Engineering and functional characterization of mouse embryonic stem cell lines optimized for differentiation into serotonergic neurons : Lmx1b transcription factor overexpression

Virginie Dolmazon

N° d'ordre : 111 – 2010   Année 2010

THÈSE DE DOCTORAT

Discipline : **Sciences de la Vie**
Spécialité : **Biologie du Développement**

Université Claude Bernard Lyon 1
École Doctorale Biologie Moléculaire Intégrée et Cognitive

Fabrication et caractérisation fonctionnelle de lignées de cellules souches embryonnaires de souris optimisées pour la différenciation en neurones sérotoninergiques : surexpression du facteur de transcription Lmx1b

Engineering and functional characterization of mouse embryonic stem cell lines optimized for differentiation into serotonergic neurons: Lmx1b transcription factor overexpression

Présentée et soutenue publiquement le 15 juillet 2010 par

Virginie DOLMAZON

Pour l'obtention du diplôme de Doctorat
(arrêté du 7 août 2006)

Membres du jury

Monsieur Edmund DERRINGTON   *Président*
Madame Isabelle DUSART   *Rapporteur*
Monsieur Frank EDENHOFER   *Rapporteur*
Monsieur Pierre GRESSENS   *Examinateur*
Monsieur Pierre SAVATIER   *Directeur de Thèse*

griffes de saphir. Que de patience lors de la 100ieme explication de tel ou tel protocole ou pour supporter mon humour.

…Véro (Vezoli), j'aurais du t'interpeller en tout début de liste puisque tu es le premier sourire que l'on rencontre en arrivant. La porte de ton bureau est toujours ouverte et on ne se rend même pas compte que l'on te dérange. Bravo !

…Les anciens, actuels, ou futurs « étudiants ». Beaucoup de personnes à faire rentrer dans ce petit paragraphe !

Tout d'abord, la « savat'team » constituée de Diana, Pierrot, PM et Vincent. Les 3 premiers, vous avez fait un périple mémorable jusqu'à Berlin. Ca a été une belle parenthèse de vous faire découvrir les rues berlinoises, et surtout de me faire nourrir de lasagnes et de gâteaux au chocolat. Petite Diana, tes surprises sur mon bureau vont me manquer pour sûr ! Muchas gracias ! Tu es la prochaine sur la liste, je ne m'inquiète pas pour toi, tu es petite mais costaude ! Petit Pierrotte, je compte sur toi pour continuer l'entraînement sans moi (;-p) et surtout je te souhaite tout plein de réussite car tu es fait pour ça. Petit PM (tu ne peux pas le nier), tant que tu conserveras ta coiffure et tes T-shirts, tout ira bien. Petit Vincent, force tranquille de la team, tu n'as pas pu venir à Berlin, il fallait bien qu'un étudiant reste à Lyon !

Yann et Magali, vous venez (presque) d'arriver mais le fantôme que j'ai été sur la fin de thèse n'a pas eu le temps de bien vous connaître.

Hongwei, you smile even during the bad days that you have experienced here! Good luck.

Irène et Guillaume, bonne route l'un avec l'autre…

Et tous les autres…Niko, Marie, Kwam, nous avons commencé ensemble ; Elodie, Julien, Louis-Jan (et tes 3 femmes, bon vent à tous les 4)…

…Les membres de l'association BIODOCS, on partage les mêmes doutes et les mêmes joies durant une thèse. Il est bon de ne pas se sentir seul.

…M. Brian Rudkin, pour ta patience, ton potentiel d'écoute, tes conseils et tes coups de pouce depuis de nombreuses années maintenant.

Et parce qu'il n'y a pas que le labo dans la vie pour s'accomplir…avec vous ce sont la Montagne, l'air frais, les voyages, les champignons, les BDs, la gastronomie, les déguisements, la philosophie de comptoir, la course, les concerts, les photos…vous me connaissez un peu/beaucoup…

…Guillaume, Mathilde, Ju, Kroll, Yo, Nel, Sev, il n'est pas bon de compter les années !

…PY, Eva, Bertrand, Will, Vince, Stef, Pascal (et Arthur !), AAaa, Emy, Sara, Seb et j'en passe. Les voisins, les (assimilés) Insaliens, les coureurs, les Lyonnais….

…Mu, pas besoin de glace, une sorte d'alter ego.

…Emilie, Emeric, les heureux propriétaires de ma résidence secondaire à la campagne et un exemple…

…Les'zéros polaires : Mariounette, Syi, Yo, Gaël, Yan, tiJu, JLM, Arno, Ben, Vaness, Fab, Rony, Phil…Plus ou moins en contact, je ne peux pas vous oublier.

…Ma famille, mon refuge, mes origines. Maman, mes Mamies, Papa, JB, Antoine…Je n'ai jamais été très expressive mais je ne pense avoir besoin de parler. Vous n'êtes pas physiquement présents, vous êtes toujours partis avec moi.

…Toi…ferme les yeux, je suis là…

A mes Grands-mères,
fières comme « Bartabac » de tous leurs petits-enfants ,
quoi qu'ils fassent...

Twenty years from now
you will be more disappointed
by the things you didn't do
than by the ones you did do.

So throw off the bowlines.
Sail away from the safe harbor.
Catch the trade winds in your sails.

Explore. Dream. Discover.

Mark Twain

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

A/P: Anteroposterior (axis of the neural tube)

AA: Ascorbic Acid (vitamin C)

AADC: aromatic L-amino acid decarboxylase

Aldh: Aldehyde dehydrogenase

ANR: Anterior Neural Ridge

BDNF: Brain-Derived Neurotrophic Factor

bFGF: basic Fibroblast Growth Factor (or FGF2)

bHLH: basic helix loop helix

BMP: Bone Morphogenetic Protein

BP: Basal Plate

cAMP: cyclic Adenosine Monophosphate

CNPase: Cyclic Nucleotide Phosphodiesterase

CNS: Central Nervous System

CO_2: carbon dioxide

cRet: RET receptor tyrosine kinase

D/V: Dorsoventral (axis of the neural tube)

DA: Dopamine/Dopaminergic

DAT: Dopamine transporter

dbcAMP: dibutyryl cyclic Adenosine Monophosphate

DBH: dopamine β-hydroxylase

DNA: Deoxyribonucleic Acid

Dox: Doxycycline

E: Embryonic day

EB: Embryoid bodies

ECC: Embryonic Carcinoma Cells

EGF: Epidermal Growth Factor

eGFP: enhanced Green Fluorescent Protein

En: Engrailed

ESC: Embryonic Stem Cells

FACS: Fluorescence Activated Cell Sorting

FCS: Fetal Calf Serum

FGF 4/8: Fibroblast Growth Factor 4/8

FN: Fibronectin

Fox: forkhead/winged helix transcription factor

FP: Floor Plate

FRT: Flp recombinase target

G418: Geneticin

Gata: GATA-motif binding transcription factor

Gbx: Gastrulation brain homeobox transcription factor

GDNF: Glial cell line-Derived Neurotrophic Factor

GFAP: Glial Fibrillary Acidic Protein

GFP: Green Fluorescent Protein

GP: Globus Pallidus

GSK-3β: Glycogen Synthase Kinase 3β

hESC: human Embryonic Stem Cells

HIV: Human Immunodeficiency Virus

hLmx1b: human Lmx1b

Hnf: Hepatocyte nuclear factor

hPLAP: human Placental Alkaline Phosphatase

ICM: Inner Cell Mass

IL: Interleukin

IsO: Isthmic Organizer

ITSFn: Insulin/Transferrin/Selenium/Fibronectin

KO: Knock-Out

LIF: Leukemia Inhibitory Factor

Lmx: LIM homeobox transcription factor

MAP: Microtubule-Associated Protein

Mash: Mouse achaete-scute homolog

mDA: mesencephalic dopaminergic (neurons)

MEF: Mouse Embryonic Fibroblasts

mESC: mouse Embryonic Stem Cells

MHB: Midbrain/Hindbrain Boundary

Ngn: Neurogenin

Nkx: NK transcription factor related

NPS: Nail-Patella Syndrome

NSC: Neural Stem Cells

Nurr1 (or Nr4a2): transcription factor of the orphan nuclear receptor family

OHT: hydroxy-tamoxifen

Otx: Orthodenticle homologue transcription factor

Pax: Paired box transcription factor

PD: Parkinson's Disease

PDL: poly-D-lysine

Phox: Paired-like homeodomain protein transcription factor

Pitx: Paired-like homeodomain transcription factor

PLO: Polyornithine

PNMT: phenylethanolamine N-methyltransferase

PTD: Protein Transduction Domain

RA: Retinoic Acid

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

RP: Roof Plate

RRF: Retrorubral Field

SDIA: Stromal cell-Derived Inducing Activity

SERT: Serotonin Transporter

Shh: Sonic hedgehog

siRNA: Small Interfering Ribonucleic Acid

SNc: Substantia Nigra pars compacta

Sox2: SRY-related HMG box 2

STAT: Signal Transducer and Activator of Transcription

TAT: Transactivator of Transcription

Tet: Tetracycline-dependent (system)

TGF: Transforming Growth Factor

TH: Tyrosine Hydroxylase

Tph: Tryptophan hydroxylase

TuJ1: βIII-tubulin protein

Vmat: Vesicular monoamine transporter

VTA: Ventral Tegmental Area

ZLI: Zona Limitans Intrathalamica

4'OHT: 4'hydroxytamoxifen

5-HT (5-hydroxytryptamine): Serotonin

6-OHDA: 6-hydroxydopamine

%: percent

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Le présent serait plein de tous les avenirs,
si le passé n'y projetait déjà une histoire.
André Gide

GENERAL INTRODUCTION

Today world is more and more affected by the burden of diseases due to dysfunction of vital organs or cellular degeneration like autoimmune diseases, diabetes, osteoporosis, Alzheimer's disease, Parkinson's disease, etc. The challenge of cell therapy is to replace in patient the damaged cells/tissues by the same specialized cells or by well-chosen precursors which could efficiently differentiate *in vivo*. For now, treatments to replace, repair, or enhance the biological function of damaged tissues in human through cell therapy are limited to very few systems (i.e. hematopoietic stem cell transplants for leukemia, treatment of burns with cultured keratinocytes).

Pluripotent embryonic stem cells may offer renewable sources of cells and tissues for replacement of damaged cells. But firstly, due to their unique self-renewal and pluripotency properties, embryonic stem cells can be used to dissect the molecular mechanisms driving *in vitro* differentiation into specialized cell types. Indeed, embryonic stem cells have been proven to have the capacity to differentiate into virtually all cell types *in vitro*. Moreover, identifying the *in vitro* differentiation mechanisms could help in exploring the developmental programmes in the embryo.

The research work presented here was performed at the Stem Cell and Brain Research Institute under the supervision of Dr. Pierre Savatier. His team focuses on the mechanisms of self-renewal and pluripotency in mouse and human embryonic stem cells, and on experimental strategies to drive the differentiation of these cells towards specialized cells of interest like dopaminergic and serotonergic neurons. We have chosen to explore the precise role of the LIM homeodomain transcription factor Lmx1b in the development of dopaminergic and serotonergic systems. Lmx1b is already known to be involved in the maintenance of the differentiated phenotype of midbrain dopaminergic neurons, and in differentiation and maintenance of hindbrain serotonergic neurons generated in the Raphe Nuclei.

In the "Introduction" part of this work, we shall review the data available on differentiation towards dopaminergic and serotonergic neurons fates. First, the cardinal properties of embryonic stem cells will be reviewed. Second, the genetic mechanisms controlling dopaminergic and serotonergic specifications *in vivo* will be explored. The role of Lmx1b in these mechanisms will be emphasized. Third, the experimental strategies used to

direct embryonic stem cells differentiation towards the neural lineage, and more specifically towards dopaminergic and serotonergic neuronal phenotypes, will be described.

In the "Results" part, we shall describe the experimental strategies used to drive and enhance the differentiation of mouse embryonic stem cells into serotonergic neurons. Two strategies were developed toward this goal. In the first approach, stable ectopic expression of Lmx1b was achieved in mouse embryonic stem cells and their derivatives. In the second approach, Lmx1b was over-expressed in mouse embryonic stem cells-derived neural precursors making use of an inducible system in order to mimic the physiological onset of Lmx1b expression.

In the last section, we shall discuss the results of this work in the light of the current knowledge of the mechanisms underlying differentiation of serotonergic and dopaminergic neurons in the mouse.

INTRODUCTION

I   EMBRYONIC STEM CELLS, ORIGIN AND CARDINAL PROPERTIES

This section briefly describes embryonic stem cell properties. Mouse embryonic stem cells are emphasized, as they are the reference for stem cell research. However, some comparisons are made with primate embryonic stem cells.

Stem cells have the potential to develop into many different cell types in the body. In many tissues, they also serve as an internal repair system, dividing to replenish other cells. Stem cells differ from other types of cells in the body by three general properties:

   i)   They are **unspecialized**. They do not have tissue-specific structures to perform specialized functions.

   ii)   They are capable of dividing and renewing themselves for long periods. This process is called long-term **self-renewal**.

   iii)   They can give rise to specialized cells under certain physiologic or experimental conditions. This process is called **differentiation**.

