.9
AW049932	RIKEN cDNA A930024E05 gene	-1.7	-2.2	-3.0
AA271544	reprimo, TP53 dependant G2 arrest mediator candidate	-1.3	-2.2	-2.3
AI854830	leucine rich repeat and fibronectin type III domain containing 5	-2.1	-2.2	-2.7
AI844298	RIKEN cDNA 6430411K14 gene	-1.7	-2.2	-2.2
AI850212	RIKEN cDNA E330009J07 gene	-1.3	-2.2	-1.7
AW123794	ephrin A3	-1.7	-2.2	-1.6
D31953	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D)	-1.4	-2.2	-1.2
AI837130	inositol 1,3,4-triphosphate 5/6 kinase	-1.5	-2.2	-1.6
AA796759	SEC24 related gene family, member D	-5.0	-2.2	-3.8

	(S. cerevisiae)			
AW046213	Down syndrome cell adhesion molecule	-1.7	-2.1	-2.2
AW121535	AW121535	-2.0	-2.1	-1.1
AI850846	cDNA sequence BC037006	-1.7	-2.1	-2.7
AF008914	somatostatin receptor type 2 (sst2)	-1.6	-2.1	-2.8
AI849563	AI849563	-1.6	-2.1	-3.4
AI835291	thymoma viral proto-oncogene 3	-1.4	-2.1	-1.7
AI852587	zinc finger protein 521	-1.5	-2.1	-1.6
D83507	neurogenic differentiation 2	-1.7	-2.1	-4.8
AI854630	Eph receptor A5	-1.5	-2.1	-1.8
AI848669	RIKEN cDNA 9630041G16 gene	-1.8	-2.0	-2.2
AI019327	AI019327	-1.9	-2.0	-4.0
AI842991	kinesin family member 21B	-1.2	-2.0	-2.0
AW048012	basic helix-loop-helix domain containing, class B5	-1.5	-2.0	-2.7
AW125453	RIKEN cDNA 1190002N15 gene	-1.6	-2.0	-2.3
AI447997	nucleosome assembly protein 1-like 5	-1.5	-2.0	-2.8
U47008	Ngfi-A binding protein 1	-1.6	-2.0	-1.5
AI593057	RIKEN cDNA E130310K16 gene	-1.4	-2.0	-2.2
AI835632	RIKEN cDNA 2310001H13 gene	-1.3	-1.9	-2.1
AI646305	RIKEN cDNA 9130024F11 gene	-1.3	-1.9	-4.0
AI462012	RIKEN cDNA D430015B01 gene	-4.3	-1.9	-3.7
X07750	thyroid hormone receptor alpha	-1.4	-1.9	-1.4
AW121490	dihydropyrimidinase-like 5	-1.4	-1.9	-1.5
AW125844	AW125844	-1.4	-1.9	-1.3
AW124534	small glutamine-rich tetratricopeptide repeat (TPR)-containing, beta	-1.3	-1.9	-1.2
AI849131	ectodermal-neural cortex 1	-1.6	-1.9	-3.0
X94404	calcium channel, voltage-dependent, beta 3 subunit	-1.4	-1.9	-1.4
AI642708	RIKEN cDNA 9030023J02 gene	-1.4	-1.9	-2.1
AI851416	RIKEN cDNA 1300018P11 gene	-1.7	-1.9	-2.5
U69599	interferon gamma receptor 2	-1.5	-1.9	-3.1
AI848868	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	-1.7	-1.9	-1.4
AI841022	thyroid hormone receptor alpha	-1.7	-1.9	-1.8
Y15907	human immunodeficiency virus type I enhancer binding protein 2	-2.6	-1.8	-7.1
AF077861	inhibitor of DNA binding 2	-1.2	-1.8	-2.3
Y07688	nuclear factor I/X	-1.8	-1.8	-1.5
AU023434	AU023434	-1.9	-1.8	-1.3
AI843031	odd Oz/ten-m homolog 4 (Drosophila)	-1.5	-1.8	-1.9
AV321418	AV321418	-1.5	-1.8	-2.7
AW124695	hypothetical protein 9330117B14	-1.4	-1.8	-3.1
X83601	pentaxin related gene	-1.4	-1.8	-1.2
D90173	NFI-B protein	-1.4	-1.8	-1.7
AF008914	somatostatin receptor type 2 (sst2)	-1.5	-1.8	-2.3
AI851272	dual specificity phosphatase 14	-1.4	-1.8	-2.1
M64278	chromogranin A	-1.6	-1.8	-1.4
AW120579	eomesodermin homolog (Xenopus laevis)	-1.7	-1.8	-2.8
AA881092	RIKEN cDNA 4631426J05 gene	-1.7	-1.8	-1.9
AW120619	podocalyxin-like 2	-1.5	-1.8	-1.6
AF054623	frizzled homolog 1 (Drosophila)	-1.5	-1.8	-1.5
AI841371	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	-1.4	-1.8	-4.7
AI844298	AI844298	-1.6	-1.8	-1.7

Annex 5: List of genes regulated in *Mash1*KO embryos

Part of the list of 266 genes up- and downregulated in the basal ganglia of *Mash1*KO embryos at E13.5.

INDEX	*NAME*	*Mash1KO/WT*
U44027	prepronociceptin	18.9
AW214353	AW214353	10.1
AW047207	AW047207	6.8
AI851155	ribonuclease P 25 subunit (human)	5.5
AI847060	BTB (POZ) domain containing 11	5.1
AI848386	neuropeptide Y	4.7
AW060572	glial cell line derived neurotrophic factor family receptor alpha 1	3.6
D26352	calbindin-28K	3.0
AF038939	paternally expressed 3	2.9
U20611	peroxiredoxin 2	2.7
M62470	thrombospondin 1	2.7
X99915	high mobility group AT-hook 2	2.6
AF017994	mesoderm specific transcript	2.6
AI839544	neuritin 1	2.5
AI851953	RIKEN cDNA 1100001I19 gene	2.5
AI646761	RIKEN cDNA 1110018J23 gene	2.5
AW060255	ankyrin repeat and SOCS box-containing protein 4	2.4
AA207825	DNA segment, Chr 8, ERATO Doi 319, expressed	2.4
AW259399	platelet-activating factor acetylhydrolase 2	2.4
AA833302	G patch domain containing 2	2.3
AA271544	reprimo, TP53 dependant G2 arrest mediator candidate	2.3
AV365678	pallidin	2.3
X94694	transcription factor AP-2, gamma	2.3
AI043090	RIKEN cDNA 3110018A08 gene	2.3
AI156452	AI156452	2.2
Y07621	neurogenin 2	2.2
AW120874	AW120874	2.2
AF092507	dipeptidylpeptidase 6	2.1
AV354117	reprimo, TP53 dependant G2 arrest mediator candidate	2.1
AW046885	FYVE, RhoGEF and PH domain containing 5	2.1
U49251	T-box brain gene 1	2.0
AW124778	RIKEN cDNA 2610034N03 gene	2.0
L12447	insulin-like growth factor binding protein 5	2.0
M13125	hemoglobin X, alpha-like embryonic chain in Hba complex	2.0
D50086	neuropilin 1	2.0
AA980253	AA980253	1.9
AA874329	RIKEN cDNA 1110008H02 gene	1.9
AW122168	RIKEN cDNA 2700069A02 gene	1.9
AA414668	ankyrin repeat and SOCS box-containing protein 4	1.9
AA797722	RIKEN cDNA 6430527G18 gene	1.9
AI835497	complexin 2	1.9
AW048155	RIKEN cDNA 2900026H06 gene	1.9
AI836610	Peg10 mRNA for paternally expressed 10, complete cds	1.9
AI314004	AI314004	1.9
AI835357	Down syndrome critical region gene 1-like 1	1.8
AI845255	zinc finger protein 423	1.8
M83749	cyclin D2	1.8
AI324025	RIKEN cDNA 4930488E11 gene	1.8
AI647526	dystrobrevin alpha	1.8

AW049175	RIKEN cDNA C630028C02 gene	1.8
AF008914	somatostatin receptor type 2 (sst2)	1.8
AW047719	HLA-B associated transcript 2	1.8
AA170647	DNA segment, Chr 12, ERATO Doi 553, expressed	1.8
Z48800	gastrulation brain homeobox 2	1.8
AI841371	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	1.8
AI646253	RIKEN cDNA 9430059P22 gene	1.8
AW050287	WD repeat domain 6	1.8
U29086	:Atonal homolog 2 (Drosophila)	1.8
M18070	prion protein	1.8
AI843094	RIKEN cDNA 1110051M20 gene	1.8
AW047822	RIKEN cDNA 6230410L23 gene /// neurotrimin	1.8
AW123803	AW123803	1.8
AI839531	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	1.7
M27938	male enhanced antigen 1	1.7
AW047710	zinc finger protein 312	1.7
AI842234	glutamate receptor, ionotropic, AMPA1 (alpha 1)	1.7
AA624586	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	1.7
X78304	signal recognition particle 9	1.7
AJ243608	melanoma antigen, family L, 2	1.7
AI844772	cDNA sequence BC058638	1.7
AI180489	par-6 partitioning defective 6 homolog gamma (C. elegans)	1.7
AW107218	RIKEN cDNA 2210415K03 gene	1.7
AW046112	neuropilin 2	1.7
X73985	calbindin 2	1.7
AW121535	AW121535	1.7
AI853002	RIKEN cDNA 1110057H19 gene	1.6
AI848790	RIKEN cDNA 2310056B04 gene	1.6
X14061	hemoglobin Y, beta-like embryonic chain	1.6
AI846552	RIKEN cDNA C030033F14 gene	1.6
AA763178	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit s	1.6
AI835976	erythrocyte protein band 4.1-like 3	1.6
AI848699	keratinocyte associated protein 2	1.6
AW124643	loss of heterozygosity, 12, chromosomal region 1 homolog (human)	1.6
AI843413	expressed sequence AW146020	1.6
AW124490	protein tyrosine phosphatase, receptor type, D	1.6
AI837711	expressed sequence AW551984	1.6
AI847023	AI847023	1.6
X59520	cholecystokinin	1.6
AW061350	synaptic vesicle glycoprotein 2 a	1.6
AJ001700	neuroserpin	1.6
AW045610	RIKEN cDNA 5330410G16 gene	1.6
AI848661	BTB (POZ) domain containing 3	1.6
AW123386	patched homolog 1	1.6
AI838622	latrophilin 2	1.6
AI844184	calbindin-28K	1.6
AI852919	catenin delta 2	1.6
AI842120	RIKEN cDNA E030034P13 gene	1.6
AI173309	RIKEN cDNA 6230416J20 gene	1.6
AI159157	expressed sequence AI194318	1.6
AI844095	Stam binding protein	1.6
AI846003	C1q and tumor necrosis factor related protein 4	1.6
AI842515	RAP2C, member of RAS oncogene family	1.6