I.1   Origin and derivation of embryonic stem cells (ESC)

ESC research in mouse dates back to the early 1970s, when embryonic carcinoma cells (ECC), the stem cells of germ line tumors (Solter et al., 1970; Stevens, 1970), were established as cell lines (Figure 1). These teratocarcinomas are malignant multidifferentiated tumors containing a significant population of undifferentiated cells: ECC (Jakob et al., 1973). Tumors were produced by grafting early mouse embryos into adult mice. Clonally isolated ECC retained the capacity for differentiation and could produce derivatives of all three primary germ layers: ectoderm, mesoderm, and endoderm. Moreover, ECC demonstrated an ability to participate in embryonic development when introduced into the inner cell mass (ICM) of early embryos at the blastocyst stage to generate chimeric mice (Mintz and Illmensee, 1975). But ECC only sporadically colonized the germ line, and they showed chromosomal aberrations and lost their ability to differentiate properly (Martin, 1980). The tumorigenic origin of ECC may explain why they were not able to retain the pluripotent capacities of early embryonic cells.
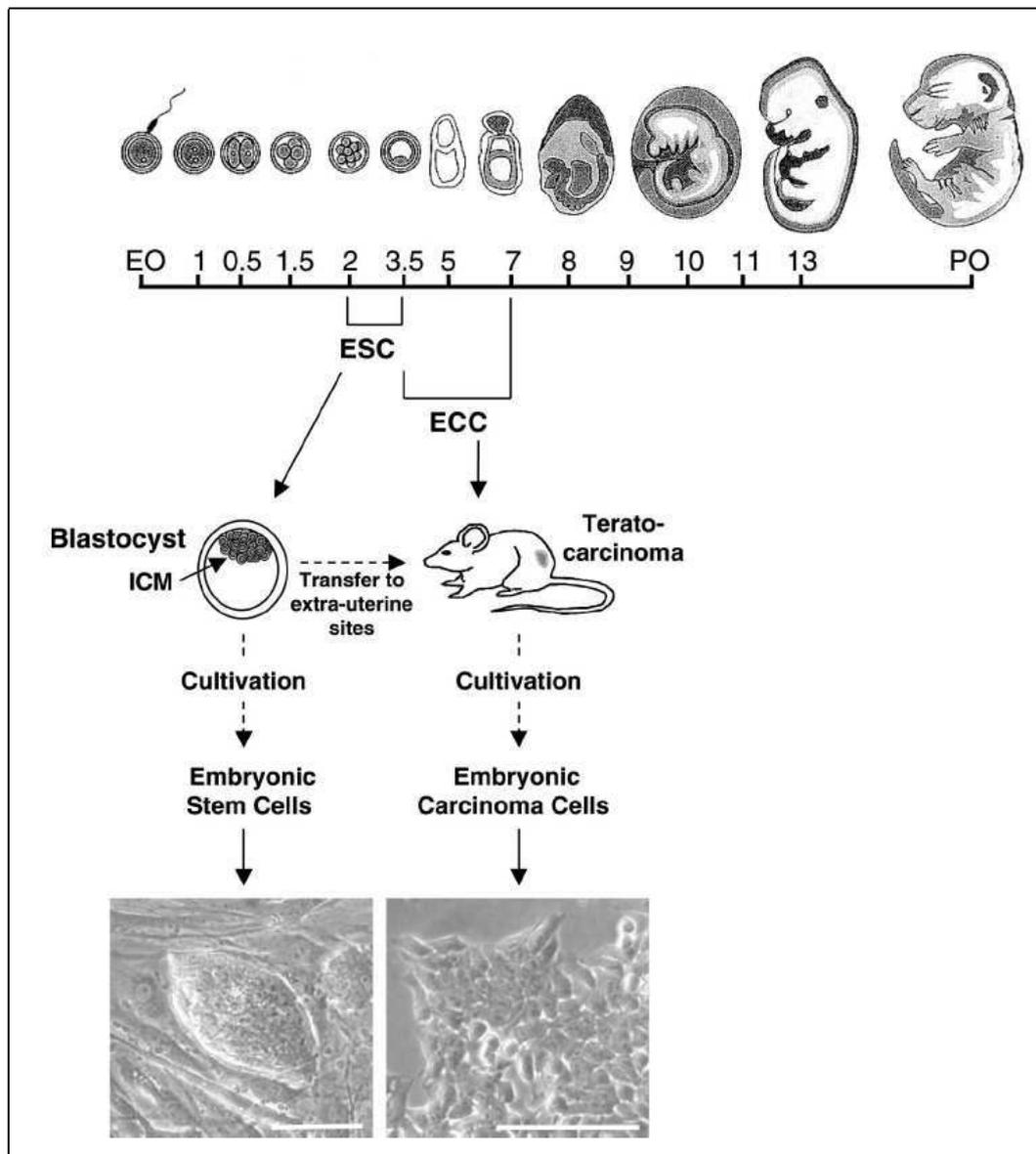
Figure 1: Developmental origin of mESC

(adapted from Wobus and Boheler, 2005)

Derivation of ESC and ECC from different embryonic stages of the mouse. See text for details. Bar = 100μm.

In 1981, two groups succeeded in cultivating *in vitro* pluripotent cell lines from the ICM of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). Evans and Kaufman employed a feeder layer of mouse embryonic fibroblast (MEF), while Martin used EC cell-conditioned medium. These **pluripotent mouse embryonic stem cells** (mESC) (Figure 1) have been maintained *in vitro* without loss of differentiation potential. They were found to develop into cells of the three germ layers (Wobus et al., 1984), and of the germ line (Toyooka et al., 2003). The pluripotency of these cells was demonstrated *in vivo* by the introduction of ESC into blastocysts. The resulting chimeric mice demonstrated that ESC could contribute to all cell lineages including germ line (Bradley et al., 1984).

The establishment of mESC lines was a major breakthrough for cell and developmental biology, followed by derivation of ESC lines from other species like rat (Buehr et al., 2008); chicken (Pain et al., 1996); dog (Hayes et al., 2008); pig (for review see Brevini et al., 2007); mink (Sukoyan et al., 1993); rhesus monkey (Thomson et al., 1995); common marmoset (Thomson et al., 1996); cynomolgus monkey (Suemori et al., 2001); and Human (Thomson et al., 1998).

I.2   *In vitro* proliferation and self-renewal

ESC can routinely be expanded to give homogenous and undifferentiated populations. Once established, mESC lines display an almost unlimited proliferation capability, and maintain a normal and stable karyotype even with continued passaging (for review see Smith, 2001).

The proliferative capacities of ESC are associated with the maintenance of ability of undifferentiated ESC to differentiate in all cell lineages. This process is called **self-renewal**.

Originally, mESC were cultured on a feeder monolayer of mitotically inactivated MEF, in medium containing fetal calf serum (FCS). It was thought that fibroblasts provide some critical factor(s), and two groups independently identified leukemia inhibitory factor (LIF) as the trophic factor responsible for this activity (Smith et al., 1988; Williams et al., 1988). Some mESC lines, like the CGR8 cell line, could be cultured without MEF on gelatinized dishes in the presence of LIF and serum. Recently, it has been shown that a growth factor, Bone Morphogenetic Protein (BMP) 4, is sufficient to sustain the undifferentiated state of mESC *in vitro* without serum (Ying et al., 2003a). So mESC could be maintained *in vitro* with LIF and BMP4, without any animal-derived products.

The signalling pathways which maintain human ESC (hESC) in the undifferentiated state are different. LIF fails to inhibit hESC differentiation. Some hESC lines are cultivated on feeder layers of MEF or feeder cells from human tissues (Thomson et al., 1998). The culture of hESC is also possible on extracellular matrix proteins, such as Matrigel (from BD Biosciences) or laminin, in presence of MEF-conditioned medium (Xu et al., 2001) and basic fibroblast growth factor (bFGF) (Amit et al., 2000).

<u>Extrinsic regulators of ESC self-renewal</u>

Maintenance of undifferentiated stem cell phenotype and pluripotency is not cell-autonomous. LIF is necessary to sustain mESC self-renewal in absence of feeder cells. LIF is

a soluble glycoprotein of the interleukin (IL) 6 family of cytokines that signal through receptor complexes to activate the signal transducer and activator of transcription (STAT) 3 (Niwa et al., 1998). The transcription factor STAT3 acts on its target genes to promote the self-renewal of mESC by blocking differentiation towards endoderm and mesoderm. The LIF/STAT3 pathway is not active in hESC (Daheron et al., 2004), explaining why LIF cannot block the spontaneous differentiation process of hESC.

In mESC, it is important to note that in the presence of LIF, and without serum or BMP4, there is a limited self-renewal and neural differentiation ensues (Ying et al., 2003a). It means that LIF is necessary, but not sufficient, to sustain mESC self-renewal. Briefly, another pathway has been described involving BMP4, a member of the transforming growth factor (TGF) β superfamily, that acts via the Smad pathway. In the presence of BMP4 alone (without LIF), cells do not self-renew properly and are driven into non-neural differentiation.

<u>Intrinsic determinants of ES cell self-renewal</u>

ESC express the epiblast/germ cell-restricted transcription factor **Oct4**. *In vivo*, zygotic expression of this transcription factor is essential for the initial development of pluripotency in the ICM. In ESC, continuous Oct4 function at appropriate levels is necessary to maintain pluripotency (Niwa et al., 2000).

Another protein, **Sox2** (SRY-related HMG box 2), plays an important role in ESC self-renewal as cofactor of Oct4 (Pesce and Scholer, 2001). In the absence of Sox2, Oct4 cannot positively regulate the expression of other ESC-specific genes. Oct4-Sox2 synergism has been reported to be mediated by direct protein-protein interaction.

A third transcription factor, **Nanog**, has been identified as a key regulator of self-renewal (Chambers et al., 2003). Forced expression of Nanog confers constitutive self-renewal in mESC without the action of the LIF/STAT3 pathway and without MEF for hESC (Darr et al., 2006).

In summary, mESC self-renewal relies on extrinsic actors, the LIF/STAT3 and BMP/Id (Ying et al., 2003a), and on intrinsic factors, Oct4, Sox2 and Nanog (Niwa et al., 2000; Chambers et al., 2003).Oct4, Sox2, and Nanog forms the so-called core pluripotency network that plays an essential role in the maintenance of pluripotency (Avilion et al., 2003; Chambers et al., 2003).

I.3   *In vivo* **pluripotency**

ESC have the capability to differentiate into derivatives of the three embryonic germ layers, namely ectoderm, mesoderm, and endoderm. This property is called **pluripotency**.

During mouse embryogenesis, the ICM of the blastocyst gives rise to both primitive endoderm (hypoblast) and primitive ectoderm (epiblast). The hypoblast subsequently gives rise to extraembryonic endoderm. The epiblast is the founder tissue of the whole embryo proper, in that it forms the three primary germ layers: mesoderm, endoderm and ectoderm, and the germ cells.

The pluripotent nature of mESC was demonstrated by their ability to contribute to all tissues of adult mice, including the germ line, following their injection into host blastocysts (Bradley et al., 1984). With this experiment, *in vitro* cultured mESC have been proved to maintain the same characteristics than ICM cells, from which they have been derived. Indeed, they are able to respond *in vivo* to the regulation factors of the embryo development. They properly proliferate, differentiate and migrate in the host embryo to become part of the entire organism.

Once injected into a host blastocyst, mESC are able to colonize the germ line and then to transmit their genotype to the progeny of the chimeric mouse. Transgenic animals obtained after genetic modifications of ESC have thus permitted to study gene functions during embryo development or adulthood (Bradley et al., 1992).

I.4   *In vitro* **pluripotency**

In vitro, when removed from the factors that maintain them as stem cells (LIF or MEF, serum or BMP4), mESC differentiate and, under appropriate conditions, generate cell-derivatives of the three embryonic germ layers.

The first experiment to mimic embryonic development *in vitro* was performed in 1985 (Doetschman et al., 1985) by culturing ESC in suspension as aggregates called embryoid bodies (EB). Initially, an outer layer of endoderm-like cells forms within the EB. After a few days, an internal cavity, which is coated with ectodermal cells, appears inside the EB. With subsequent specification of mesodermal cells, cells of the three germ layers are present in the EB. EB offer the advantage of providing a three-dimensional structure that enhances cell-cell interactions that may be important for developmental programs. The complexity of EB

formation can also be a disadvantage in understanding the signaling pathways involved in the various lineage commitments.

Two other approaches are used to initiate ESC differentiation (Figure 2). ESC can be cultured directly on stromal cells (connective tissue cells), and differentiation takes place in contact with these cells (Nakano et al., 1994). Alternatively, ESC are induced to differentiate in a monolayer on extracellular matrix proteins (Nishikawa et al., 1998). Co-culture with stromal cells provides the beneficial growth-promoting effects of the particular cell line used. However, undefined factors released by stromal cells may influence the differentiation to undesired cell types. The same is true with matrix proteins which can dramatically influence the developing cell types. An additional problem with using stromal cells method is the possible difficulty to separate the ESC-derived cells from the stromal cells.
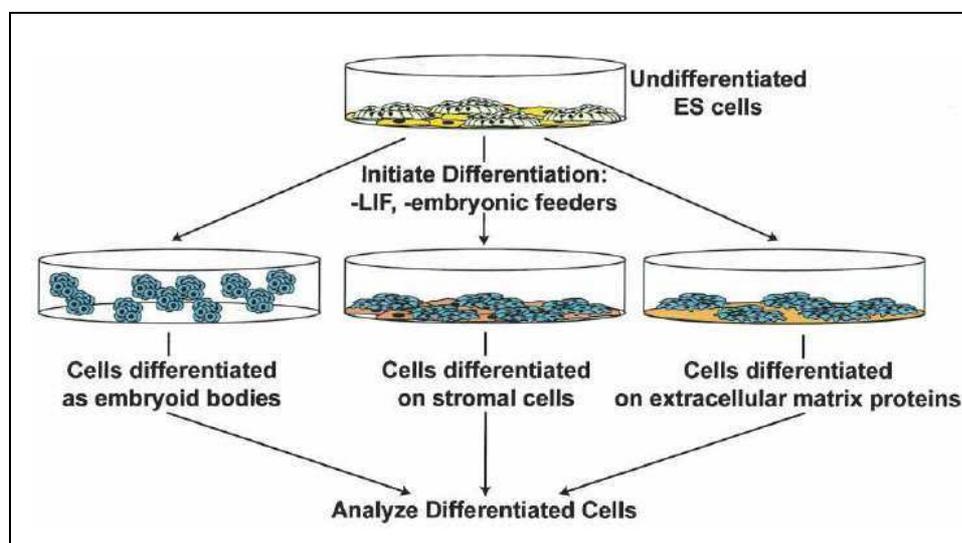
Figure 2: Three different protocols used for ESC differentiation
(from Keller, 2005)

When studying lineage-specific differentiation, it is important to keep in mind two principles/goals. First, differentiation protocol should promote **efficient** and **reproducible** differentiation of the cell type of interest. To achieve this goal, lineage development *in vitro* should mimic the developmental program that establishes the lineage in the early embryo. And second, the mature cells obtained must display **appropriate functional properties** both in culture and when transplanted into animal models.

The three differentiation methods described above have been used to generate a broad spectrum of specialized cell types from mESC (Figure 3 – Table 1 - for reviews see Keller, 2005; Wobus and Boheler, 2005).
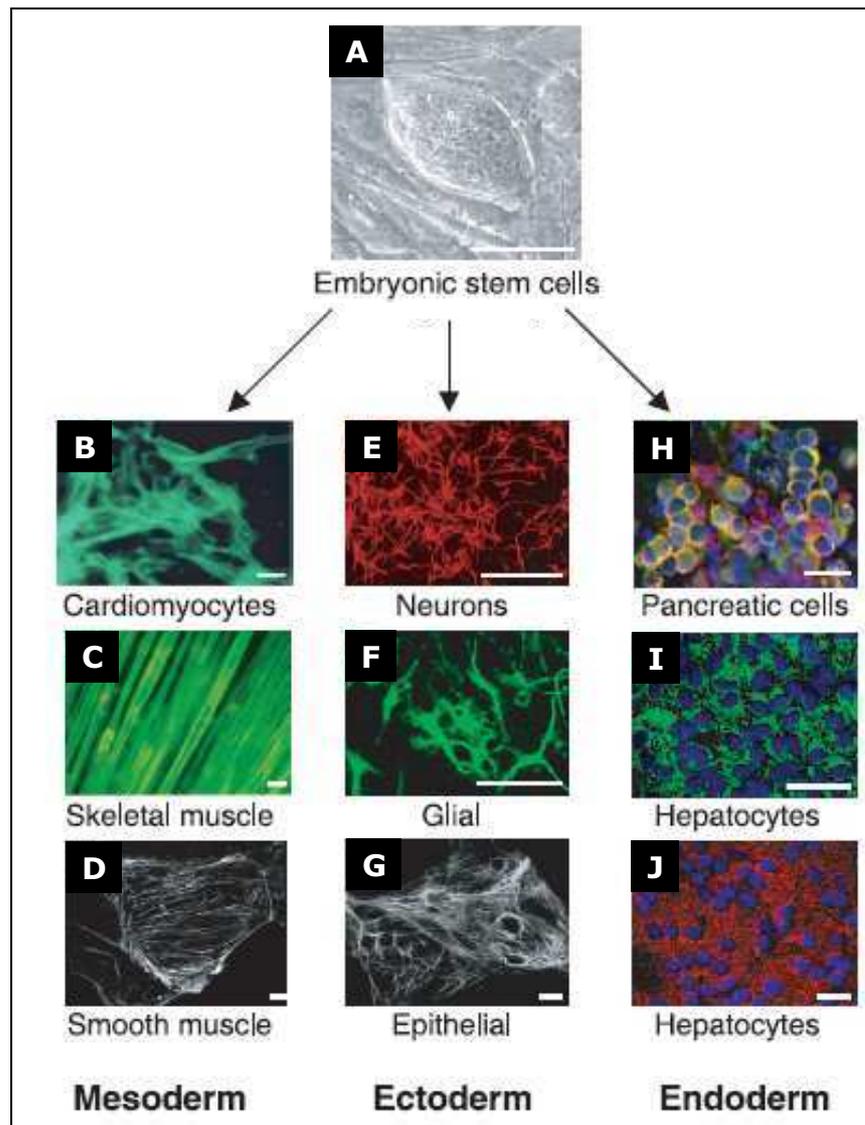
Figure 3: *In vitro* differentiation of ESC

(adapted from Wobus and Boheler, 2005)

Undifferentiated mESC (A) develop *in vitro* into differentiated cell types of all three primary germ layers (B to J).
Bars = 0.5μm (G), 20μm (H), 25μm (B, C, D), 30μm (I, J), 50μm (F) and 100μm (A, E).

Endodermal differentiation

The generation of definitive endoderm derivatives, in particular pancreas and liver cells, has been extensively studied for their potential in cell-therapy treatment of type I diabetes and liver diseases like cirrhosis (Alison et al., 2009). Progress in making endoderm-derived cell types is slow, partly because there is a lack of specific inducers for differentiation of ESC in endodermal lineages. However, differentiation has been achieved for pancreatic islet cells (Stoffel et al., 2004), hepatocytes (Hamazaki et al., 2001), thyrocytes (Lin et al., 2003), lung cells (Ali et al., 2002), and intestinal cells (Yamada et al., 2002).

Mesodermal differentiation

Mesoderm is the germ layer that develops into muscle, bone, cartilage, blood and connective tissues. Mesoderm-derived lineages, including the hematopoietic, vascular, and cardiac, are among the easiest to generate from ESC. Of these, hematopoietic development is the best characterised, and several studies have provided important insights into the embryonic origins of the hematopoietic system. *In vitro*, ESC have been successfully differentiated into hematopoeitic lineage cells (Schmitt et al., 1991; Keller et al., 1993, Nakano et al., 1994), lymphoid lineage cells (Nakano et al., 1994; Cho et al., 1999), endothelial cells (Bautch et al., 1996; Yamashita et al., 2000), skeletal muscle cells (Rohwedel et al., 1994), cardiomyocytes (Klug et al., 1996; Boheler et al., 2002), adipocytes (Dani et al., 1997), chondrocytes (Kramer et al., 2000), and osteoblasts (Buttery et al., 2001).

Ectodermal differentiation

Among the various lineages derived from the embryonic ectoderm during normal mouse development, the neuroectodermal lineage gives rise to the peripheral and central nervous systems (see section III), and the epithelial lineage is committed to become the epidermal tissues.

Epithelial differentiation from ESC has formed keratinocytes (Bagutti et al., 1996). An epidermal equivalent has also been generated after enrichment of *in vitro* differentiated keratinocytes and treatment with specific factors (Coraux et al., 2003).

Neuronal and glial cells (astrocytes, oligodendrocytes) of the nervous system are of specific importance considering possible cell therapies for neurodegenerative disorders like Parkinson's disease (PD), Huntington's disease or multiple sclerosis. The differentiation of mESC into neural cells was published independently by three groups in 1995 (Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995). The spontaneous differentiation of ESC into neural cells was rather limited but has improved significantly by a number of strategies, involving the use of retinoic acid (RA) (Bain et al., 1995; Gajovic et al., 1997), lineage selection (Li et al., 1998), and stromal cell-derived inducing activity (SDIA) (Kawasaki et al., 2000). Whereas high concentrations of RA originally promoted efficient neuronal differentiation, characterized by the expression of tissue-specific genes, proteins, ion channels, and receptor in a developmentally controlled manner, the survival and development of neurons derived in response to RA is limited. Furthermore, teratogenicity of RA makes it unsuitable for therapeutic applications (for review see Rohwedel et al., 1999). Alternative

protocols, involving multiple steps of differentiation and selection of neural progenitor cells, have been established. Neuronal cell differentiation can also be supported by the addition of neuronal differentiation factors (Okabe et al., 1996) and maintained *in vitro* by survival-promoting factors like glial cell line-derived neurotrophic factor (GDNF) (Rolletschek et al., 2001) among others.

To summarize, each of the three major neural cell types of the central nervous system – neurons, astrocytes and oligodendrocytes – can be generated, and relatively pure populations of each can be isolated when cultured under appropriate conditions: astrocytes and oligodendrocytes, see Fraichard et al., 1995; Tang et al., 2002; Brustle et al., 1999; Tropepe et al., 2001 / dopaminergic, serotonergic, GABAergic, glutamatergic and cholinergic neurons, see Bain et al., 1995; Strubing et al., 1995; Lee et al., 2000; Kawasaki et al., 2000; Finley et al., 1996. More details will be provided in section III.

Germ cell differentiation

Germ cell formation *in vitro* has been visualized only recently. ESC have been differentiated in cells morphologically similar to early ovarian follicles, and continued culture of these oocyte-like cells revealed structure similar to mouse preimplantation embryos (Hübner et al., 2003). Formation of male germ cells has also been reported. They displayed the capacity to participate in spermatogenesis *in vivo* (Toyooka et al., 2003) and to fertilize oocytes (Geijsen et al., 2004).

I.5   An outlook on ESC

ESC properties are unique.

i)   They are derived from the ICM of the pre-implantation embryo without transformation or immortalization.

ii)   They have a stable diploid karyotype.

iii)   They display high amplification and unlimited self-renewal capacities.

iv)   They are clonogenic.

v)   They are pluripotent: they are able to generate all fetal and adult cell types *in vitro* and in teratomas.

vi)   They incorporate into embryonic development and contribute to all three germ layers in chimera. They are also able to colonize the germ line.

In case of hESC, the last property above cannot be determined in humans for obvious ethical reasons.

	Differentiated cell types	References
E N D O D E R M	pancreatic islet cells	Stoffel et al., 2004
	hepatocytes	Hamazaki et al., 2001
	thyrocytes	Lin et al., 2003
	lung cells	Ali et al., 2002
	intestinal cells	Yamada et al., 2002
M E S O D E R M	hematopoeitic lineage cells	Schmitt et al., 1991; Keller et al., 1993, Nakano et al., 1994
	lymphoid lineage cells	Nakano et al., 1994; Cho et al., 1999
	endothelial cells	Bautch et al., 1996; Yamashita et al., 2000
	skeletal muscle cells	Rohwedel et al., 1994
	cardiomyocytes	Klug et al., 1996; Boheler et al., 2002
	adipocytes	Dani et al., 1997
	chondrocytes	Kramer et al., 2000
	osteoblasts	Buttery et al., 2001
E C T O D E R M	keratinocytes	Bagutti et al., 1996; Coraux et al., 2003
	neural cells	Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995
	astrocytes	Tang et al., 2002
	oligodendrocytes	Brustle et al., 1999; Tropepe et al., 2001
	dopaminergic neurons	Lee et al., 2000; Kawasaki et al., 2000;
	serotonergic neurons	Lee et al., 2000
	GABAergic neurons	Bain et al., 1995
	glutamatergic neurons	Finley et al., 1996
	cholinergic neurons	Fraichard et al., 1995
G E R M	early ovarian follicles	Hübner et al., 2003
	male germ cells	Toyooka et al., 2003; Geijsen et al., 2004

Table 1: Examples of specialized cells obtained *in vitro* from mESC

It is also important to keep in mind that ESC are not identical to the embryo cells from which they are derived, even if they share many characteristics. ESC can be cultured *in vitro* for extended period of time, but embryonic cells *in situ* exist just transitionally. ESC could then be considered as a cell culture phenomenon or even as an artifact. From this perspective, it is not surprising that they are significant differences between species, reflecting the capacity to adapt to an arbitrary set of artificial conditions.

Nevertheless, ESC derivation or "creation" has dramatically changed the developmental biology, and even the future human medicine approaches. The promises of (embryonic) stem cell research applicable to the humans are various: study embryo development by cell differentiation in culture resulting in understanding birth defects for example, identify drug targets and test potential therapeutics, and develop regenerative medicine strategies.

We have reviewed the properties of embryonic stem cells. Mouse ESC, studied as stem cell research reference cells, and primate ESC, studied for their potential in regenerative medicine, are able to self-renew for supposed unlimited period of time and to differentiate into cell-derivatives of the three embryonic germ layers under appropriate conditions *in vitro*.

In the following parts, we focus on two cell types of the neural lineage: dopaminergic and serotonergic neurons. We describe what is known about dopaminergic and serotonergic specifications *in vivo*, and differentiation protocols *in vitro* to obtain dopaminergic or serotonergic neurons from ESC.

The dopaminergic and serotonergic systems are of particular interest for regenerative medicine as they are believed to be involved in neural disorders like Parkinson's disease, or psychiatric disorders like depression, respectively.

II GENETIC MECHANISMS CONTROLLING DOPAMINERGIC AND SEROTONERGIC SPECIFICATIONS

II.1 Central nervous system (CNS) development and primary organization

Development of the CNS is a multi-step process. It arises from the dorsal epiblast of the gastrula. One of the first events in embryonic organogenesis is the formation of the neural plate from early neuroectoderm from which the CNS derives. Neural plate then folds and closes itself to constitute the neural tube through the process of neurulation.

Throughout embryonic development, the neural tube is segmented along the anteroposterior (A/P) axis (Lumsden and Krumlauf, 1996). Three main vesicles can be observed: the primary **prosencephalon**, the **mesencephalon** and the **rhombencephalon** (Figure 5a). This segmentation ultimately results in the appearances of neuromeres. The rostral-most vesicle, the primary prosencephalon (forebrain), is divided into two segments, the diencephalon and the secondary prosencephalon, which dorsally gives rise to the telencephalic vesicles later in development (Puelles, 2001; Puelles and Rubenstein, 2003). The caudal-most vesicle, the rhombencephalon (hindbrain), is subdivided into the isthmus, the rhombomeres (r1 to r7) (Lumsden, 2004), and the pseudorhombomeres. The mesencephalon (midbrain) is located between the other two vesicles, remaining a single segment or mesomere (Puelles, 2007).

Complementary to anteroposterior patterning, the neural tube is organized in longitudinal domains along the dorsoventral (D/V) axis. These are defined, ventrally to dorsally, as floor plate (FP), basal plate (BP), alar plate and roof plate (RP) (Shimamura et al., 1995). Directly underlying the neural tube is the notochord, a cord-like structure of mesodermal origin positioned ventrally in the embryo (later in development, this structure becomes the vertebral column).

Neural tube organizers

Within the three broad territories described above (forebrain, midbrain and hindbrain), specific neural cell types are generated on a precise schedule by the action of several signaling centers. Patterning along the D/V axis is accomplished through the ventral (FP) and dorsal (RP) midlines of the neural tube (Chizhikov and Millen, 2004b; Placzek and Briscoe, 2005). Patterning along the A/P axis (Lumsden and Krumlauf, 1996; Kiecker and Lumsden, 2005; for review see Liu and Joyner, 2001) is controlled by the anterior neural ridge (ANR, the most

anterior edge of the forebrain), the zona limitans intrathalamica (ZLI, boundary between the dorsal and ventral thalamus in the diencephalon), and the isthmic organizer (IsO) at the midbrain/hindbrain boundary (MHB). These signaling centers are characterized by the secretion of different factors controlling the establishment of the adjacent neural territories and the specification of the distinct neuronal populations for each territory. Diffusible signals from the centers are received by groups of cells, which turn on transcription of cell-specific genes depending of their position on the grid defined by the intersection of the different signals (Ye et al., 1998).

II.2   Dopaminergic (DA) specification

Dopamine (DA) is one of the major neurotransmitters in the CNS. The DA system has been then extensively studied due to its clinical implication in neural diseases like Parkinson's disease.

II.2.1   Dopamine belongs to the monoaminergic system

Biochemically, dopamine is a biogenic monoamine, containing an amino group ($-NH_2$) connected to an aromatic ring by two-carbon chain ($-CH_2-CH_2$) (Figure 4). Some other neurotransmitters share the same structure. They all derive from aromatic amino acids like tyrosine for catecholamines (dopamine, epinephrine, norepinephrine) or tryptophan for serotonin by the action of aromatic amino acid decarboxylase (Jaeger et al., 1984). The catecholamines and the serotonin represent the monoaminergic system of the brain. Besides acting as neurotransmitters, they are also defined as hormones. All biogenic monoamines act synergistically to maintain the organism homeostasis. They have a common origin, similar biochemical pathways, highly homologous enzymes and transporters if not the same ones, and are involved in the same physiological processes like the regulation of the body temperature, heart rate, breathing, circadian rhythm, depression, cognition, learning and emotions (Penev et al 1994, Kim et al 1997).