AW050172	RIKEN cDNA C030033F14 gene	1.6
AV103574	zinc finger protein 162	-1.7
AF079096	arginine-tRNA-protein transferase 1	-1.7
AI853444	RIKEN cDNA 2610042L04 gene	-1.7
AV161234	GPI anchor attachment protein 1	-1.7
AV358673	AV358673	-1.7
U34303	myosin heavy chain 10, non-muscle	-1.7
AW048355	PHD finger protein 17	-1.7
AV172517	AV172517	-1.7
AI834847	EF hand domain containing 2	-1.7
AI131744	RIKEN cDNA 2310009E04 gene	-1.7
X83601	pentaxin related gene	-1.8
AA062323	RIKEN cDNA 1700027D21 gene	-1.8
AI606693	E1A binding protein p400	-1.8
AI854358	RIKEN cDNA 1190002H23 gene	-1.8
X67668	high mobility group box 2	-1.8
AA472921	AA472921	-1.8
AI841562	zinc finger protein 207	-1.8
X56683	chromobox homolog 3 (Drosophila HP1 gamma)	-1.8
AJ132765	ISL1 transcription factor, LIM/homeodomain (islet 1)	-1.8
X65138	Eph receptor A4	-1.8
AW124230	tweety homolog 1 (Drosophila)	-1.8
AW121733	nuclear receptor subfamily 1, group D, member 2	-1.8
AW048812	hairy and enhancer of split 6 (Drosophila)	-1.8
AA709728	DNA segment, Chr 3, ERATO Doi 330, expressed	-1.9
AI605903	processing of precursor 1, ribonuclease P/MRP family, (S. cerevisiae)	-1.9
AW258842	RIKEN cDNA 2510049I19 gene	-1.9
AA590345	RIKEN cDNA C330027C09 gene	-1.9
AA197709	AA197709	-1.9
AI595322	RIKEN cDNA 2700099C18 gene	-1.9
AW125884	acetyl-Coenzyme A synthetase 2 (AMP forming)-like	-2.0
AA189811	RIKEN cDNA A430106J12 gene	-2.0
AV056802	claudin 10	-2.0
AA833425	RIKEN cDNA 3110003A17 gene	-2.0
AI847581	rhomboid, veinlet-like 4 (Drosophila)	-2.0
AI851210	RIKEN cDNA C030003H22 gene	-2.1
AV216394	AV216394	-2.1
AW122924	Similar to hypothetical protein FLJ39873 (LOC207787), mRNA	-2.1
AW060536	Notch-regulated ankyrin repeat protein	-2.1
AW061165	lunatic fringe gene homolog (Drosophila)	-2.2
AI645505	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	-2.2
Y12474	centrin 3	-2.3
AI842362	AI842362	-2.3
AF055638	growth arrest and DNA-damage-inducible 45 gamma	-2.3
M84487	vascular cell adhesion molecule 1	-2.3
X80903	delta-like 1 (Drosophila)	-2.3
M73748	glycoprotein 38	-2.5
AW214136	AW214136	-2.5
AV373702	ribosomal protein L9	-2.7
D32132	hairy and enhancer of split 5 (Drosophila)	-2.8
AV346105	AV346105	-3.6
AI316570	RIKEN cDNA 2310061A22 gene	-4.7
M95603	achaete-scute complex homolog-like 1 (Drosophila)	-61.9

Annex 6: Comparison between Mash1 and Ngn2 gain-of-function microarray data.

Part of the 1409 genes regulated by *Mash1* or/and *Ngn2* in E10.5 electroporated embryos. The category provides the comparison between *Ngn2* and *Mash1* gain-of-function data. MI: Mash1GOF Increased, NI: Ngn2GOF Increased, MNC: Mash1GOF No Change, NNC: Ngn2GOF No Change.

Gene Title	***Mash1GOF/WT***	***Ngn2GOF/WT***	***Category***
uterine-specific proline-rich acidic protein	38.8	1.3	MINNC
achaete-scute complex homolog-like 2 (Drosophila)	37.5	3.8	MINNC
achaete-scute complex homolog-like 1 (Drosophila)	21.7	-1.2	MINNC
carbonic anhydrase 3	7.6	1.3	MINNC
neuronal pentraxin receptor	6.9	-1.3	MINNC
ubiquitin specific protease 18	6.6	1.7	MINI
rhomboid, veinlet-like 4 (Drosophila)	6.3	6.1	MINI
cDNA sequence BC047207	6.0	2.0	NIMNC
regulator of G-protein signaling 16	5.6	5.5	MINI
inhibitor of DNA binding 1	5.5	3.3	MINI
cholinergic receptor, nicotinic, beta polypeptide 4	4.7	3.5	NIMNC
regulator of G-protein signaling 16	4.6	3.1	MINI
RIKEN cDNA 6530413N01 gene	4.1	4.1	MINI
insulin receptor substrate 4	4.0	-2.1	MINNC
Ras association (RalGDS/AF-6) domain family 4	4.0	4.1	MINI
RIKEN cDNA 2600003E23 gene	4.0	3.0	NIMNC
Adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone:B230105C16 product:unknown EST, full insert sequence	3.9	-1.5	MIND
inhibitor of DNA binding 3	3.8	1.5	MINI
0 day neonate kidney cDNA, RIKEN full-length enriched library, clone:D630033G03 product:unclassifiable, full insert sequence	3.7	2.6	NIMNC
RIKEN cDNA 5830458K16 gene	3.7	-1.1	MINNC
defensin related cryptdin, related sequence 2	3.7	2.6	NIMNC
10 days neonate cortex cDNA, RIKEN full-length enriched library, clone:A830095F23 product:unknown EST, full insert sequence	3.7	1.3	MINNC
nuclear receptor subfamily 2, group F, member 2	3.4	-1.2	MINNC
regulator of G-protein signaling 16	3.4	5.1	MINI
Rhesus blood group-associated C glycoprotein	3.3	2.3	NIMNC
dickkopf homolog 3 (Xenopus laevis)	3.3	1.5	NIMNC
BTB (POZ) domain containing 11	3.3	1.7	MINNC
nuclear receptor subfamily 2, group F, member 2	3.2	1.0	MINNC
eukaryotic translation initiation factor 5A2	3.2	-1.4	MINNC
RIKEN cDNA 1110014F12 gene	3.1	2.8	NIMNC
RIKEN cDNA 5730596K20 gene	3.1	2.2	NIMNC
DNA segment, Chr 16, Brigham & Women's Genetics 1494 expressed	3.0	1.2	MINNC
sonic hedgehog	3.0	-1.3	MINNC
insulin-like growth factor binding protein 5	2.9	-1.6	MIND
manic fringe homolog (Drosophila)	2.9	2.8	MINI
tripartite motif protein 25	2.9	-1.4	MINNC
ankyrin repeat and SAM domain containing 1	2.8	1.1	MINNC
troponin T1, skeletal, slow	2.7	1.8	MINNC
delta-like 3 (Drosophila)	2.7	2.2	MINI

microtubule-associated protein 1 A	2.7	1.7	MINNC
myosin VIIa	2.7	-1.3	MINNC
suppression of tumorigenicity 18	2.6	1.1	MINNC
BTB (POZ) domain containing 11	2.6	1.0	MINNC
glutaredoxin 1 (thioltransferase)	2.6	4.8	NIMNC
annexin A1	2.6	2.1	NIMNC
Ras association (RalGDS/AF-6) domain family 4	2.6	2.2	NIMNC
nucleolar protein 4	2.6	-1.1	MINNC
cyclin F	2.6	-1.3	MINNC
10 days neonate cortex cDNA, RIKEN full-length enriched library, clone:A830095F23 product:unknown EST, full insert sequence	2.6	-1.3	MINNC
hairy and enhancer of split 5 (Drosophila)	2.6	1.9	MINI
hypothetical protein C730009D12	2.6	1.0	MINNC
RIKEN cDNA C630016B22 gene	2.5	1.0	MINNC
hairy and enhancer of split 6 (Drosophila)	2.5	6.4	MINI
BB071890	2.4	2.4	MINI
nuclear receptor subfamily 2, group F, member 2	2.4	-1.3	MINNC
polo-like kinase 3 (Drosophila)	2.4	2.0	MINNC
cDNA sequence BC038059	2.4	-1.3	MINNC
GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein	2.4	1.7	NIMNC
SPARC related modular calcium binding 1	2.4	1.0	MINNC
myelin basic protein	2.4	-1.1	MINNC
RIKEN cDNA 1110032O16 gene	2.3	-1.9	MINNC
RIKEN cDNA 3010033P07 gene	2.3	-1.2	MINNC
RIKEN cDNA 2810401C09 gene	2.3	1.3	MINNC
Adult male corpus striatum cDNA, RIKEN full-length enriched library, clone:C030047F10 product:unknown EST, full insert sequence	2.3	1.6	MINNC
hypothetical protein 9630027E11	2.3	1.2	MINNC
ISL1 transcription factor, LIM/homeodomain (islet 1)	2.3	-2.2	MIND
RIKEN cDNA A230057G18 gene	2.3	1.7	MINI
RIKEN cDNA 4632427E13 gene	2.2	-1.3	MINNC
RIKEN cDNA 0610043B10 gene	2.2	1.1	MINNC
BM119226	2.2	2.0	MINI
ELK3, member of ETS oncogene family	2.2	1.5	NIMNC
RIKEN cDNA A530064N14 gene	2.2	1.5	NIMNC
calbindin-28K	2.2	-1.5	MINNC
homeo box, msh-like 1	2.2	1.6	NIMNC
discoidin domain receptor family, member 1	2.2	1.1	MINNC
slit homolog 2 (Drosophila)	2.2	1.0	MINNC
cell division cycle 25 homolog B (S. cerevisiae)	2.1	1.5	MINNC
homeo box, msh-like 2	2.1	1.6	NIMNC
RNA imprinted and accumulated in nucleus	2.1	1.7	MINI
aquaporin 1	2.1	1.8	NIMNC
RIKEN cDNA 5430420F09 gene	2.1	1.8	NIMNC
cellular retinoic acid binding protein I	2.1	1.8	MINI
LIM homeobox protein 6	2.1	1.2	MINNC
Transcribed sequences	2.1	2.1	NIMNC
hairy and enhancer of split 6 (Drosophila)	2.1	2.3	MINI
RIKEN cDNA 4930488B01 gene	2.1	1.1	MINNC
transmembrane channel-like gene family 7	2.1	-1.1	MINNC
delta-like 1 (Drosophila)	2.1	1.8	MINI
zinc finger, SWIM domain containing 5	2.1	1.1	MINNC
hypothetical protein LOC223773	2.1	3.2	NIMNC
12 days embryo eyeball cDNA, RIKEN full-length enriched library, clone:D230032L01 product:unknown EST, full insert sequence	2.1	2.4	NIMNC

ATP-binding cassette, sub-family A (ABC1), member 3	2.0	-1.2	MINNC
BG797225	2.0	1.3	MINNC
RIKEN cDNA 5930418K15 gene	2.0	-1.1	MINNC
ISL1 transcription factor, LIM/homeodomain (islet 1)	2.0	-3.2	MIND
MAP/microtubule affinity-regulating kinase 4	2.0	3.2	NIMNC
glycoprotein 38	2.0	-1.1	MINNC
limb-bud and heart	2.0	2.0	MINNC
cyclin-dependent kinase inhibitor 1C (P57)	2.0	1.6	MINI
RIKEN cDNA 5730596K20 gene	2.0	2.0	NIMNC
proline-rich Gla (G-carboxyglutamic acid) polypeptide 2	2.0	1.6	NIMNC
opposite strand transcription unit to Stag3	2.0	1.8	MINNC
RIKEN cDNA 5430432M24 gene	2.0	7.7	MINI
cytochrome c oxidase subunit VIb, testes-specific	2.0	1.6	MINI
interferon, alpha-inducible protein	2.0	1.8	NIMNC
complexin 2	2.0	2.1	MINI
tripartite motif protein 9	2.0	1.9	NIMNC
dickkopf homolog 3 (Xenopus laevis)	1.9	1.7	NIMNC
dihydropyrimidinase-like 4	1.9	4.0	MINI
expressed sequence AV025504	1.9	-1.6	MIND
ribosomal protein S3	1.9	-1.5	MIND
coactosin-like 1 (Dictyostelium)	1.9	1.7	MINI
Adult male urinary bladder cDNA, RIKEN full-length enriched library, clone:9530044D24 product:unknown EST, full insert sequence	1.9	-1.1	MINNC
Transcribed sequence with weak similarity to protein pir:I58401 (M.musculus) I58401 protein-tyrosine kinase	1.9	2.2	NIMNC
B-cell translocation gene 1, anti-proliferative	1.9	-1.1	MINNC
coactosin-like 1 (Dictyostelium)	1.9	2.2	NIMNC
B-cell translocation gene 2, anti-proliferative	1.9	1.8	MINI
RIKEN cDNA 2810417H13 gene	1.9	-1.1	MINNC
signal transducer and activator of transcription 1	1.9	1.6	MINNC
RIKEN cDNA B930006L02 gene	1.9	-1.3	MINNC
RIKEN cDNA A830020B06 gene	1.9	1.5	MINNC
annexin A6	1.9	2.0	NIMNC
src homology 2 domain-containing transforming protein C1	1.8	2.2	NIMNC
hairy and enhancer of split 5 (Drosophila)	1.8	2.4	MINI
caldesmon 1	1.8	1.6	MINI
phosphoglucomutase 2-like 1	1.8	1.0	MINNC
RIKEN cDNA 1110006E14 gene	1.8	-1.2	MINNC
PTEN induced putative kinase 1	1.8	1.0	MINNC
dihydropyrimidinase-like 4	1.8	2.1	MINNC
RIKEN cDNA 3110035E14 gene	1.8	5.0	NIMNC
unc-5 homolog C (C. elegans)	1.8	1.9	NIMNC
low density lipoprotein receptor-related protein associated protein 1	1.8	7.8	NIMNC
supervillin	1.8	1.1	MINNC
RIKEN cDNA A730072L08 gene	1.8	1.4	MINNC
chloride channel 5	1.8	1.6	NIMNC
WAS protein family, member 2	1.8	1.6	NIMNC
procollagen, type I, alpha 1	1.8	1.5	NIMNC
RIKEN cDNA 1700105P06 gene	1.8	2.0	NIMNC
serine (or cysteine) proteinase inhibitor, clade G, member 1	1.8	1.9	MINI
DNA segment, Chr 11, ERATO Doi 759, expressed	1.8	1.7	MINNC
RIKEN cDNA C230098O21 gene	1.8	2.0	MINI
RIKEN cDNA 2810003H13 gene	1.8	3.4	MINI
GTL2, imprinted maternally expressed untranslated mRNA	1.7	-1.1	MINNC
LIM homeobox protein 8	1.7	-2.1	MIND
syntaxin binding protein 1	1.7	1.7	NIMNC

Annex 7: List of genes regulated by Mash1 in gain and loss-of function studies.