II.2.2   Dopaminergic neuron characteristics and markers

DA neurons can be identified by the expression of proteins required for the biosynthesis, transport, synaptic packaging, release and reuptake of DA. DA is produced in two enzymatic steps from the amino acid tyrosine. Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-DOPA followed by decarboxylation to DA via the aromatic L-

amino acid decarboxylase (AADC) (Figure 4). In contrast to noradrenergic and adrenergic neurons, DA neurons never express dopamine β-hydroxylase (DBH) which catalyzes the conversion of DA to noradrenaline, nor phenylethanolamine N-methyltransferase (PNMT) which catalyzes the conversion of noradrenaline to adrenaline.
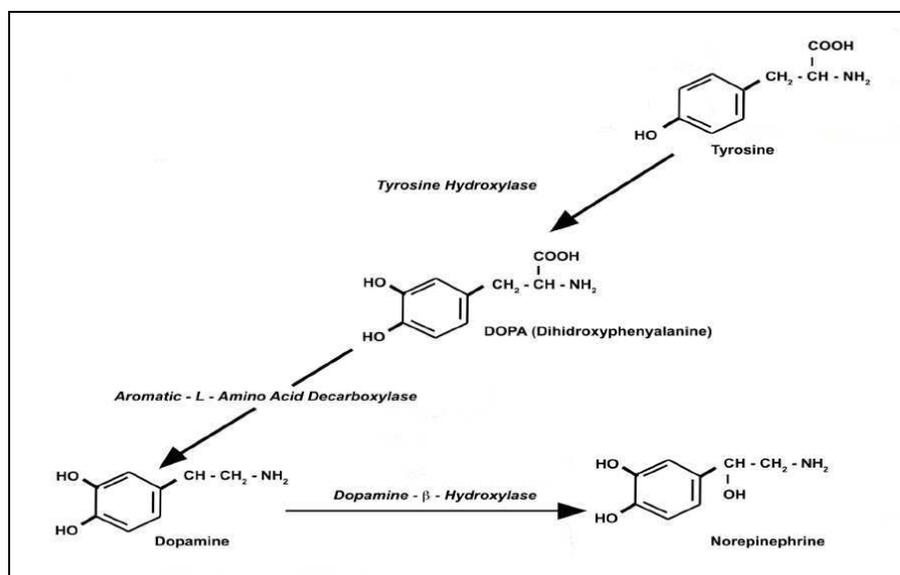
Figure 4: Biosynthesis of catecholamines dopamine and norepinephrine

TH catalyzes the rate-limiting step in DA biosynthesis but its expression is shared among all catecholaminergic neurons. Two types of transporters are essential to DA neurotransmission: the plasma membrane DA transporter (DAT) (Chen and Reith, 2000) and the vesicular monoamine transporter (Vmat) 2 (Miller et al., 1999). DAT is found exclusively in DA neurons where it terminates the action of DA by rapidly removing it from the synapse. Vmat2 loads cytoplasmic DA, as well as all other monoaminergic neurotransmitters from the presynaptic nerve terminal into vesicles for storage and subsequent release. Aldehyde dehydrogenase (Aldh) 1 is an additional dopaminergic marker expressed in both precursors and mature DA neurons (McCaffery and Drager, 1994; Wagner et al., 1999). Aldh1 is aslso called Aldh1a1 or Ahd2. The tyrosine kinase receptor cRet mediates the neurotrophic response to members of the GDNF growth factor family in motor and dopaminergic neurons, and is also used for their characterization (Durbec et al., 1996; Trupp et al., 1996).

II.2.3 Localization and type of DA neurons

The dopaminergic system of the CNS is composed of different regions. We detail here the organization of DA neurons in the murine brain.

In the murine brain, DA neurons are identified in the following structures (Figure 5):

i)   Olfactory bulb dendritic periglomerular neurons: A16 group.

ii)   Zona incerta group in the ventral thalamus: A13.

iii)   Hypoyhalamic cell groups: A12, A14 and A15.

iv)   Caudal diencephalic periaquductal gray group: A11.

v)   Meso-diencephalic tegmental groups: A8, A9 and A10.
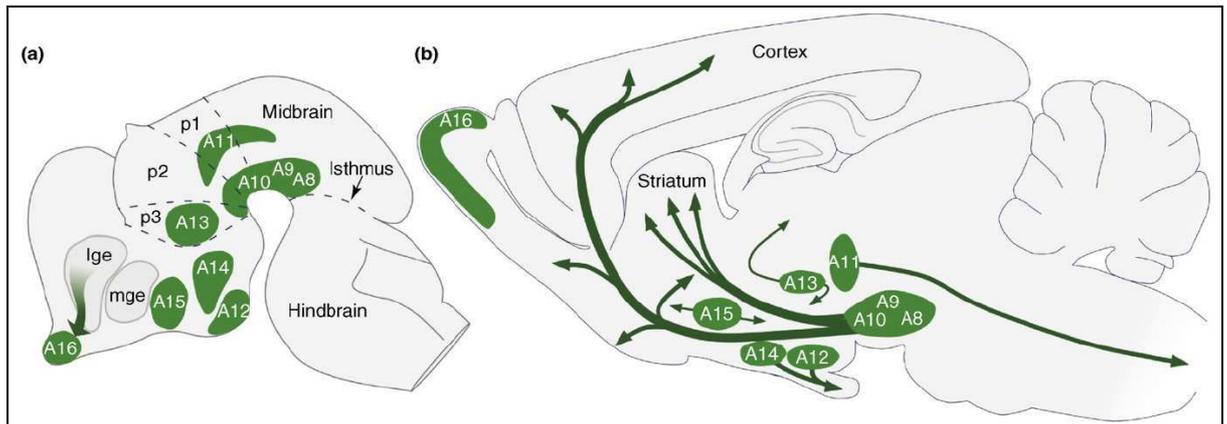
Figure 5: Distribution of the DA neuron cell groups in the developing (a) and adult (b) rodent brain

(from Bjorklund and Dunnett, 2007)

DA neurons are localized in nine distinctive cell groups. Arrows in (b): Principal projections of DA neurons.
Abbreviations: lge, lateral ganglionic eminence; mge, medial ganglionic eminence; p1-3, prosomeres 1-3.

These last three groups (A8, A9, A10) represent 75 percent (%) of all DA neurons on the adult CNS. These neurons originate from the tegmental area of the mesencephalon, so they are called **mesencephalic dopaminergic (mDA) neurons**. These groups assume distinct functions and project to specific brain regions. The A9 cell group defines neurons of the **substantia nigra pars compacta (SNc)** with main projections to the dorso-lateral striatum (caudate nucleus and putamen) through the nigrostriatal pathway, to the globus pallidus (GP), and to motor areas in the cortex. The **nigrostriatal pathway** participates in movement control as highlighted by the dramatic consequences of DA neuronal loss in PD (Lang and Lozano, 1998a,b; Barzilai and Melamed, 2003). The A10 group defines the ventral tegmental area (VTA). They project to the ventro-medial striatum, the cingulated and prefrontal cortex, and other limbic areas as part of the mesolimbicortical system that is involved in emotional behavior and reward mechanisms (Tzschentke and Schmidt, 2000). Finally, A8 neurons of the retrorubral field (RRF) partially project within the nigrostriatal projections, and also towards the SNc and the VTA. These neurons appear to be involved in the interconnection of the SNc and VTA (for review see Bjorklund and Dunnett, 2007).

Among all the DA populations in the CNS, the mDA neurons, and especially the A9 group, have been the focus of clinical interest for a long time because of their involvement in severe human neurological and psychiatric illnesses: PD, depression, schizophrenia. Parkinson's disease (PD) is an example of neurodegenerative disorder and is caused by the progressive loss of mDA neurons that innervate the striatum (Lang and Lozano, 1998a,b; Barzilai and Melamed, 2003): the A9 group of neurons from the SNc. As a consequence of neuronal loss, this illness is first clinically characterized by tremor, rigidity, bradykinesia, and postural instability (Lang and Lozano, 1998a,b). The progressive loss of voluntary and involuntary muscle control produces a number of secondary symptoms also associated with PD: constipation, difficult swallowing, excessive salivation and hypophonia are among them. Some other like anxiety, depression and isolation are psychosocial symptoms. The cause of the selective loss of A9 DA neurons is still not well understood, although several mechanisms have been proposed such as increased oxidative stress or mitochondrial dysfunction (Dawson and Dawson, 2003; Inamdar et al., 2007). The selective vulnerability within the groups of mDA neurons may root in the molecular make-up of these neurons, which originates from specific differentiation routes during development (for review see Smits et al., 2006).

It is interesting to note that most cases of PD are classified as sporadic and occur in people after age 50 with no apparent history of this disorder in their family. However, juvenile onset PD also exists in some people before age 20. Around 15% of people with PD have a family history. They are referred to as familial cases. These familial cases are caused by mutations or alterations of genes that have not yet been all identified and that increase the risk of developing the disease.

II.2.4   Developmental program of mDA neurons

We will now detail the developmental program governing the emergence of mDA neurons during mammalian embryogenesis. They are generated near the FP in the ventral midline of the neural tube, anterior of the IsO in the midbrain (Figure 6). Their developmental programme can be subdivided into three distinct processes: (1) the regionalization or induction of a progenitor field within the neuroepithelium competent to generate mDA precursors at early stages of neural development (from embryonic day (E) 8.5 to E10.5 in mouse); (2) the specification of a mDA neuronal fate in these precursors at intermediate stages (from E10.5 to E12.5 in mouse); and (3) the terminal differentiation or acquisition of the mature phenotype of mDA neurons at late stages of neural development (from E12.5 in

mouse) (for review see Wallen and Perlmann, 2003; Vitalis et al., 2004; Nakamura et al., 2005; Burbach and Smidt, 2006; Prakash and Wurst, 2006; Smits et al., 2006; Abeliovich and Hammond, 2007; Puelles, 2007; Smidt and Burbach, 2007; Gale and Li, 2008).
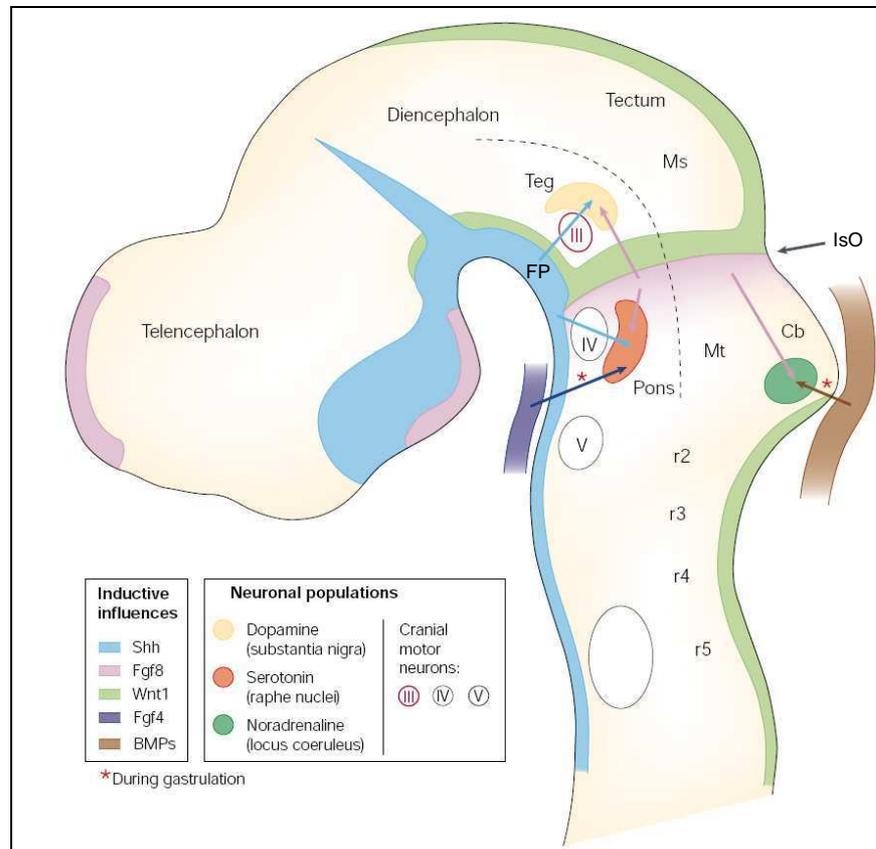
Figure 6: Signals that influence mDA and 5-HT neurons generation along the A/P and D/V axes

(adapted from Wurst and Bally-Cuif, 2001)

Sagittal view of a mouse neural tube at E11, anterior to the left. Secreted factors (arrows) that control neuronal identities originate from the neural plate at the MHB (FGF8 by the IsO) and floor plate (Shh), as well as from non-neural tissues during gastrulation (FGF4 from the primitive streak). DA neurons from the midbrain tegmental area respond to a combination of Shh and FGF8. Serotonergic neurons from the hindbrain require early FGF4 signalling, followed by Shh and FGF8. Abbreviations: 5-HT, serotonergic; BMP, bone morphogenetic protein; Cb, cerebellum; FGF, fibroblast growth factor; FP, floor plate; IsO, isthmic organizer; Ms, mesencephalon; Mt, metencephalon; r, rhombomeres; Shh, sonic hedgehog; Teg, tegmentum; Tel, telencephalon.

II.2.4.1 Specification of the mDA neuronal field

Mesencephalic organization is initiated by the positioning of two key signaling centers: the **floor plate organizer** at the ventral midline of the neural tube and the **isthmic organizer** at the caudal extreme of the mesencephalon (Figure 6). These organizers instruct the fate of adjacent neural progenitors by the secretion of diffusible factors along the D/V and A/P axes of the neural tube. mDA neurons originate from the specific region where these signals intersect each other. Concurrently to these signals, cell-intrinsic factors within the progenitors progressively restrict cell fate choices.

The lipid-modified glycoprotein **Sonic hedgehog (Shh)** is the main patterning molecule along the D/V axis (for review see Ingham and McMahon, 2001; Ingham, 2008). It is secreted by the FP organizer and the underlying notochord. Its expression is controlled by the transcriptional regulator hepatocyte nuclear factor (Hnf) 3 and starts at the closure of the neural tube (E9 in mice) (Epstein et al., 1999). Shh have been shown to be necessary to instruct the ventral fate of progenitors *in vivo* and *in vitro* (Yamada et al., 1991; Hynes et al., 1995b). As a proof, explants from E9 rat embryos, including midbrain and forebrain, were isolated and presumptive floor plate was removed. Remaining tissue was cultured in the presence of Shh, which was able to induce DA neurons in explants (Hynes et al., 1995a). Shh acts within receptive cells through a Patched receptor complex that transduces Shh signaling to Smoothened membrane protein and then to Gli family transcription factors (Ingham, 2008). It has also been shown that another neurotrophically acting molecule, TGFβ, is expressed in early structures such as the notochord and the floor plate (Flanders et al., 1991), and in the area in which mDA neurons develop (Farkas et al., 2003). Unlike what was first thought, TGFβ does not act downstream of Shh, but both TGFβ and Shh appear to be required for the induction of the mDA region by a cooperative mode of action (Farkas et al., 2003).

Another key factor, which determines the position of the mDA progenitor field along the A/P axis, is the **fibroblast growth factor (FGF) 8** secreted by the IsO (Figure 7) (Ye et al., 1998). FGF8 is also broadly expressed in the developing embryo, in particular in cardiac mesoderm underlying the midbrain/hindbrain region. Two different and independent pathways are necessary for the onset of FGF8 expression in the IsO. First is a pathway downstream of the paired domain protein (Pax) 2, also involving Pax5 (Asano and Gruss, 1992; Rowitch and McMahon, 1995). A second mechanism relies on the combined action of orthodenticle homologue (Otx) 2 and gastrulation brain homeobox (Gbx) 2, two transcription factors expressed early in embryonic development and known to be involved in the positioning of the isthmus. From E5.5 in mice, Otx2 is expressed in the forebrain and midbrain while Gbx2 can be found in the prospective hindbrain region (Figure 7), where Gbx2 inhibits the action of Otx2 (Nakamura et al., 2005). The meeting point of the territories that express these two genes seems to identify the future position of the MHB. Pax2 has been shown to be expressed in both posterior Otx2- and anterior Gbx2-expression domains. Otx2 inhibits FGF8 expression cell autonomously, but is able to stimulate its expression in neighboring cells. Therefore FGF8 appears in the anterior hindbrain in the region of Gbx2-

expression, but adjacent to the Otx2 expression domain (Brodski et al., 2003; Nakamura et al., 2005).
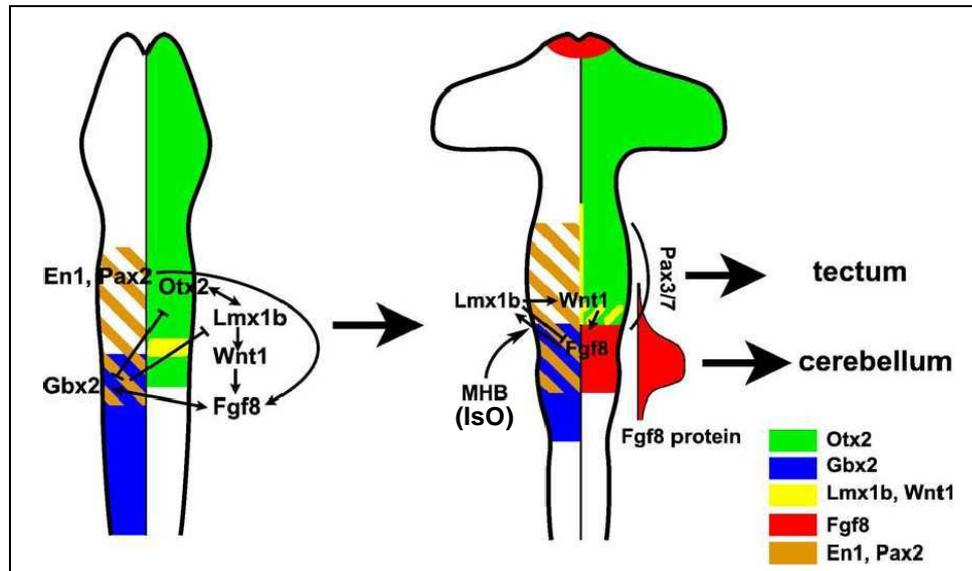
Figure 7: Isthmus related genes and organizing activity
(adapted from Nakamura et al., 2005)
See text for details.