A total of 25 genes are upregulated in the basal ganglia of Mash1 electroporated embryos (E10.5) and in the same time downregulated in the basal ganglia of Mash1KO embryos (E13.5).
(*) fold change inferior to 1.2 times. Acc#: Gen bank accession number.

Name	Acc #	Mash1GOF	Mash1KO bg/WT
Mash1	NM_008553	22.9	-61.9
U94828*	U94828	4.5	-1.2
Rhbdl4	BM118307	3.3	-2.0
BF472132*	BF472132	3.1	-1.3
Tnnt1	NM_011618	2.8	-1.6
Mfng	NM_008595	2.5	-1.7
Isl1	NM_021459	2.5	-1.8
Gp38	NM_010329	1.9	-2.5
Dll1	NM_007865	1.8	-2.3
Hes6*	AI326893	1.8	-1.4
Nrarp	NM_025980	1.6	-2.1
Hes5	AV337579	1.6	-2.8
Lmo1	NM_057173	1.6	-1.2
Btg2*	NM_007570	1.6	-1.3
BM246377	BM246377	1.6	-1.6
Hdac5*	NM_010412	1.6	-1.3
AK014009*	AK014009	1.5	-1.4
Lhx8	D49658	1.5	-1.6
BF462770*	BF462770	1.4	-2.1
AW121365*	AW121365	1.4	-1.7
Ddah1*	AW556888	1.3	-1.3
Elavl4*	AK014133	1.3	-1.4
Xist*	L04961	1.3	-1.6
Gadd45g*	AK007410	1.3	-2.3
Actb*	NM_007393	1.2	-1.5

Annex 8: List of genes regulated by Ngn2 in gain and loss-of function studies.

A total of 89 genes are upregulated in the cortex of Ngn2 electroporated embryos (E10.5) and in the same time downregulated in the cortex of Mash1_Ngn2DKO embryos (E13.5).

(*) fold change inferior to 1.2 times. Acc#: Gen bank accession number.

Name	Acc #	Ngn2GOF	Mash1_Ngn2DKO
Sult4a1	NM_013873	8.2	-1.9
AK005776	AK005776	5.7	-2.8
Rhbdl4	BM118307	5.5	-1.7
U94828*	U94828	4.6	-1.4
Rnf14*	AK010162	4.1	-1.3
Wnt7b	NM_009528	3.0	-2.6
BB258560	BB258560	2.9	-2.4
Ebf3	NM_010096	2.6	-1.5
BE979968	BE979968	2.6	-1.9
Ier5l	NM_030244	2.6	-1.6
Gab1	NM_021356	2.5	-1.5
Centg3	AF459091	2.5	-1.6
Mfng	NM_008595	2.3	-1.5
Plxna2	BB085537	2.2	-1.6
C130099A20Rik	BB183525	2.1	-1.5
Podxl2	BB461988	2.1	-1.8
Hes5	AV337579	2.1	-4.0
C230098O21Rik	BG075755	2.1	-1.8
NM_009708	NM_009708	2.0	-2.6
Atp6v0a1	U13836	2.0	-1.5
Ywhag*	NM_018871	2.0	-1.4
Syt4	NM_009308.2	2.0	-4.2
Hes6	AI326893	2.0	-1.5
Cap1	NM_007598	2.0	-1.8
Pcbp4*	NM_021567	1.9	-1.4
BQ177224	BQ177224	1.9	-2.0
Igsf8	AF411055	1.9	-1.6
Rhoe	BC009002	1.8	-1.8
NM_009250	NM_009250	1.8	-3.8
Elavl2	AY035380	1.8	-2.3
Elavl4	NM_010488	1.8	-2.2
Rab3d	BM230485	1.8	-3.1
Serping1	NM_009776	1.8	-2.5
Mtus1*	AW554551	1.8	-1.4
Fgd4*	AF402611	1.8	-1.2
Chst8	AK005217	1.7	-2.4
Stxbp1	NM_009295	1.7	-2.6
Kif21b	AV122249	1.7	-2.0
Cpeb4	BG070342	1.7	-1.9
NM_009217	NM_009217	1.6	-2.1
Rbm9	AF229055	1.6	-2.5
Mtap2	NM_008632	1.6	-2.6
Tmem2*	BC019745	1.6	-1.4
Spnb3	AF026489	1.6	-3.3
Dll1	NM_007865	1.6	-1.8
Elavl3	U29149	1.6	-2.5

Kif5c	NM_008449	1.6	-1.9
Freq	BE990928	1.6	-2.3
AV328634	AV328634	1.6	-2.6
Dusp14	AK009744	1.6	-1.8
BE956483	BE956483	1.5	-2.9
Gabarapl1	AF180518	1.5	-1.4
Adar	AF291876	1.5	-1.5
Lrrc4b	AW491122	1.5	-1.7
Dusp4*	AK012530	1.4	-3.1
Tubb3*	NM_023279	1.4	-1.6
Sox11*	BG072739	1.4	-1.5
Slc15a2*	NM_021301	1.4	-1.8
BM245880 *	BM245880	1.4	-1.9
BM114349 *	BM114349	1.4	-2.0
AK014196 *	AK014196	1.4	-2.6
AK011360 *	AK011360	1.4	-1.2
Plk2*	BM234765	1.4	-2.1
Cacna2d1*	BI106777	1.4	-2.4
BM117827 *	BM117827	1.4	-1.4
Calm3*	BB396904	1.4	-1.2
Arhgef2*	BE865221	1.4	-1.7
Gng2*	BC021599	1.4	-2.3
Gap43*	BB622036	1.4	-2.0
Cmkor1*	BC015254	1.4	-2.9
AV024662 *	AV024662	1.4	-1.8
Prkcb1*	X59274	1.4	-1.3
Nhlh1*	NM_010916	1.4	-2.7
Cdk5*	NM_007668	1.4	-1.4
Ntrk3*	NM_008746.2	1.3	-6.7
Dner*	AF370126	1.3	-1.8
BQ175875 *	BQ175875	1.3	-1.9
Tmsb10*	NM_025284	1.3	-1.2
Vcam1*	NM_011693	1.3	-3.2
Cbfa2t3h*	NM_009824	1.3	-2.9
Idb2*	BF019883	1.3	-1.9
BC023182 *	BC023182	1.3	-1.3
AV131166 *	AV131166	1.3	-1.4
Apbb1*	AF206720	1.3	-1.5
Spin*	BM228780	1.2	-1.4
AI464302 *	AI464302	1.2	-2.3
Nedd4l*	BB759157	1.2	-1.7
Hey1*	NM_010423	1.2	-2.8
Ddah2*	NM_016765	1.2	-1.4

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Acampora,D., Avantaggiato,V., Tuorto,F., Barone,P., Reichert,H., Finkelstein,R., and Simeone,A. (1998). Murine Otx1 and Drosophila otd genes share conserved genetic functions required in invertebrate and vertebrate brain development. Development *125*, 1691-1702.

Akamatsu,W., Okano,H.J., Osumi,N., Inoue,T., Nakamura,S., Sakakibara,S., Miura,M., Matsuo,N., Darnell,R.B., and Okano,H. (1999). Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. Proc. Natl. Acad. Sci. U. S. A *96*, 9885-9890.

Akazawa,C., Ishibashi,M., Shimizu,C., Nakanishi,S., and 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. J. Biol. Chem. *270*, 8730-8738.

Akazawa,C., Sasai,Y., Nakanishi,S., and Kageyama,R. (1992). Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J. Biol. Chem. *267*, 21879-21885.

Alifragis,P., Liapi,A., and Parnavelas,J.G. (2004). Lhx6 regulates the migration of cortical interneurons from the ventral telencephalon but does not specify their GABA phenotype. J. Neurosci. *24*, 5643-5648.

Altman,R.B. and Raychaudhuri,S. (2001). Whole-genome expression analysis: challenges beyond clustering. Curr. Opin. Struct. Biol. *11*, 340-347.

Anchan,R.M. and Reh,T.A. (1995). Transforming growth factor-beta-3 is mitogenic for rat retinal progenitor cells in vitro. J. Neurobiol. *28*, 133-145.

Anderson,S.A., Eisenstat,D.D., Shi,L., and Rubenstein,J.L. (1997a). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science *278*, 474-476.

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

Artavanis-Tsakonas,S., Rand,M.D., and Lake,R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Backman,M., Machon,O., Mygland,L., van den Bout,C.J., Zhong,W., Taketo,M.M., and Krauss,S. (2005). Effects of canonical Wnt signaling on dorso-ventral specification of the mouse telencephalon. Dev. Biol. *279*, 155-168.

Bae,S., Bessho,Y., Hojo,M., and Kageyama,R. (2000). The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development *127*, 2933-2943.

Bailey,A.M. and Posakony,J.W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes Dev. *9*, 2609-2622.

Baladron,V., Ruiz-Hidalgo,M.J., Nueda,M.L., Diaz-Guerra,M.J., Garcia-Ramirez,J.J., Bonvini,E., Gubina,E., and Laborda,J. (2005). dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. Exp. Cell Res. *303*, 343-359.

Banfi,S., Borsani,G., Rossi,E., Bernard,L., Guffanti,A., Rubboli,F., Marchitiello,A., Giglio,S., Coluccia,E., Zollo,M., Zuffardi,O., and Ballabio,A. (1996). Identification and mapping of human cDNAs homologous to Drosophila mutant genes through EST database searching. Nat. Genet. *13*, 167-174.

Baraban,S.C. and Tallent,M.K. (2004). Interneuron Diversity series: Interneuronal neuropeptides--endogenous regulators of neuronal excitability. Trends Neurosci. *27*, 135-142.

Barres,B.A., Lazar,M.A., and Raff,M.C. (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development *120*, 1097-1108.

Bartholoma,A. and Nave,K.A. (1994). NEX-1: a novel brain-specific helix-loop-helix protein with autoregulation and sustained expression in mature cortical neurons. Mech. Dev. *48*, 217-228.

Beckers,J., Caron,A., Hrabe,d.A., Hans,S., Campos-Ortega,J.A., and Gossler,A. (2000). Distinct regulatory elements direct delta1 expression in the nervous system and paraxial mesoderm of transgenic mice. Mech. Dev. *95*, 23-34.

Belandia,B., Powell,S.M., Garcia-Pedrero,J.M., Walker,M.M., Bevan,C.L., and Parker,M.G. (2005). Hey1, a mediator of notch signaling, is an androgen receptor corepressor. Mol. Cell Biol. *25*, 1425-1436.

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

Ben Arie,N., Bellen,H.J., Armstrong,D.L., McCall,A.E., Gordadze,P.R., Guo,Q., Matzuk,M.M., and Zoghbi,H.Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. Nature *390*, 169-172.

Ben Arie,N., Hassan,B.A., Bermingham,N.A., Malicki,D.M., Armstrong,D., Matzuk,M., Bellen,H.J., and Zoghbi,H.Y. (2000). Functional conservation of atonal and Math1 in the CNS and PNS. Development *127*, 1039-1048.

Bertrand,N., Castro,D.S., and Guillemot,F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. *3*, 517-530.

Bettenhausen,B., Hrabe,d.A., Simon,D., Guenet,J.L., and Gossler,A. (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. Development *121*, 2407-2418.

Bishop,K.M., Garel,S., Nakagawa,Y., Rubenstein,J.L., and O'Leary,D.D. (2003). Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding. J. Comp Neurol. *457*, 345-360.

Bishop,K.M., Goudreau,G., and O'Leary,D.D. (2000). Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science *288*, 344-349.

Boda,B., Alberi,S., Nikonenko,I., Node-Langlois,R., Jourdain,P., Moosmayer,M., Parisi-Jourdain,L., and Muller,D. (2004). The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. J. Neurosci. *24*, 10816-10825.

Bonni,A., Sun,Y., Nadal-Vicens,M., Bhatt,A., Frank,D.A., Rozovsky,I., Stahl,N., Yancopoulos,G.D., and Greenberg,M.E. (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science *278*, 477-483.

Bramblett,D.E., Copeland,N.G., Jenkins,N.A., and Tsai,M.J. (2002). BHLHB4 is a bHLH transcriptional regulator in pancreas and brain that marks the dimesencephalic boundary. Genomics *79*, 402-412.

Brand,M., Jarman,A.P., Jan,L.Y., and Jan,Y.N. (1993). asense is a Drosophila neural precursor gene and is capable of initiating sense organ formation. Development *119*, 1-17.

Bray,S. (1998). Notch signalling in Drosophila: three ways to use a pathway. Semin. Cell Dev. Biol. *9*, 591-597.

Brazma,A. and Vilo,J. (2000). Gene expression data analysis. FEBS Lett. *480*, 17-24.

Briscoe,J. and Ericson,J. (2001). Specification of neuronal fates in the ventral neural tube. Curr. Opin. Neurobiol. *11*, 43-49.

Brott,B.K., Pinsky,B.A., and Erikson,R.L. (1998). Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. Proc. Natl. Acad. Sci. U. S. A *95*, 963-968.

Brunelli,S., Innocenzi,A., and Cossu,G. (2003). Bhlhb5 is expressed in the CNS and sensory organs during mouse embryonic development. Gene Expr. Patterns. *3*, 755-759.

Brunskill,E.W., Witte,D.P., Shreiner,A.B., and Potter,S.S. (1999). Characterization of npas3, a novel basic helix-loop-helix PAS gene expressed in the developing mouse nervous system. Mech. Dev. *88*, 237-241.

Bush,A., Hiromi,Y., and Cole,M. (1996). Biparous: a novel bHLH gene expressed in neuronal and glial precursors in Drosophila. Dev. Biol. *180*, 759-772.

Cabrera,C.V. and Alonso,M.C. (1991). Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila. EMBO J. *10*, 2965-2973.

Cadigan,K.M. and Nusse,R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286-3305.

Campbell,K. and Gotz,M. (2002). Radial glia: multi-purpose cells for vertebrate brain development. Trends Neurosci. *25*, 235-238.

Campuzano,S. and Modolell,J. (1992). Patterning of the Drosophila nervous system: the achaete-scute gene complex. Trends Genet. *8*, 202-208.

Cannizzaro,C., Tel,B.C., Rose,S., Zeng,B.Y., and Jenner,P. (2003). Increased neuropeptide Y mRNA expression in striatum in Parkinson's disease. Brain Res. Mol. Brain Res. *110*, 169-176.

Cao,Y. and Dulac,C. (2001). Profiling brain transcription: neurons learn a lesson from yeast. Curr. Opin. Neurobiol. *11*, 615-620.

Casarosa,S., Fode,C., and Guillemot,F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525-534.

Cattaneo,E. and McKay,R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature *347*, 762-765.

Cau,E., Gradwohl,G., Casarosa,S., Kageyama,R., and Guillemot,F. (2000). Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development *127*, 2323-2332.

Cau,E., Gradwohl,G., Fode,C., and Guillemot,F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development *124*, 1611-1621.

Chen,H., Thiagalingam,A., Chopra,H., Borges,M.W., Feder,J.N., Nelkin,B.D., Baylin,S.B., and Ball,D.W. (1997). Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proc. Natl. Acad. Sci. U. S. A *94*, 5355-5360.

Chen,P., Johnson,J.E., Zoghbi,H.Y., and Segil,N. (2002). The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. Development *129*, 2495-2505.

Chiang,C., Litingtung,Y., Lee,E., Young,K.E., Corden,J.L., Westphal,H., and Beachy,P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature *383*, 407-413.

Chien,C.T., Hsiao,C.D., Jan,L.Y., and Jan,Y.N. (1996). Neuronal type information encoded in the basic-helix-loop-helix domain of proneural genes. Proc. Natl. Acad. Sci. U. S. A *93*, 13239-13244.

Cho,R.J., Campbell,M.J., Winzeler,E.A., Steinmetz,L., Conway,A., Wodicka,L., Wolfsberg,T.G., Gabrielian,A.E., Landsman,D., Lockhart,D.J., and Davis,R.W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell *2*, 65-73.

Choi,K.W. and Benzer,S. (1994). Rotation of photoreceptor clusters in the developing Drosophila eye requires the nemo gene. Cell *78*, 125-136.

Chu,S., DeRisi,J., Eisen,M., Mulholland,J., Botstein,D., Brown,P.O., and Herskowitz,I. (1998). The transcriptional program of sporulation in budding yeast. Science *282*, 699-705.

Cobos,I., Broccoli,V., and Rubenstein,J.L. (2005). The vertebrate ortholog of Aristaless is regulated by Dlx genes in the developing forebrain. J. Comp Neurol. *483*, 292-303.

Conti,L., Cataudella,T., and Cattaneo,E. (2003). Neural stem cells: a pharmacological tool for brain diseases? Pharmacol. Res. *47*, 289-297.

Cross,J.C., Baczyk,D., Dobric,N., Hemberger,M., Hughes,M., Simmons,D.G., Yamamoto,H., and Kingdom,J.C. (2003). Genes, development and evolution of the placenta. Placenta *24*, 123-130.

Crossley,P.H., Martinez,S., Ohkubo,Y., and Rubenstein,J.L. (2001). Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. Neuroscience *108*, 183-206.

Dale,J.K., Vesque,C., Lints,T.J., Sampath,T.K., Furley,A., Dodd,J., and Placzek,M. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. Cell *90*, 257-269.

Davis,R.L. and Turner,D.L. (2001). Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene *20*, 8342-8357.

de la Pompa,J.L., Wakeham,A., Correia,K.M., Samper,E., Brown,S., Aguilera,R.J., Nakano,T., Honjo,T., Mak,T.W., Rossant,J., and Conlon,R.A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. Development *124*, 1139-1148.

De Strooper,B., Annaert,W., Cupers,P., Saftig,P., Craessaerts,K., Mumm,J.S., Schroeter,E.H., Schrijvers,V., Wolfe,M.S., Ray,W.J., Goate,A., and Kopan,R. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature *398*, 518-522.

Diatchenko,L., Lau,Y.F., Campbell,A.P., Chenchik,A., Moqadam,F., Huang,B., Lukyanov,S., Lukyanov,K., Gurskaya,N., Sverdlov,E.D., and Siebert,P.D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. U. S. A *93*, 6025-6030.

Dominguez,M. and Campuzano,S. (1993). asense, a member of the Drosophila achaete-scute complex, is a proneural and neural differentiation gene. EMBO J. *12*, 2049-2060.

Donoviel,D.B., Donoviel,M.S., Fan,E., Hadjantonakis,A., and Bernstein,A. (1998). Cloning and characterization of Sel-1l, a murine homolog of the C. elegans sel-1 gene. Mech. Dev. *78*, 203-207.

Dorflinger,U., Pscherer,A., Moser,M., Rummele,P., Schule,R., and Buettner,R. (1999). Activation of somatostatin receptor II expression by transcription factors MIBP1 and SEF-2 in the murine brain. Mol. Cell Biol. *19*, 3736-3747.

Dougherty,J.D. and Geschwind,D.H. (2002). Subtraction-coupled custom microarray analysis for gene discovery and gene expression studies in the CNS. Chem. Senses *27*, 293-298.

Dubois,L., Bally-Cuif,L., Crozatier,M., Moreau,J., Paquereau,L., and Vincent,A. (1998). XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in Xenopus. Curr. Biol. *8*, 199-209.

Durand,B. and Raff,M. (2000). A cell-intrinsic timer that operates during oligodendrocyte development. Bioessays *22*, 64-71.

Egger,B., Leemans,R., Loop,T., Kammermeier,L., Fan,Y., Radimerski,T., Strahm,M.C., Certa,U., and Reichert,H. (2002). Gliogenesis in Drosophila: genome-wide analysis of downstream genes of glial cells missing in the embryonic nervous system. Development *129*, 3295-3309.

Eib,D.W., Holling,T.M., Zwijsen,A., Dewulf,N., de Groot,E., van den Eijnden-van Raaij AJ, Huylebroeck,D., and Martens,G.J. (2000). Expression of the follistatin/EGF-containing transmembrane protein M7365 (tomoregulin-1) during mouse development. Mech. Dev. *97*, 167-171.

Eisen,M.B., Spellman,P.T., Brown,P.O., and Botstein,D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A *95*, 14863-14868.

Eisenstat,D.D., Liu,J.K., Mione,M., Zhong,W., Yu,G., Anderson,S.A., Ghattas,I., Puelles,L., and Rubenstein,J.L. (1999). DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. J. Comp Neurol. *414*, 217-237.

Englund,C., Fink,A., Lau,C., Pham,D., Daza,R.A., Bulfone,A., Kowalczyk,T., and Hevner,R.F. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J. Neurosci. *25*, 247-251.

Ericson,J., Muhr,J., Placzek,M., Lints,T., Jessell,T.M., and Edlund,T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. Cell *81*, 747-756.

Faedo,A., Quinn,J.C., Stoney,P., Long,J.E., Dye,C., Zollo,M., Rubenstein,J.L., Price,D.J., and Bulfone,A. (2004). Identification and characterization of a novel transcript down-regulated in Dlx1/Dlx2 and up-regulated in Pax6 mutant telencephalon. Dev. Dyn. *231*, 614-620.

Fan,C.M. and Tessier-Lavigne,M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. Cell *79*, 1175-1186.

Farah,M.H., Olson,J.M., Sucic,H.B., Hume,R.I., Tapscott,S.J., and Turner,D.L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693-702.

Ferreiro,B., Kintner,C., Zimmerman,K., Anderson,D., and Harris,W.A. (1994). XASH genes promote neurogenesis in Xenopus embryos. Development *120*, 3649-3655.

Ferreiro,B., Skoglund,P., Bailey,A., Dorsky,R., and Harris,W.A. (1993). XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. Mech. Dev. *40*, 25-36.

Fisher,A.L. and Caudy,M. (1998). Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. Genes Dev. *12*, 1931-1940.

Fode,C., Gradwohl,G., Morin,X., Dierich,A., LeMeur,M., Goridis,C., and Guillemot,F. (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron *20*, 483-494.

Fode,C., Ma,Q., Casarosa,S., Ang,S.L., Anderson,D.J., and Guillemot,F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev. *14*, 67-80.