In *Fgf8* null mutants, severe defects lead to early death around E9.5 (Sun et al., 1999). Furthermore, ectopic TH-expressing neurons have been obtained in ventral forebrain explants by coculturing them with isthmic tissue expressing FGF8 and production of mDA neurons in ventral midbrain explants can be abolished by a blocking receptor of FGF8 (Ye et al., 1998).

In concert with FGF8, Wnt signaling, through the secreted factor Wnt1 (Castelo-Branco et al., 2003), is crucial for the establishment of the midbrain-hindbrain region. Wnt1 is thought to be required for the maintenance of FGF8 expression (Lee et al., 1997). Wnt signaling is moreover involved in activation of *engrailed* (*En*) genes, which are expressed across the MHB and necessary for later stages of mDA neuronal development (Danielian et al., 1996). In a recent study (Chung et al., 2009), Wnt1 has been shown to collaborate with the transcription factor LIM homeobox (Lmx) 1a in an autoregulatory loop (see section II.2.4.3 for details). Another transcription factor, Lmx1b, expressed in the anterior embryo as early as E7.5, sees its expression restricted to the isthmus around E9 in mice. Lmx1b is essential for the inductive activity of the IsO but its exact role at this stage is unclear. Knock-out (KO) experiments for Lmx1b have shown that this transcription factor is required for the initiation of FGF8 expression and for the maintenance of Wnt1 expression (Guo et al., 2007). In this study, FGF8, Wnt1 and isthmus-related transcription factors (En1, En2, Pax2) are not expressed in *Lmx1b* mutant mice, causing a severe reduction of the tectum and cerebellum. Another study

demonstrated that the expression of Lmx1b is induced by FGF8 (Alexandre et al., 2006). See section II.4 for further details on transcription factor Lmx1b.

Finally, the position of the IsO (Brodski et al., 2003) that expresses FGF8 is a determining parameter for the generation of mDA progenitors. Influencing the position of the IsO indirectly acts on the emergence of mDA neurons through the ablation or the expansion of the midbrain. Otx2-deficient mice lack the mid- and forebrain, whereas *Gbx2* null mutants show a failure of anterior hindbrain development and display a posterior expansion of the midbrain (Brodski et al., 2003).

In summary, early signaling from organizers generates a permissive region that is defined by a specific pattern of gene expression in the mesodiencephalon ventricular zone. This induces mitotic cells in this region to become postmitotic young neurons that are destined to become fully differentiated mDA neurons.

II.2.4.2 Transcriptional regulation in the specification of mitotic mDA precursors (Figure 8)

The following molecular markers, mainly transcription factors, are involved in the specification of mesencephalic precursors and characterize these precursors. None of them is unique to the mDA population, although some are also implicated in the mDA fate determination.

Sox proteins

Members of the Sox family do not act uniquely in the formation of mDA neurons. They are general regulators of neural development in regard of stem cell maintenance as well as in induction of gliogenic versus neurogenic fates (Pevny and Placzek, 2005). While Sox 1/2/3 (SoxB1 group) partially inhibit neuronal proliferation by blocking the capacity of proneural bHLH proteins to induce downstream events of neuronal differentiation, members of the SoxB2 group, such as Sox21, rather induce neural genes like the one coding for the transcription factor neurogenin (Ngn) 1 and promote progression of neurogenesis (Sandberg et al., 2005).

Otx2

Otx2 is a homeodomain transcription factor that plays an essential role in the positioning of the isthmus as already discussed above. Otx2 is also expressed within the mDA precursors and is required for their generation. Indeed, Otx2 is thought to repress the homeodomain protein Nkx2.2 (Figure 8), which is a negative regulator in mDA neuron

formation program (Prakash et al., 2006). Experiments showed that mDA neurons production is reduced in mice in which Otx2 is conditionally deleted after E9.5 (Puelles et al., 2004). Interestingly, in the absence of Otx2, mDA neurons are replaced by serotonergic neurons (see section II.3).

Lmx1a and Msx1

The transcription factor Lmx1a is another marker of mDA precursors and is required for their generation as proved by loss-of-function experiments with siRNA (small interfering ribonucleic acid) for Lmx1a in chick embryo (Andersson et al., 2006b). A similar requirement of Lmx1a in the mouse has not been reported. Moreover, *dreher* mutant mice carrying a mutation in the Lmx1a locus showed only modest developmental defect of the mDA neurons (Ono et al., 2007). Lmx1a expression within the FP cells, around E9 in mice, appears to be directly induced by Shh (Andersson et al., 2006b). The cascade downstream of Lmx1a is complex. Over-expression of Lmx1a induces the expression of Msx1, another homeodomain factor (Figure 8). Msx1 is not sufficient to induce mDA neurons after ectopic expression in chick midbrain (Andersson et al., 2006b) but is a transcriptional repressor that inhibits expression of the homeodomain factor Nkx6.1 required for the generation of motor neurons (Muhr et al., 2001; Vallstedt et al., 2001). Thus Msx1 suppresses alternative ventral cell fates in the DA progenitor domain. Ultimatly, Lmx1a-Msx1 cascade induces proneural factor neurogenin (Ngn) 2 (see below). Msx1 alone is neither necessary nor sufficient for mDA neurons generation, suggesting additional pathways. A recent study (Chung et al., 2009) showed that Wnt1, as well early expressed in the IsO, and Lmx1a form an autoregulatory loop, and that Lmx1a directly regulates two critical factors of mDA neuron terminal differentiation: Nurr1 and Pitx3 (see below). These last findings demonstrate the role of Lmx1a in the acquisition of the DA phenotype.

Foxa1/2

Members of the Foxa subfamily of forkhead/winged helix transcription factors (Fox), Foxa1 and Foxa2 are expressed in floor plate progenitors as early as E8. First, they have been shown to regulate neuronal specification in mDA precursors by regulating the expression of proneural bHLH transcription factor Ngn2 (Ferri et al., 2007). Another recent study reported that Foxa1/2 play an earlier role in specifying mDA precursor identity by inhibiting the expression of Nkx2.2 and positively regulating the expression of Lmx1a (Lin et al., 2009).

<u>Ngn2</u>

Ngn2 is a member of the family of bHLH genes that are principally involved in gliogenic versus neurogenic fate decisions during neuronal patterning. Neurogenins promote neurogenesis and neural subtype specification (for review see Bertrand et al., 2002). Ngn2 expression is regulated by Otx2 in mDA precursors, as its expression is absent in Otx2-null animals (Vernay et al., 2005). Moreover, Ngn2 is normally maintained in postmitotic precursors (Andersson et al., 2006a; Kele et al., 2006), suggesting additional functions in maturing cells. *Ngn2*-null mutant mice display a severe reduction in postmitotic mDA neurons markers (Andersson et al., 2006a) such as Nurr1 (see section II.2.4.3) and TH. Ngn2 thus appears to be required for the normal generation of mDA precursors and their differentiation into mature mDA neurons.
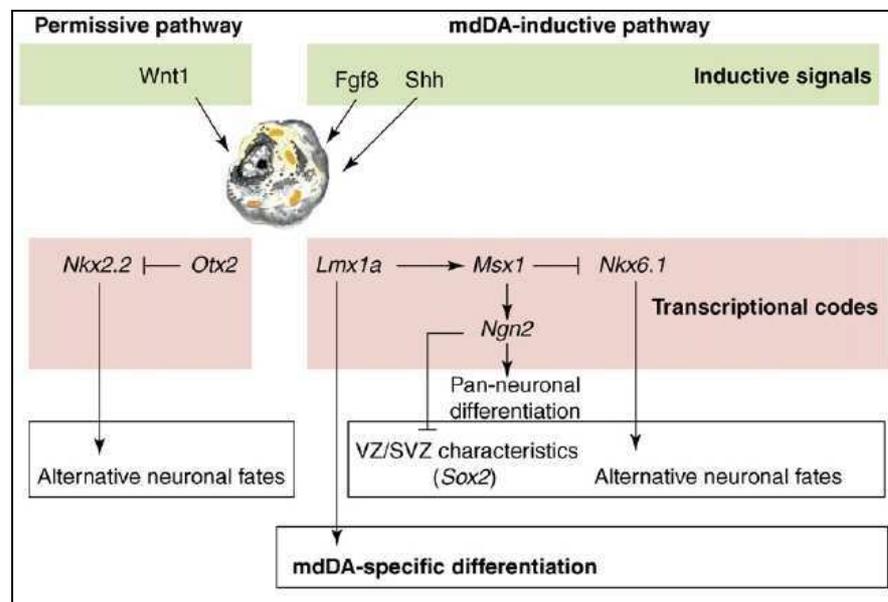
Figure 8: Inductive signals and transcriptional codes in the mDA neuronal specification of neuroepithelial stem cells

(from Burbach and Smidt, 2006)

See text for details. Inductive signals are shown on a green background, transcription factors are on pink.
Abbreviations: VZ/SVZ, ventricular/subventricular zones.

II.2.4.3 Transcriptional regulation for the development of postmitotic mDA neurons (Figure 9)

The genes mentioned above are involved in the specification of mDA precursors. Several other genes are also required for the development and the maintenance of the DA neurotransmitter phenotype and the survival of mDA neurons. As mDA precursors exit the cell cycle (around E11 in mouse), they migrate away from the ventricular surface. DA cells

first migrate ventrally along the radial glia, and then laterally along tangential axons to reach the SNc area (Kawano et al., 1995). Early phenotypic markers of mDA neurons, such as TH and other enzymes of the DA biosynthesis pathway (see section II.2.2), are then induced (Wallen et al., 1999). Subsequent maturation of mDA neurons from E12.5 is characterized by the expression of the DA transporter DAT (see section II.2.2) among others. [To note: the section II.4 is dedicated to the transcription factor Lmx1b that is not detailed in this part.]
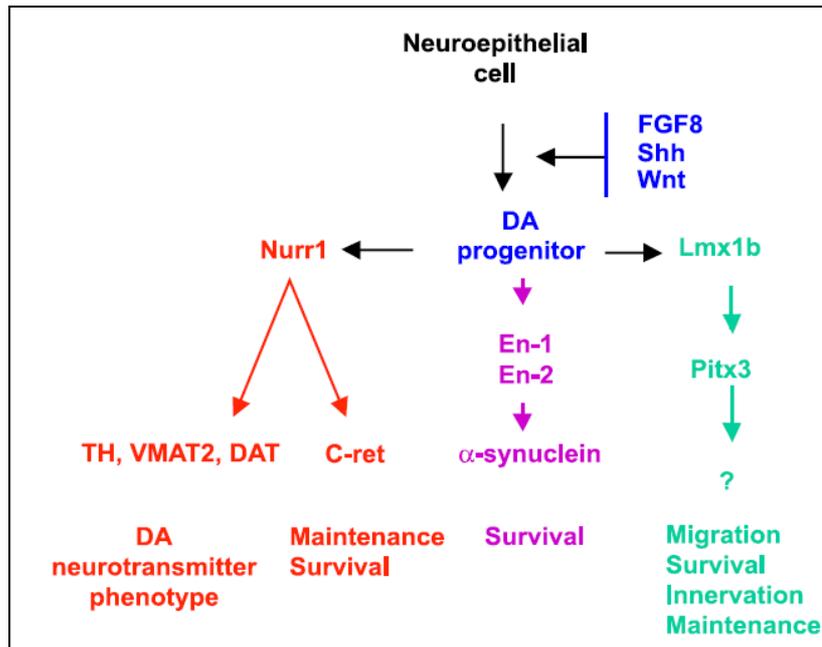
Figure 9: Transcriptional control of mDA neurons terminal differentiation
(from Vitalis et al., 2005)

Nurr1

Orphan nuclear receptor family transcription factor Nurr1 (or Nr4a2) was first identified in 1992 (Law et al., 1992). Expression of Nurr1 is initiated at E10.5 in mouse, and just precedes TH expression (Wallen and Perlmann, 2003). At this stage of development, expression of Nurr1 is restricted to the midbrain, but subsequently expression extents to other CNS regions including the cortex and the hippocampus. Evidence that Nurr1 defines an important cell-intrinsic component of postmitotic DA neuron specification and maturation comes from Nurr1-deficient animals, which fail to express mDA neuron markers like TH, cRet, Vmat2, AADC and the DA transporter DAT (Wallen et al., 1999). By birth, TH-positive cell are absent in the midbrain of these animals. Nurr1 pathway may be involved in the definition of neurotransmitter identity of mDA neuron systems. However, other early markers of mDA neurons, such as Pitx3, Lmx1b, En1, Aldh2 (see below), remain unaltered in Nurr1-

deficient animals, showing that the mDA cell fate is not totally abrogated (Castillo et al., 1998) and is surely maintained by the action of other pathway(s).

Pitx3

The paired-like homeodomain transcription factor Pitx3, a member of the Pitx subfamily, was identified by two groups at about the same time (Semina et al., 1997; Smidt et al., 1997). Pitx3 is transiently expressed in the eye lens and skeletal muscle (E12-E18), and uniquely and constitutively expressed in mDA neurons. This expression site is maintained until adulthood in mouse and human (Smidt et al., 1997). Double labeling studies (Smidt et al., 1997; Smidt et al., 2000; Smidt et al., 2004) as well as Pitx3-Green fluorescent protein (GFP) knock-in in animals (Zhao et al., 2004a; Maxwell et al., 2005) have shown that, during development and in the adult stage, there is an almost 100% overlap in TH and Pitx3 expression within the mDA neurons. The naturally occurring aphakia mice harbor a deletion in Pitx3 but display initially normal expression of TH (E11.5 in mouse). However, by E12, a deficit appears in lateral mDA cells that represent the future SN (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004). By birth, there is a rather specific loss of TH expression and other mDA markers in the SN of aphakia mice, whereas the VTA is relatively preserved. Pitx3 is present both in SNc and VTA dopaminergic populations, but expression of Pitx3 in SNc cells just precedes TH expression, whereas expression of Pitx3 in VTA cells is coincident with TH (Maxwell et al., 2005). Interestingly, a similar pattern of mDA neurons deficiency (SN more than VTA) is seen in PD. In summary, Pitx3 and upstream Lmx1b (see section II.4) are suggested to be part of a pathway promoting survival and maintenance of mDA neurons (Figure 9).