Foster,G.A., Schultzberg,M., Hokfelt,T., Goldstein,M., Hemmings,H.C., Jr., Ouimet,C.C., Walaas,S.I., and Greengard,P. (1987). Development of a dopamine- and cyclic adenosine 3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system, and its relationship to the arrival of presumptive dopaminergic innervation. J. Neurosci. *7*, 1994-2018.

Fukuchi-Shimogori,T. and Grove,E.A. (2001). Neocortex patterning by the secreted signaling molecule FGF8. Science *294*, 1071-1074.

Furuta,Y., Piston,D.W., and Hogan,B.L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development *124*, 2203-2212.

Gaasterland,T. and Bekiranov,S. (2000). Making the most of microarray data. Nat. Genet. *24*, 204-206.

Gabay,L., Lowell,S., Rubin,L.L., and Anderson,D.J. (2003). Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. Neuron *40*, 485-499.

Gao,F.B., Durand,B., and Raff,M. (1997). Oligodendrocyte precursor cells count time but not cell divisions before differentiation. Curr. Biol. *7*, 152-155.

Garcia-Dominguez,M., Poquet,C., Garel,S., and Charnay,P. (2003). Ebf gene function is required for coupling neuronal differentiation and cell cycle exit. Development *130*, 6013-6025.

Gasa,R., Mrejen,C., Leachman,N., Otten,M., Barnes,M., Wang,J., Chakrabarti,S., Mirmira,R., and German,M. (2004). Proendocrine genes coordinate the pancreatic islet differentiation program in vitro. Proc. Natl. Acad. Sci. U. S. A *101*, 13245-13250.

Geschwind,D.H., Ou,J., Easterday,M.C., Dougherty,J.D., Jackson,R.L., Chen,Z., Antoine,H., Terskikh,A., Weissman,I.L., Nelson,S.F., and Kornblum,H.I. (2001). A genetic analysis of neural progenitor differentiation. Neuron *29*, 325-339.

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

Ghysen,A. and Dambly-Chaudiere,C. (1988). From DNA to form: the achaete-scute complex. Genes Dev. *2*, 495-501.

Ghysen,A., Dambly-Chaudiere,C., Jan,L.Y., and Jan,Y.N. (1993). Cell interactions and gene interactions in peripheral neurogenesis. Genes Dev. *7*, 723-733.

Giagtzoglou,N., Alifragis,P., Koumbanakis,K.A., and Delidakis,C. (2003). Two modes of recruitment of E(spl) repressors onto target genes. Development *130*, 259-270.

Gillies,K. and Price,D.J. (1993). The fates of cells in the developing cerebral cortex of normal and methylazoxymethanol acetate-lesioned mice. Eur. J. Neurosci. *5*, 73-84.

Gomez-Skarmeta,J.L., Diez,d.C., Calle-Mustienes,E., Ferre-Marco,D., and Modolell,J. (1996). Araucan and caupolican, two members of the novel iroquois complex, encode homeoproteins that control proneural and vein-forming genes. Cell *85*, 95-105.

Gomez-Skarmeta,J.L., Glavic,A., Calle-Mustienes,E., Modolell,J., and Mayor,R. (1998). Xiro, a Xenopus homolog of the Drosophila Iroquois complex genes, controls development at the neural plate. EMBO J. *17*, 181-190.

Goodman,R.H. and Smolik,S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553-1577.

Gotz,M. (2003). Glial cells generate neurons--master control within CNS regions: developmental perspectives on neural stem cells. Neuroscientist. *9*, 379-397.

Goulding,S.E., White,N.M., and Jarman,A.P. (2000a). cato encodes a basic helix-loop-helix transcription factor implicated in the correct differentiation of Drosophila sense organs. Dev. Biol. *221*, 120-131.

Goulding,S.E., zur,L.P., and Jarman,A.P. (2000b). amos, a proneural gene for Drosophila olfactory sense organs that is regulated by lozenge. Neuron *25*, 69-78.

Gradwohl,G., Dierich,A., LeMeur,M., and Guillemot,F. (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc. Natl. Acad. Sci. U. S. A *97*, 1607-1611.

Gradwohl,G., Fode,C., and Guillemot,F. (1996). Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. Dev. Biol. *180*, 227-241.

Gross,R.E., Mehler,M.F., Mabie,P.C., Zang,Z., Santschi,L., and Kessler,J.A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron *17*, 595-606.

Grove,E.A., Tole,S., Limon,J., Yip,L., and Ragsdale,C.W. (1998). The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. Development *125*, 2315-2325.

Guillemot,F., Caspary,T., Tilghman,S.M., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., Anderson,D.J., Joyner,A.L., Rossant,J., and Nagy,A. (1995). Genomic imprinting of Mash2, a mouse gene required for trophoblast development. Nat. Genet. *9*, 235-242.

Guillemot,F. and Joyner,A.L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mech. Dev. *42*, 171-185.

Guillemot,F., Lo,L.C., Johnson,J.E., Auerbach,A., Anderson,D.J., and Joyner,A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463-476.

Gumbiner,B.M. (1998). Propagation and localization of Wnt signaling. Curr. Opin. Genet. Dev. *8*, 430-435.

Gupta,A., Tsai,L.H., and Wynshaw-Boris,A. (2002). Life is a journey: a genetic look at neocortical development. Nat. Rev. Genet. *3*, 342-355.

Hagiwara,N., Klewer,S.E., Samson,R.A., Erickson,D.T., Lyon,M.F., and Brilliant,M.H. (2000). Sox6 is a candidate gene for p100H myopathy, heart block, and sudden neonatal death. Proc. Natl. Acad. Sci. U. S. A *97*, 4180-4185.

Hamada-Kanazawa,M., Ishikawa,K., Ogawa,D., Kanai,M., Kawai,Y., Narahara,M., and Miyake,M. (2004). Suppression of Sox6 in P19 cells leads to failure of neuronal differentiation by retinoic acid and induces retinoic acid-dependent apoptosis. FEBS Lett. *577*, 60-66.

Hamasaki,T., Leingartner,A., Ringstedt,T., and O'Leary,D.D. (2004). EMX2 regulates sizes and positioning of the primary sensory and motor areas in neocortex by direct specification of cortical progenitors. Neuron *43*, 359-372.

Hartenstein,V. and Posakony,J.W. (1989). Development of adult sensilla on the wing and notum of Drosophila melanogaster. Development *107*, 389-405.

Hartenstein,V. and Posakony,J.W. (1990). A dual function of the Notch gene in Drosophila sensillum development. Dev. Biol. *142*, 13-30.

Haspel,J. and Grumet,M. (2003). The L1CAM extracellular region: a multi-domain protein with modular and cooperative binding modes. Front Biosci. *8*, s1210-s1225.

Hassan,B.A., Bermingham,N.A., He,Y., Sun,Y., Jan,Y.N., Zoghbi,H.Y., and Bellen,H.J. (2000). atonal regulates neurite arborization but does not act as a proneural gene in the Drosophila brain. Neuron *25*, 549-561.

Hatakeyama,J. and Kageyama,R. (2004). Retinal cell fate determination and bHLH factors. Semin. Cell Dev. Biol. *15*, 83-89.

Hatten,M.E. (1999). Central nervous system neuronal migration. Annu. Rev. Neurosci. *22*, 511-539.

He,W., Ingraham,C., Rising,L., Goderie,S., and Temple,S. (2001). Multipotent stem cells from the mouse basal forebrain contribute GABAergic neurons and oligodendrocytes to the cerebral cortex during embryogenesis. J. Neurosci. *21*, 8854-8862.

Hebert,J.M., Mishina,Y., and McConnell,S.K. (2002). BMP signaling is required locally to pattern the dorsal telencephalic midline. Neuron *35*, 1029-1041.

Heins,N., Malatesta,P., Cecconi,F., Nakafuku,M., Tucker,K.L., Hack,M.A., Chapouton,P., Barde,Y.A., and Gotz,M. (2002). Glial cells generate neurons: the role of the transcription factor Pax6. Nat. Neurosci. *5*, 308-315.

Heitzler,P., Bourouis,M., Ruel,L., Carteret,C., and 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*, 161-171.

Helms,A.W. and Johnson,J.E. (2003). Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol. *13*, 42-49.

Herbert,J., Wilcox,J.N., Pham,K.T., Fremeau,R.T., Jr., Zeviani,M., Dwork,A., Soprano,D.R., Makover,A., Goodman,D.S., Zimmerman,E.A., and . (1986). Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. Neurology *36*, 900-911.

Hevner,R.F., Shi,L., Justice,N., Hsueh,Y., Sheng,M., Smiga,S., Bulfone,A., Goffinet,A.M., Campagnoni,A.T., and Rubenstein,J.L. (2001). Tbr1 regulates differentiation of the preplate and layer 6. Neuron *29*, 353-366.

Hiol,A., Davey,P.C., Osterhout,J.L., Waheed,A.A., Fischer,E.R., Chen,C.K., Milligan,G., Druey,K.M., and Jones,T.L. (2003). Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue. J. Biol. Chem. *278*, 19301-19308.

Hirsch,M.R., Tiveron,M.C., Guillemot,F., Brunet,J.F., and Goridis,C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development *125*, 599-608.

Horne,M.C., Donaldson,K.L., Goolsby,G.L., Tran,D., Mulheisen,M., Hell,J.W., and Wahl,A.F. (1997). Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J. Biol. Chem. *272*, 12650-12661.

Hu,Y., Wang,T., Stormo,G.D., and Gordon,J.I. (2004). RNA interference of achaete-scute homolog 1 in mouse prostate neuroendocrine cells reveals its gene targets and DNA binding sites. Proc. Natl. Acad. Sci. U. S. A *101*, 5559-5564.

Huang,H.P., Liu,M., El Hodiri,H.M., Chu,K., Jamrich,M., and Tsai,M.J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol. Cell Biol. *20*, 3292-3307.

Hubank,M. and Schatz,D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. *22*, 5640-5648.

Hughes,T.R., Marton,M.J., Jones,A.R., Roberts,C.J., Stoughton,R., Armour,C.D., Bennett,H.A., Coffey,E., Dai,H., He,Y.D., Kidd,M.J., King,A.M., Meyer,M.R., Slade,D., Lum,P.Y., Stepaniants,S.B., Shoemaker,D.D., Gachotte,D., Chakraburtty,K., Simon,J., Bard,M., and Friend,S.H. (2000). Functional discovery via a compendium of expression profiles. Cell *102*, 109-126.

Hyodo-Miura,J., Urushiyama,S., Nagai,S., Nishita,M., Ueno,N., and Shibuya,H. (2002). Involvement of NLK and Sox11 in neural induction in Xenopus development. Genes Cells *7*, 487-496.

Imaki,J., Yoshida,K., and Yamashita,K. (1994). A developmental study of cyclic AMP-response element binding protein (CREB) by in situ hybridization histochemistry and immunocytochemistry in the rat neocortex. Brain Res. *651*, 269-274.

Ishibashi,M., Ang,S.L., Shiota,K., Nakanishi,S., Kageyama,R., and 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 Dev. *9*, 3136-3148.

Ishii,Y., Nakamura,S., and Osumi,N. (2000). Demarcation of early mammalian cortical development by differential expression of fringe genes. Brain Res. Dev. Brain Res. *119*, 307-320.

Ishitani,T., Ninomiya-Tsuji,J., Nagai,S., Nishita,M., Meneghini,M., Barker,N., Waterman,M., Bowerman,B., Clevers,H., Shibuya,H., and Matsumoto,K. (1999). The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature *399*, 798-802.

Isshiki,T., Pearson,B., Holbrook,S., and Doe,C.Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell *106*, 511-521.

Iyer,V.R., Eisen,M.B., Ross,D.T., Schuler,G., Moore,T., Lee,J.C., Trent,J.M., Staudt,L.M., Hudson,J., Jr., Boguski,M.S., Lashkari,D., Shalon,D., Botstein,D., and Brown,P.O. (1999). The transcriptional program in the response of human fibroblasts to serum. Science *283*, 83-87.