First, it was believed that this pathway was independent of the Nurr1-dependent pathway regulating a subset of phenotypic markers like TH, Vmat2 or DAT. However, experiments of over-expression of both Pitx3 and Nurr1 at the neural precursor stage in mESC- and hESC-derivatives indicated that Pitx3 and Nurr1 cooperatively induce the late maturation of mDA neurons (Martinat et al., 2006). The mechanism of this cooperation was elucidated in a recent study, in which Pitx3 was proved to be a crucial regulator of Nurr1-mediated transcription (Jacobs et al., 2009).

En1/En2

The *engrailed* genes belong to the family of homeobox genes. The two mouse *En* genes, *En1* and *En2*, are expressed during early neural development (see section II.2.4.1) across the

MHB in the caudal midbrain and rostral hindbrain, and thus in a region where mDA neurons arise. Furthermore, both En1 and En2 continue to be expressed within the SN and VTA of the postnatal and adult mouse brain (Davis and Joyner, 1988; Simon et al., 2001; Simon et al., 2004). Experiments with *En* mutants have shown that both En proteins together are required for the proper development of mDA neurons at later stages, that is to say for survival and maintenance of these cells, but not for their induction or initial differentiation. *En* genes can also partly compensate for each other (Simon et al., 2001; Simon et al., 2004).

The Figure 10 summarizes the inductive signals and transcriptional codes involved in the developmental program of mDA neurons. The mDA progenitor domain is defined at early stages of neural development. FGF8/Wnt1-regulated network and Shh-controlled genetic cascade maintain the expression of Otx2 in the ventral midbrain, which in turn represses Nkx2.2 in this domain. The induction of Lmx1a and Msx1 follows and these two transcription factor repress Nkx6.1. Thereby, a $Wnt1^+$, $Otx2^+$, $Lmx1a^+$, $Msx1^+$, but $Nkx2.2^-$ and $Nkx6.1^-$ territory is established in the neuroepithelium of the ventral midbrain from which mDA precursors expressing Aldh1 and Nurr1 develop. Aldh1 is an interesting marker as it was reported as the unique specific marker of proliferating DA precursor cells from E9.5 (Wallen et al., 1999) and of TH-positive mesencephalic neurons (McCaffery and Drager, 1994). The *Aldh1* gene codes for an aldehyde dehydrogenase that is involved in the synthesis of retinoic acid from vitamin A. Failure to establish the transcriptional code results in a fate-switch of mDA precursors into other identities such as serotonergic neurons described below. At intermediate stages of neural development, Lmx1a/Msx1-controlled genetic cascade confers the generic neuronal identity to the precursors by activation of the proneural gene *Ngn2*, and enables specific mDA differentiation by suppression of alternative neuronal fates. The early stage of mDA-specific differentiation (E9-E10 in mice) sees the onset of expression of the first gene for DA synthesis coding for AADC. Subsequently, transcription factors for terminal differentiation are induced: Nurr1 at E10.5 and Pitx3 at E11.5; which are required to induce and sustain the expression of mature mDA neuron markers: TH at E11.5, Vmat2 at E12.5 and DAT at E14.
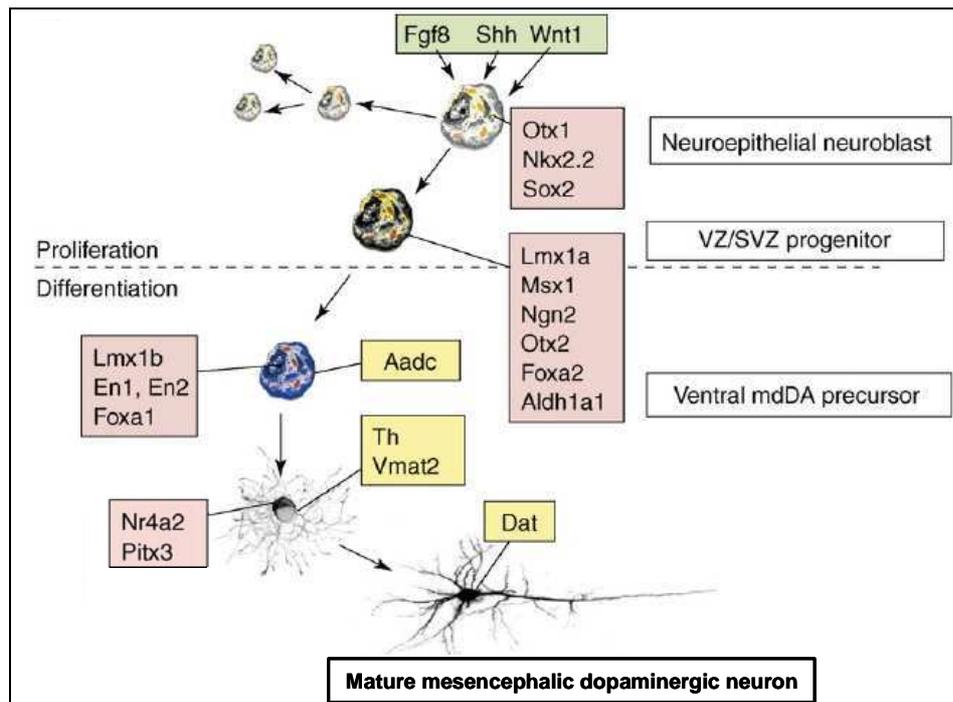
Figure 10: Induction and differentiation of mDA neurons

(adapted from Burbach and Smidt, 2006)

See text for details. Inductive signals are shown on a green background, transcription factors are on pink and enzymes of the DA neuron phenotype are on yellow. Abbreviations: Nr4a2, Nurr1; VZ/SVZ, ventricular/subventricular zones.

II.3 Serotonergic specification

In 1930s and 1940s, the amine serotonin was first extracted from the smooth muscle of rat uterus and first called enteramin, and then from beef serum with its present name serotonin (Rapport et al., 1948). Its presence in mammalian brain was demonstrated in 1953 (Twarog and Page, 1953). The study of central serotonergic system is of great interest because it is believed to be involved in the physiopathology of psychiatric diseases like anxiety disorders, drug addiction and autism (for review see Lucki, 1998). The 5-HT system has also been linked with Parkinson's disease itself. In fact, 5-HT neurons are able to release without control DA from its precursor L-DOPA, increasing L-DOPA induced dyskinesia that is a side effect of the first PD drug (Carlsson et al., 2007).

II.3.1 Serotonergic neuron characteristics and markers

Serotonin or 5-hydroxytryptamine (5-HT) is a biogenic monoamine (see section II.2.1). 5-HT is synthesized in two steps from the essential amino acid tryptophan (Figure 11). The first step is the rate limiting hydroxylation of tryptophan to 5-hydroxytryptophan by

tryptophan hydroxylase (Tph). The second step is the immediate decarboxylation of 5-hydroxytryptophan to 5-HT by a non-specific aromatic amino acid decarboxylase (AADC).
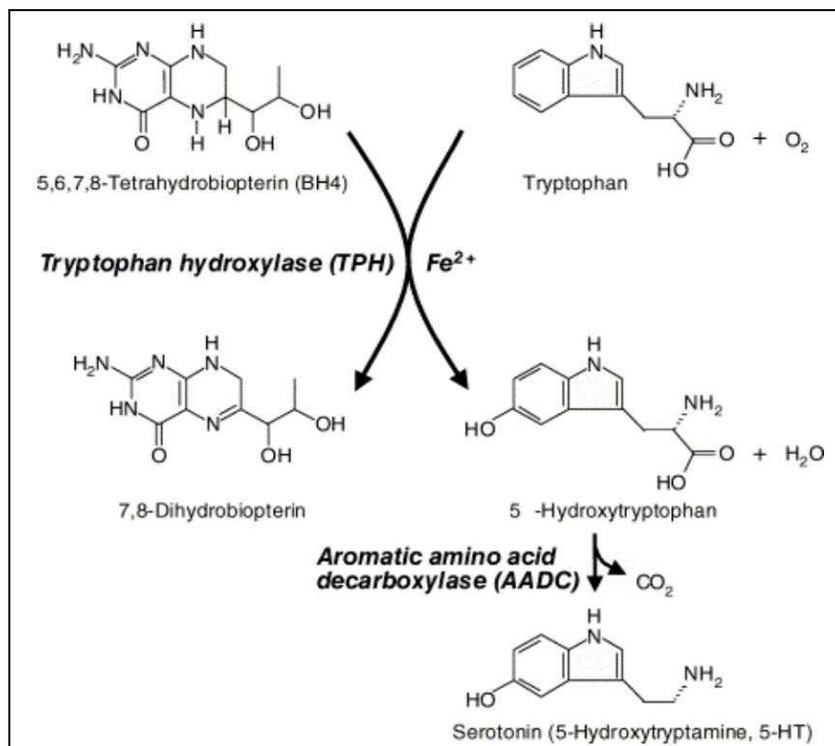
Figure 11: Biosynthesis of serotonin

The generation of the neurotransmitter serotonin in the brain relies on the Tph2 isoform, whereas the isoform Tph1 is present in enterochromaffin cells of the gut for the synthesis of the serotonin outside the brain (Walther et al., 2003; Walther and Bader, 2003). The serotonergic neuron phenotype is further defined by the presence of the non specific Vmat2 (Fon et al., 1997), the specific serotonin transporter (SERT) (Blakely et al., 1991) and different serotonin receptors like the 5-HT2C receptor.

II.3.2   Localization of 5-HT neurons

The neurons that release 5-HT are located in a restricted zone: most are found in the **raphe nuclei**, on the midline of the hindbrain, with a smaller number in the reticular formation. The raphe nuclei serotonergic neurons form two clusters, a rostral with groups B6-B9 innervating the brain, and a caudal with groups B1-B5 innervating the spinal cord (Figure 12). The total number of serotonergic neurons is small but they provide a relatively dense innervation to all the brain areas and the spinal cord by an extensive and diffuse collateralization of their axons. 5-HT neurons are implicated in a wild range of processes, such as mood, vegetative homeostasis, and movement control (Harvey et al., 2004, for review

see Lucki, 1998). Because of their early formation in development and their widespread distribution, 5-HT neurons are also thought to be involved in the modulation of development of other fibers thereby playing a central role in the organization of neurotransmitter networks in the CNS (Lambe et al., 2000).
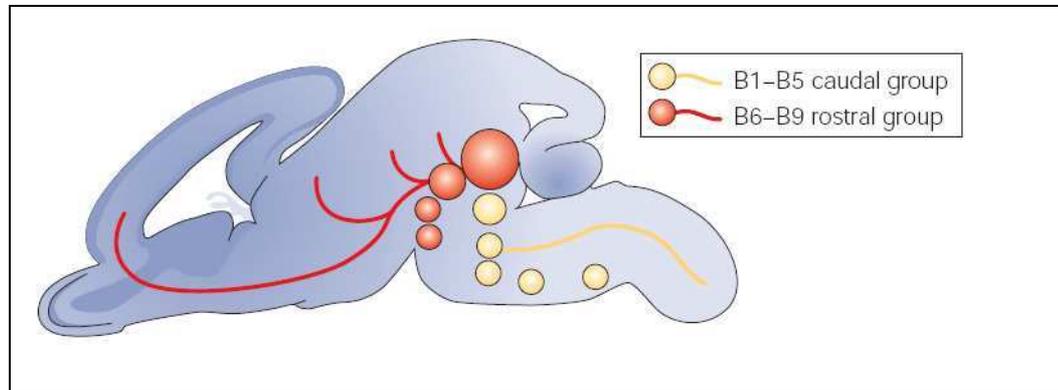
Figure 12: Organization of the 5-HT neurons in the brainstem

(from Gaspar et al., 2003)

II.3.3   Developmental program of 5-HT neurons

Serotonergic neurons are generated early in development (from E10 to E12 in mice), but the full maturation of the axon terminal network requires more time and is achieved only after birth in rodents (Lidov and Molliver, 1982) (for review see Hynes and Rosenthal, 1999; Gaspar et al., 2003; Nakamura et al., 2005; Alenina et al., 2006).

II.3.3.1   Specification of the 5-HT neuronal field

As already discussed in section II.1 and II.2, it is believed that the organizing centers (FP along the D/V axis and IsO along the A/P axis) establish an epigenetic grid of Cartesian coordinates and that neural precursors assume distinct cell fates according to their location on this grid (Ye et al., 1998). mDA and 5-HT neurons occupy adjacent, non-overlapping domains, with mDA neurons rostral to the MHB and 5-HT neurons caudal to it (Figure 6).

The development of 5-HT neurons appears to be regulated along the D/V axis by **Shh**, like in mDA neurons development (see section II.2.4.1). 5-HT neurons can be induced ectopically in explants culture in the dorsal hindbrain (Ye et al., 1998), but not in other locations. *In vivo*, the notochord and the FP, both of which produce Shh, induce the production of 5-HT precursors (Yamada et al., 1991). In addition, all groups of 5-HT neurons fail to develop in the presence of Shh-blocking antibodies (Ye et al., 1998). Thus, Shh

controls the development of 5-HT neurons along the D/V axis of the neural tube, but cannot induce them ectopically along the A/P axis.

Along the A/P axis, the inductive signal by **FGF8** (see section II.2.4.1) also enables the development of rostral, but not caudal, 5-HT neurons, as shown by the blockade of FGF8 that prevent the development of these neurons (Ye et al., 1998). Moreover, animals with severely reduced levels of FGF8 have deficits in the rostral hindbrain (and the caudal midbrain) (Meyers et al., 1998).

In summary, rostral hindbrain 5-HT neurons, like mDA neurons, have been shown to depend on both Shh and FGF8 for their development (Ye et al., 1998). These two molecules, however, cannot induce 5-HT neurons in ectopic locations, suggesting that a third signal may be involved. A candidate for the third signal is **FGF4**, as it induces ectopic 5-HT neurons when added to ventral midbrain explants, a tissue that contains endogenous FGF8 and Shh (Ye et al., 1998). Under these conditions, endogenous mDA no longer develop. These findings suggest that FGF4, in combination with Shh and FGF8, induces 5-HT neurons to develop in the hindbrain, whereas its absence in midbrain tissue, which only contains Shh and FGF8, allows the development of mDA neurons. FGF4 is not localized in the hindbrain, but is highly expressed in the primitive streak, a region juxtaposed to the posterior neural plate during early development (Niswander and Martin, 1992; Ye et al., 1998). It is possible that FGF4 acts to pre-pattern the future hindbrain and that the later exposure to Shh and FGF8 confers a 5-HT phenotype on the precursors (Figure 6).

II.3.3.2 Transcriptional regulation for the differentiation of 5-HT neurons

5-HT neurons are originated from the rhombomeres r1 to r7 of the hindbrain. The development of specific cell types within each rhombomere is time and space specific. In fact, in the r1-r7 zone, branchiomotor and visceromotor precursors are previously produced (before E10) (Pattyn et al., 2003). Exceptions are r1, which never generates motor neurons, and r4, which carries on producing motor neurons and never gets serotonergic (Figure 13). r4 thus separates two zones of 5-HT fate forming the basis for the two clusters of adult 5-HT neurons discussed above (see section II.3.2). [To note: the section II.4 is dedicated to the transcription factor Lmx1b that is not detailed in this part.]
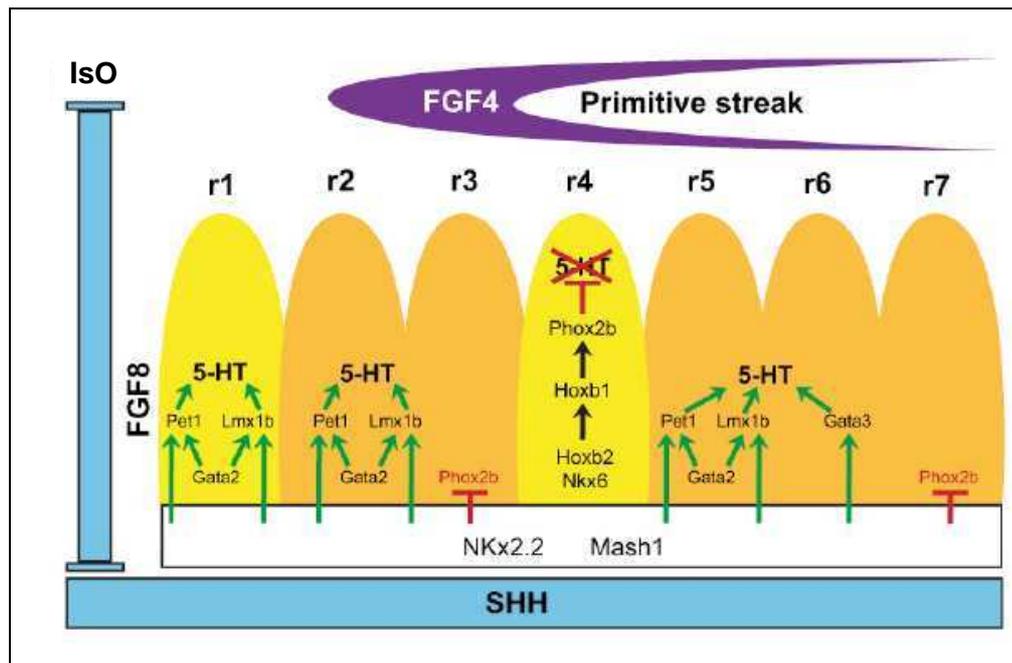
Figure 13: Induction and differentiation of 5-HT neurons *in vivo*

(adapted from Alenina et al., 2006)

See text for details.

En1/En2

The *engrailed* genes are not only involved in mDA neurons development as discussed above, but also in 5-HT neurons development (Simon et al., 2005). However, the effects of the lack of En expression are less severe for serotonergic neurons. Mutant mice for both *engrailed* genes show a specific loss of the rostral part of the raphe nuclei, whereas the caudal part is not affected. *En* genes were also shown to be functionally redundant. In mutant mice, the loss of serotonergic cells appears as early as E11, indicating that *En* genes are necessary for the early proper development of serotonergic neurons, and not only for their survival and maintenance like for the mDA neurons.

Phox2b

Paired-like homeodomain protein (Phox) 2b is a transcription factor repressing serotonergic differentiation in r2-r7. Phox2b-deficient mice lack all visceromotor neuron precursors and serotonergic neurons are extensively produced in r2-r7 instead (Pattyn et al., 2003), confirming that Phox2b is a central repressor of serotonergic fate. The formation of 5-HT neurons is enabled in r2-r3 and r5-r7 through inhibition of Phox2b by NK transcription factor related (Nkx) 2.2, whereas in r4, Hoxb1, Nkx6.1 and Nkx6.2 sustain its expression and thereby block serotonergic differentiation (Samad et al., 2004).

Nkx2.2

Nkx2.2 is expressed transiently in all serotonergic precursors from E10.5 in mice. Mice lacking this factor do not express Gata3, Lmx1b, and Pet1 (see below) in caudal raphe nuclei and no 5-HT neurons develop in this area in contrast to the rostral raphe nuclei where all these factors and 5-HT neurons persist (Briscoe et al., 1999; Ding et al., 2003b; Pattyn et al., 2003). Together with Lmx1b and Pet1, it can induce ectopically the development of 5-HT neurons in the chick neural tube (Cheng et al., 2003).

Mash1

Mouse achaete-scute homolog (Mash) 1 is a bHLH transcription factor, which is already detected in r1-r7 during motor neuron generation but become only essential when 5-HT neurons are developed. In Mash1-deficient mice, no cells expressing the downstream factors Pet1, Lmx1b, Gata2 and Gata3, and also no 5-HT neurons appear (Pattyn et al., 2004). However, Nkx2.2, Phox2b and Shh retain their normal pattern of expression in these mice.

Gata2 and Gata3

Six GATA-motif binding transcription factors (Gata) exist in vertebrates, characterized by C4-type zinc-finger motifs. Two of them, Gata2 and Gata3, are expressed in the developing brain (Patient and McGhee, 2002). Experiments in chicks showed that Gata2 is necessary and sufficient for the induction of Lmx1b, Pet1 and 5-HT neurons in r1, but not more caudally (Craven et al., 2004). In hindbrain explant cultures of Gata2-deficient mice, no 5-HT neurons are developed indicating that Gata2 may be also pivotal for 5-HT differentiation in general. In contrast, Gata3 is not required for the differentiation of the rostral 5-HT neurons (van Doorninck et al., 1999) and appears unable to substitute for the loss of Gata2 in r1. However, in Gata3-deficient mice, around 80% of 5-HT neurons in the caudal cluster and 30% in the rostral one are missing (Pattyn et al., 2004). Nevertheless, the expression of Pet1 and Lmx1b was unchanged in Gata3 KO mice showing that these factors act in parallel.

Pet1

The transcription factor Pet1 is a specific marker for all 5-HT neurons from E11 (in mice) until adulthood (Hendricks et al., 1999). This specificity was confirmed by the use of the Pet1 promoter to target marker genes exclusively to 5-HT neurons in transgenic mice (Scott et al., 2005). Pet1 binding sites are found in the promoter regions of several genes

expressed in 5-HT neurons such as the ones coding for AADC and SERT (Hendricks et al., 1999). In mice lacking Pet1, 70% of 5-HT neurons fail to differentiate, whereas in the remaining Pet1-deficient neurons diminished expression of Vmat2, Tph2 and SERT was observed (Hendricks et al., 2003). These animals survive but show anxiety-like and aggressive behavior.

To summarize the action of the transcription factors involved in serotonergic differentiation, 5-HT neurons are produced in rhombomeres r1-r7, except in r4 due to the persistence of the expression of Phox2b (Figure 13). In all other rhombomeres, except r1 where it is never expressed, Phox2b is switched off at E10.5 by Nkx2.2. Mash1 and Nkx2.2 activate the transcription factors Gata2, Gata3, Lmx1b and Pet1, which together define the serotonergic cell type by activating marker genes such as the ones coding for Tph2, AADC, SERT and Vmat. Thereby, Gata3 is only essential for the development of the caudal cluster but not the rostral one, and the function of Pet1, which is nevertheless expressed very specifically in all 5-HT neurons and until adulthood, is partially redundant since Pet1-deficient mice still retain about 30% of 5-HT cells. Thus, only Mash1, Lmx1b and probably Gata2 are indispensable for all 5-HT neurons to develop properly.

II.4   The transcription factor Lmx1b is involved in both dopaminergic and serotonergic development

Lmx1b is a LIM (for Lin11-Isl1-Mec3) homeodomain transcription factor characterized by two zinc-finger domains at the NH2-terminus, a central DNA-binding homeodomain, and a putative transcriptional activation domain at the COOH-terminus (Curtiss and Heilig, 1998) (Figure 14). Lmx1b is highly conserved among vertebrates in terms of sequence similarities and functions. In Table 2, the multiple roles of Lmx1b in vertebrate development are summarized. *Lmx1b* was reported (for review see Dai et al., 2009) as a key gene for dorsoventral patterning of vertebrate limb (Vogel et al., 1995), formation of the skull (Chen et al., 1998b), differentiation and functioning of kidney podocytes (Chen et al., 1998a) and development of anterior-derived tissues in the eye (Pressman et al., 2000). Moreover, Lmx1b mutations in humans cause an autosomal dominant inherited disease called nail-patella syndrome (NPS), which is characterized by abnormalities of the arms and legs as well as kidney disease and glaucoma (Vollrath et al., 1998; Dreyer et al., 2000).
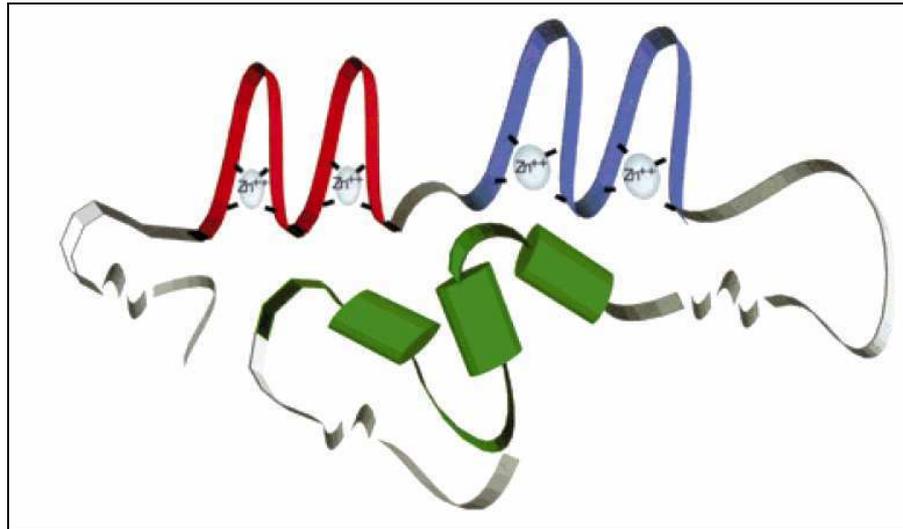
Figure 14: Configuration of a LIM homeodomain protein

(from Curtiss and Heilig, 1998)

Zinc-finger domains (LIM1 and LIM2) are depicted in red and blue, and homeodomains are in green. The broken gray lines indicate that the length of these regions vary between different proteins.

Lmx1b also plays important roles in the development of the CNS ranging from regulating early regionalization of the neural tube, to controlling the differentiation and maintaining the survival and function of several types of neurons including dopaminergic and serotonergic neurons. The roles of Lmx1b in dopaminergic AND serotonergic differentiation programs are developed below (Figure 15).

Region	Localization of *Lmx1b*	Normal functions of *Lmx1b* in vertebrate development	Phenotype of *Lmx1b* mutation
Limb	Dorsal mesenchyme of developing limbs	I. Critical for the specification of dorsal limb cell fates and consequently dorsoventral patterning of limbs	I. Loss of dorsoventral patterning of limbs in *Lmx1b⁻/⁻* mice
		II. Specify the trajectories of spinal motor neurons into dorsal limb	II. *LMX1B* mutations in human cause NPS characterized by developmental defects in dorsal limb structures
Skeleton	Developing skull and mesenchyme	Molecular mechanism is unknown	I. Hypoplastic or absent patellae and severe joint abnormalities in *Lmx1b⁻/⁻* mice and NPS patients
			II. Calvaria bones are missing or severely reduced, and the sutures are severely anomalous in *Lmx1b⁻/⁻* mice
Eye	Anterior portion of developing eye	Regulating development of anterior portion of eye, while the mechanism is unclear	I. Abnormal development of the anterior compartment of eye in *Lmx1b⁻/⁻* mice
			II. Ocular malformations including microcornea and sclerocornea in NPS patients
Kidney	Glomerulus	Required for the initial differentiation and the maintenance of podocytes	I. Defective differentiation of podocytes in *Lmx1b⁻/⁻* mice
			II. Proteinuria and end-stage renal failure in 30% of NPS patients
Brain	Isthmus, midbrain dopaminergic neuron, serotonergic neuron, glutamatergic neurons in dorsal horn neuron	I. Essential for the inductive activity of isthmic organizer and consequently mid/hindbrain development	I. Severe reduction of the tectum and cerebellum in *Lmx1b⁻/⁻* mice
		II. *Lmx1b* is not required for differentiation of midbrain dopaminergic neurons	II. Loss of midbrain dopaminergic neurons in *Lmx1b⁻/⁻* mice is due to the disruption of inductive activity of isthmic organizer?
		III. Essential for differentiation and survival of central 5-HT neurons	III. Loss of central serotonergic neurons in *Lmx1b⁻/⁻* or Pet1-Cre; *Lmx1b* conditional knockout mice
		IV. Required for differentiation and migration of spinal dorsal horn neurons	IV. No synaptic contacts is formed between primary nociceptive afferents and dorsal horn neurons in *Lmx1b⁻/⁻* mice

Table 2: Multiple roles for Lmx1b in vertebrate development

(adapted from Dai et al., 2009)

II.4.1   Lmx1b in the dopaminergic differentiation

Lmx1b is highly related to another protein that plays a critical role in DA neurons development: Lmx1a (see section II.2.4.2). They display a 61% amino acid identity (Hobert and Westphal, 2000).

As already discussed in section II.2.4.1, it has been shown that Lmx1b is first essential early in development for the inductive activity of the IsO (O'hara et al., 2005; Guo et al., 2007). Lmx1b is then expressed broadly in the midbrain, and not only in DA precursor cells (Asbreuk et al., 2002). At the stage of DA precursors' specification, Lmx1a siRNA experiments (Andersson et al., 2006b) provided the evidence that Lmx1b cannot compensate for the loss of the structurally related Lmx1a in the specification of DA neurons in chick embryos. However, in *dreher* mutant mice (Lmx1a KO mice), even if the number of DA neurons is reduced, the phenotype is not as severe as in chick embryos and most DA neurons differentiate normally (Ono et al., 2007). These results suggest the existence of compensating factor(s) of Lmx1a loss in mice. Because *Lmx1a* and *Lmx1b* are homolog genes and are coexpressed in the DA precursors, it is possible that they act in the DA specification in a combinational manner, and that Lmx1b can partially compensates for Lmx1a loss (Chung et al., 2009).

Other experiments with *dreher* mutant mice have shown that Lmx1b has some functional redundancy to Lmx1a. In *dreher* mice, Lmx1b, which is normally not expressed in roof plate progenitors, is able to partially rescue roof plate development (Chizhikov and Millen, 2004a).