Jafar-Nejad,H., Acar,M., Nolo,R., Lacin,H., Pan,H., Parkhurst,S.M., and Bellen,H.J. (2003). Senseless acts as a binary switch during sensory organ precursor selection. Genes Dev. *17*, 2966-2978.

Jan,Y.N. and Jan,L.Y. (1994). Genetic control of cell fate specification in Drosophila peripheral nervous system. Annu. Rev. Genet. *28*, 373-393.

Jarman,A.P., Grau,Y., Jan,L.Y., and Jan,Y.N. (1993). atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell *73*, 1307-1321.

Jaszai,J. and Brand,M. (2002). Cloning and expression of Ventrhoid, a novel vertebrate homologue of the Drosophila EGF pathway gene rhomboid. Mech. Dev. *113*, 73-77.

Jensen,C.H., Meyer,M., Schroder,H.D., Kliem,A., Zimmer,J., and Teisner,B. (2001a). Neurons in the monoaminergic nuclei of the rat and human central nervous system express FA1/dlk. Neuroreport *12*, 3959-3963.

Jensen,C.H., Meyer,M., Schroder,H.D., Kliem,A., Zimmer,J., and Teisner,B. (2001b). Neurons in the monoaminergic nuclei of the rat and human central nervous system express FA1/dlk. Neuroreport *12*, 3959-3963.

Jimenez,F. and Campos-Ortega,J.A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster. Neuron *5*, 81-89.

Jimenez,F. and Modolell,J. (1993). Neural fate specification in Drosophila. Curr. Opin. Genet. Dev. *3*, 626-632.

Joel,D. and Weiner,I. (2000). The connections of the dopaminergic system with the striatum in rats and primates: an analysis with respect to the functional and compartmental organization of the striatum. Neuroscience *96*, 451-474.

Johnson,J.E., Birren,S.J., and Anderson,D.J. (1990). Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. Nature *346*, 858-861.

Johnson,J.E., Birren,S.J., Saito,T., and Anderson,D.J. (1992). DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer. Proc. Natl. Acad. Sci. U. S. A *89*, 3596-3600.

Johnson,R.L., Laufer,E., Riddle,R.D., and Tabin,C. (1994). Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. Cell *79*, 1165-1173.

Kageyama,R. and Nakanishi,S. (1997). Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr. Opin. Genet. Dev. *7*, 659-665.

Kane,C.J., Brown,G.J., and Phelan,K.D. (1996). Transforming growth factor-beta 2 both stimulates and inhibits neurogenesis of rat cerebellar granule cells in culture. Brain Res. Dev. Brain Res. *96*, 46-51.

Kanekar,S., Perron,M., Dorsky,R., Harris,W.A., Jan,L.Y., Jan,Y.N., and Vetter,M.L. (1997). Xath5 participates in a network of bHLH genes in the developing Xenopus retina. Neuron *19*, 981-994.

Kario,E., Marmor,M.D., Adamsky,K., Citri,A., Amit,I., Amariglio,N., Rechavi,G., and Yarden,Y. (2004). Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. J. Biol. Chem.

Karsten,S.L., Kudo,L.C., Jackson,R., Sabatti,C., Kornblum,H.I., and Geschwind,D.H. (2003). Global analysis of gene expression in neural progenitors reveals specific cell-cycle, signaling, and metabolic networks. Dev. Biol. *261*, 165-182.

Kasashima,K., Terashima,K., Yamamoto,K., Sakashita,E., and Sakamoto,H. (1999). Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. Genes Cells *4*, 667-683.

Kim,P., Helms,A.W., Johnson,J.E., and Zimmerman,K. (1997). XATH-1, a vertebrate homolog of Drosophila atonal, induces a neuronal differentiation within ectodermal progenitors. Dev. Biol. *187*, 1-12.

Kohtz,J.D., Baker,D.P., Corte,G., and Fishell,G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. Development *125*, 5079-5089.

Kondo,T. and Raff,M. (2000). The Id4 HLH protein and the timing of oligodendrocyte differentiation. EMBO J. *19*, 1998-2007.

Kortenjann,M., Nehls,M., Smith,A.J., Carsetti,R., Schuler,J., Kohler,G., and Boehm,T. (2001). Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk. Eur. J. Immunol. *31*, 3580-3587.

Kotani,M., Tajima,Y., Osanai,T., Irie,A., Iwatsuki,K., Kanai-Azuma,M., Imada,M., Kato,H., Shitara,H., Kubo,H., and Sakuraba,H. (2003). Complementary DNA cloning and characterization of RANDAM-2, a type I membrane molecule specifically expressed on glutamatergic neuronal cells in the mouse cerebrum. J. Neurosci. Res. *73*, 603-613.

Koyano-Nakagawa,N., Kim,J., Anderson,D., and Kintner,C. (2000). Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. Development *127*, 4203-4216.

Koyano-Nakagawa,N., Wettstein,D., and Kintner,C. (1999). Activation of Xenopus genes required for lateral inhibition and neuronal differentiation during primary neurogenesis. Mol. Cell Neurosci. *14*, 327-339.

Kramatschek,B. and Campos-Ortega,J.A. (1994). Neuroectodermal transcription of the Drosophila neurogenic genes E(spl) and HLH-m5 is regulated by proneural genes. Development *120*, 815-826.

Kruger,M. and Braun,T. (2002). The neuronal basic helix-loop-helix transcription factor NSCL-1 is dispensable for normal neuronal development. Mol. Cell Biol. *22*, 792-800.

Kruger,M., Ruschke,K., and Braun,T. (2004). NSCL-1 and NSCL-2 synergistically determine the fate of GnRH-1 neurons and control necdin gene expression. EMBO J. *23*, 4353-4364.

Kuhn,H.G., Winkler,J., Kempermann,G., Thal,L.J., and Gage,F.H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J. Neurosci. *17*, 5820-5829.

Lamar,E., Deblandre,G., Wettstein,D., Gawantka,V., Pollet,N., Niehrs,C., and Kintner,C. (2001a). Nrarp is a novel intracellular component of the Notch signaling pathway. Genes Dev. *15*, 1885-1899.

Lamar,E., Kintner,C., and Goulding,M. (2001b). Identification of NKL, a novel Gli-Kruppel zinc-finger protein that promotes neuronal differentiation. Development *128*, 1335-1346.

Lavdas,A.A., Grigoriou,M., Pachnis,V., and Parnavelas,J.G. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J. Neurosci. *19*, 7881-7888.

Lecourtois,M. and 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 Dev. *9*, 2598-2608.

Lee,E.C., Yu,D., Martinez,d., V, Tessarollo,L., Swing,D.A., Court DL, Jenkins,N.A., and Copeland,N.G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics *73*, 56-65.

Lee,J., Wu,Y., Qi,Y., Xue,H., Liu,Y., Scheel,D., German,M., Qiu,M., Guillemot,F., Rao,M., and Gradwohl,G. (2003a). Neurogenin3 participates in gliogenesis in the developing vertebrate spinal cord. Dev. Biol. *253*, 84-98.

Lee,J.E. (1997). Basic helix-loop-helix genes in neural development. Curr. Opin. Neurobiol. *7*, 13-20.

Lee,J.E., Hollenberg,S.M., Snider,L., Turner,D.L., Lipnick,N., and Weintraub,H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science *268*, 836-844.

Lee,K., Villena,J.A., Moon,Y.S., Kim,K.H., Lee,S., Kang,C., and Sul,H.S. (2003b). Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). J. Clin. Invest *111*, 453-461.

Lee,K.J. and Jessell,T.M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. Annu. Rev. Neurosci. *22*, 261-294.

Lee,S.K., Lee,B., Ruiz,E.C., and Pfaff,S.L. (2005). Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev. *19*, 282-294.

Lee,S.K. and Pfaff,S.L. (2003). Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. Neuron *38*, 731-745.

Letinic,K., Zoncu,R., and Rakic,P. (2002). Origin of GABAergic neurons in the human neocortex. Nature *417*, 645-649.

Leung,Y.F. and Cavalieri,D. (2003). Fundamentals of cDNA microarray data analysis. Trends Genet. *19*, 649-659.

Leyns,L., Gomez-Skarmeta,J.L., and Dambly-Chaudiere,C. (1996). iroquois: a prepattern gene that controls the formation of bristles on the thorax of Drosophila. Mech. Dev. *59*, 63-72.

Li,W., Cogswell,C.A., and LoTurco,J.J. (1998). Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. J. Neurosci. *18*, 8853-8862.

Lin,M., Chang,J.K., Shankar,D., and Sakamoto,K.M. (2003a). The role of p55CDC in cell cycle control and mammalian cell proliferation, differentiation, and apoptosis. Exp. Mol. Pathol. *74*, 123-128.

Lin,S.P., Youngson,N., Takada,S., Seitz,H., Reik,W., Paulsen,M., Cavaille,J., and Ferguson-Smith,A.C. (2003b). Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. Nat. Genet. *35*, 97-102.

Lindsell,C.E., Boulter,J., diSibio,G., Gossler,A., and Weinmaster,G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol. Cell Neurosci. *8*, 14-27.

Lindsell,C.E., Shawber,C.J., Boulter,J., and Weinmaster,G. (1995). Jagged: a mammalian ligand that activates Notch1. Cell *80*, 909-917.

Lisitsyn,N.A. (1995). Representational difference analysis: finding the differences between genomes. Trends Genet. *11*, 303-307.

Liu,K.J. and Harland,R.M. (2003). Cloning and characterization of Xenopus Id4 reveals differing roles for Id genes. Dev. Biol. *264*, 339-351.

Lo,L., Dormand,E., Greenwood,A., and Anderson,D.J. (2002). Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development *129*, 1553-1567.

Lo,L., Guillemot,F., Joyner,A.L., and Anderson,D.J. (1994). MASH-1: a marker and a mutation for mammalian neural crest development. Perspect. Dev. Neurobiol. *2*, 191-201.

Lo,L., Sommer,L., and Anderson,D.J. (1997). MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. Curr. Biol. *7*, 440-450.

Lo,L., Tiveron,M.C., and Anderson,D.J. (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*, 609-620.

Lockhart,D.J. and Winzeler,E.A. (2000). Genomics, gene expression and DNA arrays. Nature *405*, 827-836.

Lois,C. and Alvarez-Buylla,A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145-1148.

LoTurco,J. (2004). Doublecortin and a tale of two serines. Neuron *41*, 175-177.

Lu,Q.R., Sun,T., Zhu,Z., Ma,N., Garcia,M., Stiles,C.D., and Rowitch,D.H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell *109*, 75-86.

Luskin,M.B. and Shatz,C.J. (1985). Studies of the earliest generated cells of the cat's visual cortex: cogeneration of subplate and marginal zones. J. Neurosci. *5*, 1062-1075.

Lyden,D., Young,A.Z., Zagzag,D., Yan,W., Gerald,W., O'Reilly,R., Bader,B.L., Hynes,R.O., Zhuang,Y., Manova,K., and Benezra,R. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature *401*, 670-677.

Lyons,G.E., Micales,B.K., Schwarz,J., Martin,J.F., and Olson,E.N. (1995). Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. J. Neurosci. *15*, 5727-5738.

Ma,Q., Chen,Z., del,B.B., I, de la Pompa,J.L., and Anderson,D.J. (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron *20*, 469-482.

Ma,Q., Fode,C., Guillemot,F., and Anderson,D.J. (1999). Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev. *13*, 1717-1728.

Ma,Q., Kintner,C., and Anderson,D.J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. Cell *87*, 43-52.

Malatesta,P., Hack,M.A., Hartfuss,E., Kettenmann,H., Klinkert,W., Kirchhoff,F., and Gotz,M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. Neuron *37*, 751-764.

Malatesta,P., Hartfuss,E., and Gotz,M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development *127*, 5253-5263.

Mallamaci,A., Muzio,L., Chan,C.H., Parnavelas,J., and Boncinelli,E. (2000). Area identity shifts in the early cerebral cortex of Emx2-/- mutant mice. Nat. Neurosci. *3*, 679-686.

Marin,O., Anderson,S.A., and Rubenstein,J.L. (2000). Origin and molecular specification of striatal interneurons. J. Neurosci. *20*, 6063-6076.

Mattar,P., Britz,O., Johannes,C., Nieto,M., Ma,L., Rebeyka,A., Klenin,N., Polleux,F., Guillemot,F., and Schuurmans,C. (2004). A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev. Biol. *273*, 373-389.

Mei,B., Zhao,L., Chen,L., and Sul,H.S. (2002). Only the large soluble form of preadipocyte factor-1 (Pref-1), but not the small soluble and membrane forms, inhibits adipocyte differentiation: role of alternative splicing. Biochem. J. *364*, 137-144.

Meneghini,M.D., Ishitani,T., Carter,J.C., Hisamoto,N., Ninomiya-Tsuji,J., Thorpe,C.J., Hamill,D.R., Matsumoto,K., and Bowerman,B. (1999). MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. Nature *399*, 793-797.

Miyata,T., Kawaguchi,A., Okano,H., and Ogawa,M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727-741.

Mizuguchi,R., Sugimori,M., Takebayashi,H., Kosako,H., Nagao,M., Yoshida,S., Nabeshima,Y., Shimamura,K., and Nakafuku,M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron *31*, 757-771.

Mlodzik,M. (1999). Planar polarity in the Drosophila eye: a multifaceted view of signaling specificity and cross-talk. EMBO J. *18*, 6873-6879.

Modolell,J. (1997). Patterning of the adult peripheral nervous system of Drosophila. Perspect. Dev. Neurobiol. *4*, 285-296.

Moon,Y.S., Smas,C.M., Lee,K., Villena,J.A., Kim,K.H., Yun,E.J., and Sul,H.S. (2002). Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol. Cell Biol. *22*, 5585-5592.

Moore,A.W., Barbel,S., Jan,L.Y., and Jan,Y.N. (2000). A genomewide survey of basic helix-loop-helix factors in Drosophila. Proc. Natl. Acad. Sci. U. S. A *97*, 10436-10441.

Murre,C., McCaw,P.S., Vaessin,H., Caudy,M., Jan,L.Y., Jan,Y.N., Cabrera,C.V., Buskin,J.N., Hauschka,S.D., Lassar,A.B., and . (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

Murrin,L.C. and Ferrer,J.R. (1984). Ontogeny of the rat striatum: correspondence of dopamine terminals, opiate receptors and acetylcholinesterase. Neurosci. Lett. *47*, 155-160.

Muzio,L., DiBenedetto,B., Stoykova,A., Boncinelli,E., Gruss,P., and Mallamaci,A. (2002). Conversion of cerebral cortex into basal ganglia in Emx2(-/-) Pax6(Sey/Sey) double-mutant mice. Nat. Neurosci. *5*, 737-745.

Nakashima,K., Yanagisawa,M., Arakawa,H., Kimura,N., Hisatsune,T., Kawabata,M., Miyazono,K., and Taga,T. (1999). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science *284*, 479-482.

Nieto,M., Schuurmans,C., Britz,O., and Guillemot,F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401-413.

Niwa,H., Yamamura,K., and Miyazaki,J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene *108*, 193-199.

Noctor,S.C., Flint,A.C., Weissman,T.A., Dammerman,R.S., and Kriegstein,A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714-720.

Nolo,R., Abbott,L.A., and Bellen,H.J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. Cell *102*, 349-362.

Novitch,B.G., Chen,A.I., and Jessell,T.M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron *31*, 773-789.

O'Leary,D.D. and Nakagawa,Y. (2002). Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. Curr. Opin. Neurobiol. *12*, 14-25.

Ogawa,M., Miyata,T., Nakajima,K., Yagyu,K., Seike,M., Ikenaka,K., Yamamoto,H., and Mikoshiba,K. (1995). The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron *14*, 899-912.

Ohnuma,S., Hopper,S., Wang,K.C., Philpott,A., and Harris,W.A. (2002). Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina. Development *129*, 2435-2446.

Ohnuma,S., Philpott,A., Wang,K., Holt,C.E., and Harris,W.A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. Cell *99*, 499-510.

Ohtsuka,T., Ishibashi,M., Gradwohl,G., Nakanishi,S., Guillemot,F., and Kageyama,R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. *18*, 2196-2207.

Ohtsuka,T., Sakamoto,M., Guillemot,F., and Kageyama,R. (2001). Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. J. Biol. Chem. *276*, 30467-30474.

Okano,H. (2002). Stem cell biology of the central nervous system. J. Neurosci. Res. *69*, 698-707.

Osumi,N. and Inoue,T. (2001). Gene transfer into cultured mammalian embryos by electroporation. Methods *24*, 35-42.

Otero,J.J., Fu,W., Kan,L., Cuadra,A.E., and Kessler,J.A. (2004). Beta-catenin signaling is required for neural differentiation of embryonic stem cells. Development *131*, 3545-3557.

Panto,M.R., Zappala,A., Tuorto,F., and Cicirata,F. (2004). Role of the Otx1 gene in cell differentiation of mammalian cortex. Eur. J. Neurosci. *19*, 2893-2902.

Parras,C., Garcia-Alonso,L.A., Rodriguez,I., and Jimenez,F. (1996). Control of neural precursor specification by proneural proteins in the CNS of Drosophila. EMBO J. *15*, 6394-6399.

Parras,C.M., Galli,R., Britz,O., Soares,S., Galichet,C., Battiste,J., Johnson,J.E., Nakafuku,M., Vescovi,A., and Guillemot,F. (2004). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J. *23*, 4495-4505.

Parras,C.M., Schuurmans,C., Scardigli,R., Kim,J., Anderson,D.J., and Guillemot,F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. Genes Dev. *16*, 324-338.

Patapoutian,A. and Reichardt,L.F. (2000). Roles of Wnt proteins in neural development and maintenance. Curr. Opin. Neurobiol. *10*, 392-399.

Pattyn,A., Simplicio,N., van Doorninck,J.H., Goridis,C., Guillemot,F., and Brunet,J.F. (2004). Ascl1/Mash1 is required for the development of central serotonergic neurons. Nat. Neurosci. *7*, 589-595.

Pavlidis,P. (2003). Using ANOVA for gene selection from microarray studies of the nervous system. Methods *31*, 282-289.

Perron,M., Opdecamp,K., Butler,K., Harris,W.A., and Bellefroid,E.J. (1999). X-ngnr-1 and Xath3 promote ectopic expression of sensory neuron markers in the neurula ectoderm and have distinct inducing properties in the retina. Proc. Natl. Acad. Sci. U. S. A *96*, 14996-15001.

Peyrefitte,S., Kahn,D., and Haenlin,M. (2001). New members of the Drosophila Myc transcription factor subfamily revealed by a genome-wide examination for basic helix-loop-helix genes. Mech. Dev. *104*, 99-104.

Pirot,P., van Grunsven,L.A., Marine,J.C., Huylebroeck,D., and Bellefroid,E.J. (2004). Direct regulation of the Nrarp gene promoter by the Notch signaling pathway. Biochem. Biophys. Res. Commun. *322*, 526-534.

Powell,L.M., Zur Lage,P.I., Prentice,D.R., Senthinathan,B., and Jarman,A.P. (2004). The proneural proteins Atonal and Scute regulate neural target genes through different E-box binding sites. Mol. Cell Biol. *24*, 9517-9526.

Qian,X., Davis,A.A., Goderie,S.K., and Temple,S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron *18*, 81-93.

Qian,X., Shen,Q., Goderie,S.K., He,W., Capela,A., Davis,A.A., and Temple,S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron *28*, 69-80.

Quackenbush,J. (2002). Microarray data normalization and transformation. Nat. Genet. *32 Suppl*, 496-501.

Rabitsch,K.P., Toth,A., Galova,M., Schleiffer,A., Schaffner,G., Aigner,E., Rupp,C., Penkner,A.M., Moreno-Borchart,A.C., Primig,M., Esposito,R.E., Klein,F., Knop,M., and Nasmyth,K. (2001). A screen for genes required for meiosis and spore formation based on whole-genome expression. Curr. Biol. *11*, 1001-1009.

Ramain,P., Khechumian,R., Khechumian,K., Arbogast,N., Ackermann,C., and Heitzler,P. (2000). Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. Mol. Cell *6*, 781-790.

Raychaudhuri,S., Sutphin,P.D., Chang,J.T., and Altman,R.B. (2001). Basic microarray analysis: grouping and feature reduction. Trends Biotechnol. *19*, 189-193.

Redies,C., Kovjanic,D., Heyers,D., Medina,L., Hirano,S., Suzuki,S.T., and Puelles,L. (2002). Patch/matrix patterns of gray matter differentiation in the telencephalon of chicken and mouse. Brain Res. Bull. *57*, 489-493.

Reeves,N. and Posakony,J.W. (2005). Genetic Programs Activated by Proneural Proteins in the Developing Drosophila PNS. Dev. Cell *8*, 413-425.

Reynolds,B.A., Tetzlaff,W., and Weiss,S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. *12*, 4565-4574.

Rodriguez,I., Hernandez,R., Modolell,J., and Ruiz-Gomez,M. (1990). Competence to develop sensory organs is temporally and spatially regulated in Drosophila epidermal primordia. EMBO J. *9*, 3583-3592.

Ross,S.E., Greenberg,M.E., and Stiles,C.D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13-25.

Saito,T., Sawamoto,K., Okano,H., Anderson,D.J., and Mikoshiba,K. (1998). Mammalian BarH homologue is a potential regulator of neural bHLH genes. Dev. Biol. *199*, 216-225.

Sasai,Y., Kageyama,R., Tagawa,Y., Shigemoto,R., and Nakanishi,S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev. *6*, 2620-2634.

Sauvageot,C.M. and Stiles,C.D. (2002). Molecular mechanisms controlling cortical gliogenesis. Curr. Opin. Neurobiol. *12*, 244-249.

Scardigli,R., Baumer,N., Gruss,P., Guillemot,F., and Le,R., I (2003). Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. Development *130*, 3269-3281.

Scardigli,R., Schuurmans,C., Gradwohl,G., and Guillemot,F. (2001). Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. Neuron *31*, 203-217.

Schmidt,J.V., Matteson,P.G., Jones,B.K., Guan,X.J., and Tilghman,S.M. (2000). The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes Dev. *14*, 1997-2002.

Schulze,A. and Downward,J. (2001). Navigating gene expression using microarrays--a technology review. Nat. Cell Biol. *3*, E190-E195.

Schuster-Gossler,K., Simon-Chazottes,D., Guenet,J.L., Zachgo,J., and Gossler,A. (1996). Gtl2lacZ, an insertional mutation on mouse chromosome 12 with parental origin-dependent phenotype. Mamm. Genome *7*, 20-24.

Schuurmans,C., Armant,O., Nieto,M., Stenman,J.M., Britz,O., Klenin,N., Brown,C., Langevin,L.M., Seibt,J., Tang,H., Cunningham,J.M., Dyck,R., Walsh,C., Campbell,K., Polleux,F., and Guillemot,F. (2004). Sequential phases of cortical specification involve Neurogenin-dependent and -independent pathways. EMBO J. *23*, 2892-2902.

Schuurmans,C. and Guillemot,F. (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. Curr. Opin. Neurobiol. *12*, 26-34.

Segev,E., Halachmi,N., Salzberg,A., and Ben Arie,N. (2001). Nato3 is an evolutionarily conserved bHLH transcription factor expressed in the CNS of Drosophila and mouse. Mech. Dev. *106*, 197-202.

Seibt,J., Schuurmans,C., Gradwhol,G., Dehay,C., Vanderhaeghen,P., Guillemot,F., and Polleux,F. (2003). Neurogenin2 specifies the connectivity of thalamic neurons by controlling axon responsiveness to intermediate target cues. Neuron *39*, 439-452.

Sherlock,G. (2000). Analysis of large-scale gene expression data. Curr. Opin. Immunol. *12*, 201-205.

Simpson,P. (1998). Introduction: Notch signalling and choice of cell fates in development. Semin. Cell Dev. Biol. *9*, 581-582.

Singson,A., Leviten,M.W., Bang,A.G., Hua,X.H., and Posakony,J.W. (1994). Direct downstream targets of proneural activators in the imaginal disc include genes involved in lateral inhibitory signaling. Genes Dev. *8*, 2058-2071.

Skeath,J.B. and Doe,C.Q. (1996). The achaete-scute complex proneural genes contribute to neural precursor specification in the Drosophila CNS. Curr. Biol. *6*, 1146-1152.

Smart,I.H. (1976). A pilot study of cell production by the ganglionic eminences of the developing mouse brain. J. Anat. *121*, 71-84.

Smas,C.M., Chen,L., Zhao,L., Latasa,M.J., and Sul,H.S. (1999). Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. J. Biol. Chem. *274*, 12632-12641.

Smas,C.M., Kachinskas,D., Liu,C.M., Xie,X., Dircks,L.K., and Sul,H.S. (1998). Transcriptional control of the pref-1 gene in 3T3-L1 adipocyte differentiation. Sequence requirement for differentiation-dependent suppression. J. Biol. Chem. *273*, 31751-31758.

Smas,C.M. and Sul,H.S. (1993). Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell *73*, 725-734.

Sommer,L., Shah,N., Rao,M., and Anderson,D.J. (1995). The cellular function of MASH1 in autonomic neurogenesis. Neuron *15*, 1245-1258.

Soria,J.M., Taglialatela,P., Gil-Perotin,S., Galli,R., Gritti,A., Verdugo,J.M., and Bertuzzi,S. (2004). Defective postnatal neurogenesis and disorganization of the rostral migratory stream in absence of the Vax1 homeobox gene. J. Neurosci. *24*, 11171-11181.

Spassky,N., Heydon,K., Mangatal,A., Jankovski,A., Olivier,C., Queraud-Lesaux,F., Goujet-Zalc,C., Thomas,J.L., and Zalc,B. (2001). Sonic hedgehog-dependent emergence of oligodendrocytes in the telencephalon: evidence for a source of oligodendrocytes in the olfactory bulb that is independent of PDGFRalpha signaling. Development *128*, 4993-5004.

Stathopoulos,A., Van Drenth,M., Erives,A., Markstein,M., and Levine,M. (2002). Whole-genome analysis of dorsal-ventral patterning in the Drosophila embryo. Cell *111*, 687-701.

Stenman,J., Toresson,H., and Campbell,K. (2003). Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. J. Neurosci. *23*, 167-174.

Stoykova,A., Treichel,D., Hallonet,M., and Gruss,P. (2000). Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. J. Neurosci. *20*, 8042-8050.

Struhl,G. and Greenwald,I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature *398*, 522-525.

Strutt,D.I., Weber,U., and Mlodzik,M. (1997). The role of RhoA in tissue polarity and Frizzled signalling. Nature *387*, 292-295.

Stuhmer,T., Anderson,S.A., Ekker,M., and Rubenstein,J.L. (2002a). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. Development *129*, 245-252.

Stuhmer,T., Puelles,L., Ekker,M., and Rubenstein,J.L. (2002b). Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. Cereb. Cortex *12*, 75-85.

Sun,Y., Jan,L.Y., and Jan,Y.N. (1998). Transcriptional regulation of atonal during development of the Drosophila peripheral nervous system. Development *125*, 3731-3740.

Sun,Y., Nadal-Vicens,M., Misono,S., Lin,M.Z., Zubiaga,A., Hua,X., Fan,G., and Greenberg,M.E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365-376.

Sussel,L., Marin,O., Kimura,S., and Rubenstein,J.L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development *126*, 3359-3370.

Taglialatela,P., Soria,J.M., Caironi,V., Moiana,A., and Bertuzzi,S. (2004). Compromised generation of GABAergic interneurons in the brains of Vax1-/- mice. Development *131*, 4239-4249.

Takahashi,T., Nowakowski,R.S., and Caviness,V.S., Jr. (1995). Early ontogeny of the secondary proliferative population of the embryonic murine cerebral wall. J. Neurosci. *15*, 6058-6068.

Takebayashi,H., Ohtsuki,T., Uchida,T., Kawamoto,S., Okubo,K., Ikenaka,K., Takeichi,M., Chisaka,O., and Nabeshima,Y. (2002). Non-overlapping expression of Olig3 and Olig2 in the embryonic neural tube. Mech. Dev. *113*, 169-174.

Takebayashi,K., Takahashi,S., Yokota,C., Tsuda,H., Nakanishi,S., Asashima,M., and Kageyama,R. (1997). Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to Drosophila proneural gene atonal. EMBO J. *16*, 384-395.

Talikka,M., Perez,S.E., and Zimmerman,K. (2002). Distinct patterns of downstream target activation are specified by the helix-loop-helix domain of proneural basic helix-loop-helix transcription factors. Dev. Biol. *247*, 137-148.

Tamayo,P., Slonim,D., Mesirov,J., Zhu,Q., Kitareewan,S., Dmitrovsky,E., Lander,E.S., and Golub,T.R. (1999). Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. Proc. Natl. Acad. Sci. U. S. A *96*, 2907-2912.

Tanaka,M., Gertsenstein,M., Rossant,J., and Nagy,A. (1997). Mash2 acts cell autonomously in mouse spongiotrophoblast development. Dev. Biol. *190*, 55-65.

Tavazoie,S., Hughes,J.D., Campbell,M.J., Cho,R.J., and Church,G.M. (1999). Systematic determination of genetic network architecture. Nat. Genet. *22*, 281-285.

Temple,S. (2001). The development of neural stem cells. Nature *414*, 112-117.

Temple,S. (2003). Embryonic stem cell self-renewal, analyzed. Cell *115*, 247-248.

Teng,J., Takei,Y., Harada,A., Nakata,T., Chen,J., and Hirokawa,N. (2001). Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. J. Cell Biol. *155*, 65-76.

Thorpe,C.J. and Moon,R.T. (2004). nemo-like kinase is an essential co-activator of Wnt signaling during early zebrafish development. Development *131*, 2899-2909.

Tietjen,I., Rihel,J.M., Cao,Y., Koentges,G., Zakhary,L., and Dulac,C. (2003). Single-cell transcriptional analysis of neuronal progenitors. Neuron *38*, 161-175.

Toresson,H., Parmar,M., and Campbell,K. (2000a). Expression of Meis and Pbx genes and their protein products in the developing telencephalon: implications for regional differentiation. Mech. Dev. *94*, 183-187.

Toresson,H., Potter,S.S., and Campbell,K. (2000b). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development *127*, 4361-4371.

Tucker,R.P. (1990). The roles of microtubule-associated proteins in brain morphogenesis: a review. Brain Res. Brain Res. Rev. *15*, 101-120.

Ungos,J.M., Karlstrom,R.O., and Raible,D.W. (2003). Hedgehog signaling is directly required for the development of zebrafish dorsal root ganglia neurons. Development *130*, 5351-5362.

Vairapandi,M., Balliet,A.G., Hoffman,B., and Liebermann,D.A. (2002). GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J. Cell Physiol *192*, 327-338.

van der,K.D. and Fishell,G. (1987). Neuronal birthdate underlies the development of striatal compartments. Brain Res. *401*, 155-161.

Van Doren,M., Bailey,A.M., Esnayra,J., Ede,K., and Posakony,J.W. (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. Genes Dev. *8*, 2729-2742.

Van Doren,M., Powell,P.A., Pasternak,D., Singson,A., and Posakony,J.W. (1992). Spatial regulation of proneural gene activity: auto- and cross-activation of achaete is antagonized by extramacrochaetae. Genes Dev. *6*, 2592-2605.

van Ham,M., Croes,H., Schepens,J., Fransen,J., Wieringa,B., and Hendriks,W. (2003). Cloning and characterization of mCRIP2, a mouse LIM-only protein that interacts with PDZ domain IV of PTP-BL. Genes Cells *8*, 631-644.

Vernon,A.E., Devine,C., and Philpott,A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in Xenopus. Development *130*, 85-92.

Verzi,M.P., Anderson,J.P., Dodou,E., Kelly,K.K., Greene,S.B., North,B.J., Cripps,R.M., and Black,B.L. (2002). N-twist, an evolutionarily conserved bHLH protein expressed in the developing CNS, functions as a transcriptional inhibitor. Dev. Biol. *249*, 174-190.

Villares,R. and Cabrera,C.V. (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*, 415-424.

Wang,S.W., Kim,B.S., Ding,K., Wang,H., Sun,D., Johnson,R.L., Klein,W.H., and Gan,L. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev. *15*, 24-29.

Wang,V.Y., Hassan,B.A., Bellen,H.J., and Zoghbi,H.Y. (2002). Drosophila atonal fully rescues the phenotype of Math1 null mice: new functions evolve in new cellular contexts. Curr. Biol. *12*, 1611-1616.

Welford,S.M., Gregg,J., Chen,E., Garrison,D., Sorensen,P.H., Denny,C.T., and Nelson,S.F. (1998). Detection of differentially expressed genes in primary tumor tissues using representational differences analysis coupled to microarray hybridization. Nucleic Acids Res. *26*, 3059-3065.

Wen,X., Fuhrman,S., Michaels,G.S., Carr,D.B., Smith,S., Barker,J.L., and Somogyi,R. (1998). Large-scale temporal gene expression mapping of central nervous system development. Proc. Natl. Acad. Sci. U. S. A *95*, 334-339.

Wichterle,H., Turnbull,D.H., Nery,S., Fishell,G., and Alvarez-Buylla,A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development *128*, 3759-3771.

Wilson,S.W. and Rubenstein,J.L. (2000). Induction and dorsoventral patterning of the telencephalon. Neuron *28*, 641-651.

Woods,C., Montcouquiol,M., and Kelley,M.W. (2004). Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat. Neurosci. *7*, 1310-1318.

Wu,L., Aster,J.C., Blacklow,S.C., Lake,R., Artavanis-Tsakonas,S., and Griffin,J.D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat. Genet. *26*, 484-489.

Wylie,A.A., Murphy,S.K., Orton,T.C., and Jirtle,R.L. (2000). Novel imprinted DLK1/GTL2 domain on human chromosome 14 contains motifs that mimic those implicated in IGF2/H19 regulation. Genome Res. *10*, 1711-1718.

Xu,Q., Cobos,I., De La,C.E., Rubenstein,J.L., and Anderson,S.A. (2004). Origins of cortical interneuron subtypes. J. Neurosci. *24*, 2612-2622.

Yokota,Y. (2001). Id and development. Oncogene *20*, 8290-8298.

Young,R.A. (2000). Biomedical discovery with DNA arrays. Cell *102*, 9-15.

Yun,K., Fischman,S., Johnson,J., Hrabe,d.A., Weinmaster,G., and Rubenstein,J.L. (2002). Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development *129*, 5029-5040.

Zaki,P.A., Quinn,J.C., and Price,D.J. (2003). Mouse models of telencephalic development. Curr. Opin. Genet. Dev. *13*, 423-437.

Zhao,Y., Marin,O., Hermesz,E., Powell,A., Flames,N., Palkovits,M., Rubenstein,J.L., and Westphal,H. (2003). The LIM-homeobox gene Lhx8 is required for the development of many cholinergic neurons in the mouse forebrain. Proc. Natl. Acad. Sci. U. S. A *100*, 9005-9010.

Zhou,Q. and Anderson,D.J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell *109*, 61-73.

Zhou,Q., Choi,G., and Anderson,D.J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron *31*, 791-807.

Zimmerman,K., Shih,J., Bars,J., Collazo,A., and Anderson,D.J. (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*, 221-232.

Zine,A., Aubert,A., Qiu,J., Therianos,S., Guillemot,F., Kageyama,R., and de Ribaupierre,F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. J. Neurosci. *21*, 4712-4720.

Zur Lage,P.I., Powell,L.M., Prentice,D.R., McLaughlin,P., and Jarman,A.P. (2004). EGF receptor signaling triggers recruitment of Drosophila sense organ precursors by stimulating proneural gene autoregulation. Dev. Cell *7*, 687-696.