Lmx1b is later specifically expressed in DA neurons (Smidt et al., 2000). Lmx1b KO mice express Nurr1 (from E10.5 in mouse) and TH at early time points, but fail to express the mDA neuron marker Pitx3, a paired-like homeodomain protein, and the mDA population is lost after birth (Smidt et al., 2000), suggesting that Lmx1b is involved in the maintenance of the DA phenotype. Whether this defect is due to cell-autonomous Lmx1b activity in the mDA neurons, or to a consequence of alterations at the MHB where Lmx1b is present at earlier stage is not sure. But the absence of Pitx3 expression in the *Lmx1b*-null mutants was interpreted to suggest that Lmx1b may be a transcriptional activator of the *Pitx3* gene, thus defining a second pathway in mDA cell fate specification which acts independently of the Nurr1-dependent neurotransmitter specifying pathway (Smidt et al., 2000). New findings have shown that these pathways are interconnected (Jacobs et al., 2009).

To finish, Lmx1b also continues to be expressed in mDA neurons through the adulthood (Asbreuk et al., 2002). However, no study has been reported so far using mDA neuron-

specific Lmx1b deletion in postnatal brain, and thus little is known about the function of Lmx1b in the adult brain.
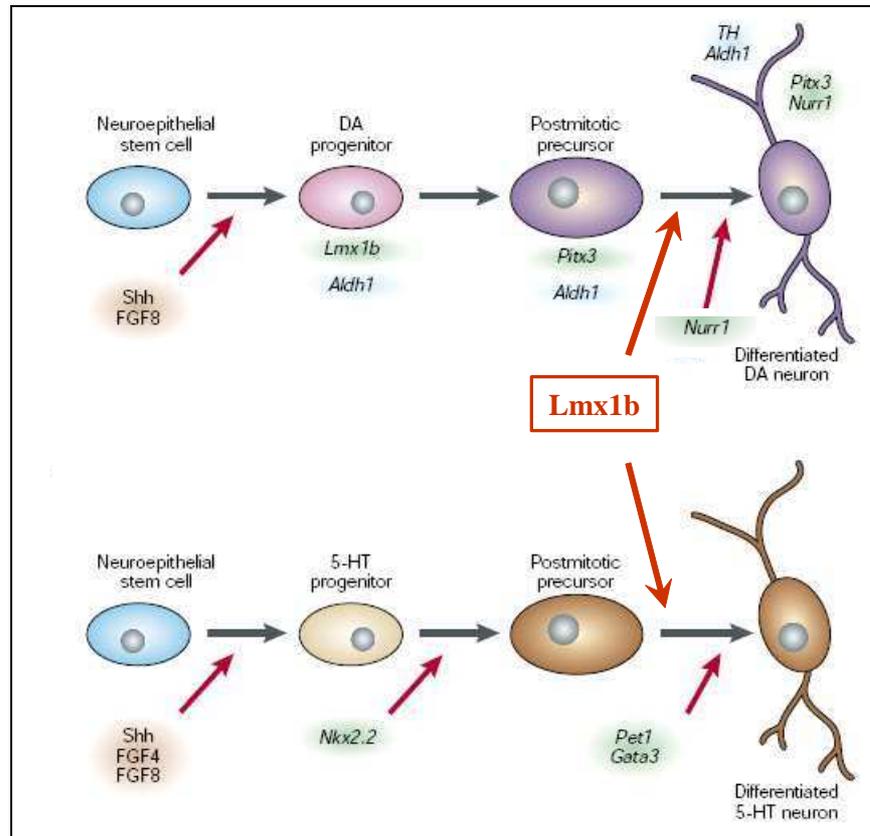
Figure 15: Lmx1b in the generation of DA and 5-HT neurons
(adapted from Goridis and Rohrer, 2002)

II.4.2   <u>Lmx1b in the serotonergic differentiation</u>

Lmx1b is required for the formation of the entire serotonin system in the hindbrain: its deletion in mice leads to the absence of 5-HT neurons in the brain (Briscoe et al., 1999; Cheng et al., 2003; Ding et al., 2003b). It is expressed in the developing serotonergic neurons together with Pet1 starting around E11 (in mice) in the rostral 5-HT cluster, and one day after in the caudal one consistent with the delayed appearance of 5-HT neurons in the latter region (Cheng et al., 2003). Its ablation does not affect the expression of Nkx2.2, Gata3 and Shh, and only partially the one of Pet1, putting these factors upstream or in parallel to Lmx1b (Cheng et al., 2003; Ding et al., 2003b). However, conditional deletion of Lmx1b at later stage of development showed that Pet1 expression is dependent of Lmx1b expression (Zhao et al., 2006). Although initiation of Pet1 expression is independent of Lmx1b (Cheng et al., 2003), Lmx1b seems to be required for Pet1 maintenance but does not require Pet1 for its own maintenance (Zhao et al., 2006). In conclusion, Lmx1b is essential not only for the generation

of 5-HT neurons but also for maintaining their differentiation and survival during postnatal development.

The specification mechanism towards DA neurons has been extensively studied, mostly due to their implication in PD burden, about which the public is well aware now. The specification mechanism towards 5-HT neurons was not as well studied so far, but becomes more and more of interest because of the wide range of action of serotonergic system. Despite specification mechanisms are not fully understood yet, they help a lot to establish and improve specific differentiation protocols to obtain DA and 5-HT neurons from mouse and primate ESC *in vitro*.

III DIFFERENTIATION OF ESC TOWARDS THE NEURAL LINEAGE: focus on dopaminergic and serotonergic neuronal phenotypes

In 1981, Dr. Martin and colleagues have already demonstrated the ability of mouse ES cells to spontaneously differentiate towards many cell types *in vitro*, including neurons (Martin et al., 1981). Differentiation of mouse and primate (human and monkey) ES cells towards the neural lineage is constituted by progression through stages resembling embryonic neurogenesis and results in the generation of different neuronal subtypes, astrocytes and oligodendrocytes (Figure 16). Whereas the generation of neurons among a mixed population of cells is easily obtainable, their directed differentiation towards specific neuronal lineages remains more challenging. This can be achieved through modulation of the extracellular and/or intracellular environment that will be discussed in section III.2 with a particular interest for dopaminergic and serotonergic differentiation patterns (for review see Perrier and Studer, 2003; Schwartz et al., 2008; Suter and Krause, 2008; Erceg et al., 2009).
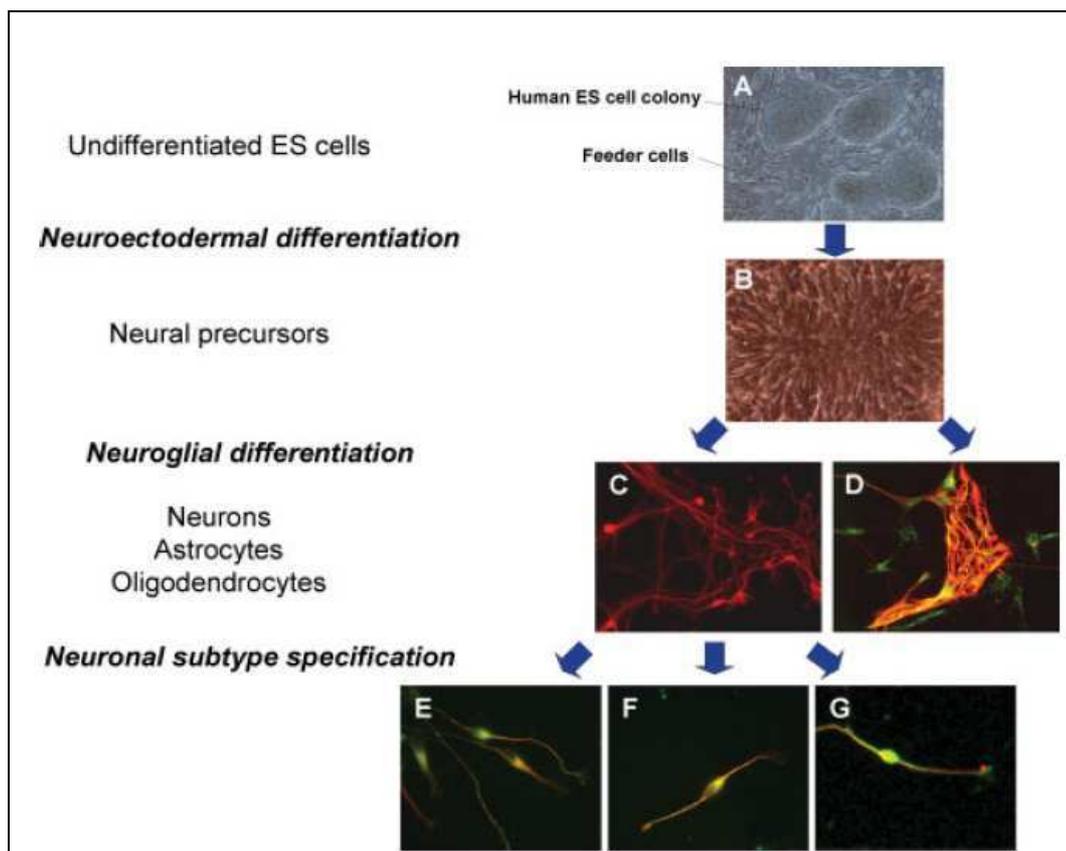
Figure 16: Neural differentiation steps of human ES cells

(from Suter and Krause, 2008)

Differentiation of ES cells (A) towards neurons involves two major steps. The first step is induction of neuroectodermal differentiation. Neuroectoderm is characterized by a columnar shape and later by rosette-like structures

resembling neural tube (B). These neural progenitors will then further differentiate into neurons, which express markers such as β–tubulin (C, red), but also into astrocytes expressing glial fibrillary acidic protein (GFAP) (D, red) and oligodendrocytes expressing cyclic nucleotide phosphodiesterase (CNPase) (D, green). Mature neurons are further specified into diverse neuronal subtypes: glutamatergic (E), GABAergic (F) or dopaminergic (G) neurons for instance.

III.1 From ESC to neural differentiated cells in three stages

Independently of the specie, ESC differentiation towards the neural lineage can be divided in three major steps: (1) induction of differentiation (EB formation for instance), (2) differentiation of primitive neuroepithelial cells (generation of rosettes or neuroepithelial colonies) followed by amplification of neural precursors/neural stem cells, and (3) generation of phenotype-specific differentiated cells (mature neurons like dopaminergic or serotonergic neurons for instance).

III.1.1 Step 1: Induction of differentiation

The most widely used method for inducing differentiation of ESC is to enzymatically or mechanically lift them and place them into low-adherence culture dishes without substrate, feeder cells or mitogens. The result is the formation of floating, spherical clusters of cells known as embryoid bodies (EB) (Figure 2). EB have been shown to be constituted by cells of all three germ layers (Doetschman et al., 1985; Tropepe et al., 2001; Yan et al., 2005).
Non-EB approaches have also been used (Figure 2). One way to direct differentiation is to co-culture ESC with stromal cells (see section III.2.1.4). Some cell lines have been found to produce factors that enhance a specific lineage phenotype like DA lineage (Kawasaki et al., 2000; Kawasaki et al., 2002). This method and the influence of stromal cells upon ESC are called stromal cell-derived inducing activity (SDIA). Another way is the use of specific growth factors and/or antagonists to accelerate differentiation towards one lineage of interest in adherent monolayer culture without support cells (Ying et al., 2003b). This induction step duration varies with the species: from 4 days with mESC derivatives till 10 days with hESC derivatives.

III.1.2 Step 2: Neural induction

The EB are differentiated to neuroepithelial cells in simple, serum-free culture media. EB are first plated onto laminin or poly-D-lysine (PDL)-laminin coated dishes to generate an adherent culture. Neural induction of EB gives formation of clusters of small and columnar cells surrounding a central cell-free zone. These neural rosettes (Figure 16), which resemble the morphology of the very early neural tube in cross-section, express early neural markers

such as Nestin and Musashi-1 (Pankratz et al., 2007; Elkabetz et al., 2008). In case of induction of differentiation by non-EB approaches, neural rosettes for human and monkey ESC derivatives, or neuroepithelial colonies for mouse ESC derivatives, are also generated on stromal cells or on monolayer culture. The typical yield of neuroepithelial cells, defined by immunostaining for the neuroepithelial transcription factors Pax6, Sox1 and Sox2, is about 90% of the total cells constituting the rosettes/colonies (Pankratz et al., 2007; Elkabetz et al., 2008). At this stage, early markers of neural cells can also be detected: the intermediate filament protein Nestin and the binding protein Musashi-1 (Okabe et al., 1996; Schwartz et al., 2008). The formation of rosettes allows control of developmental stages and generation of primitive neuroepithelial cells which can be further induced to neuronal or glial progenitors with forebrain, mid/hindbrain, and spinal cord identities.

Rosettes or neuroepithelial colonies can be maintained and expanded in culture in the presence of standard medium containing growth factors like bFGF (see section III.2.1.2), which is the predominant mitogen of choice (Pankratz et al., 2007). Epidermal growth factor (EGF) and ascorbic acid (AA, vitamin C) have also been used in combination with bFGF. hESC-derived rosettes are typically expanded over a 7- to 10-day period and neuroepithelial colonies (for mouse) over a 3- to 5-day period prior to their isolation and plating for induction of terminal differentiation (Barberi et al., 2003; Elkabetz et al., 2008).

At this point, it is interesting to mention another protocol that has been widely used to isolate and expand in culture neural stem cells from mouse brain of different ages. When culture from brain tissue dissection is under appropriate conditions and density, continued cell division generates non-adherent spherical clusters of cells, commonly referred to as neurospheres (Reynolds and Weiss, 1992; Pacey et al.). These spheres can be further dissociated and the neural stem cells obtained can be expanded in culture or differentiated depending on the conditions.

III.1.3 Step 3: Neuronal (or glial) differentiation

The primitive neuroepithelial cells from rosettes are plated on an adhesive substrate like PDL-laminin, polyornithine (PLO)-laminin, fibronectin (FN) or Matrigel, an extracellular matrix protein-rich basement membrane. The media used are serum-free and supplemented with defined combination of growth factors depending on the phenotype of interest (see section III.2 for more details). The mitogen bFGF is used in the first part of the neuronal or glial differentiation, and is then withdrawn for inducing the terminal differentiation. βIII-

tubulin protein (TuJ1) and microtubule-associated protein (MAP) 2 are mature neuron specific markers (Okabe et al., 1996; Schwartz et al., 2008).

III.2 Directed differentiation of ESC through modulation of the extracellular and/or intracellular environments

Extracellular factors, such as growth factors, extracellular matrix components or contact with other cell types, act on ES cells through interactions with cell membrane components. These interactions trigger intracellular signaling cascades and result in the modification of the cell fate. Alternatively, the intracellular environment can be directly modified through transgenic expression of proteins, or by the addition to the culture medium of cell-permeant proteins or small organic molecules, which will bind to intracellular targets to modify their activity. The modifications of the extracellular or intracellular environments exposed below are a non-exhaustive list of all the methods currently used to study or orient the ESC differentiation.

III.2.1 <u>Extracellular environment</u>

III.2.1.1 Supplements

Maintenance of ESC in an undifferentiated state is generally performed in the presence of feeder cells, except for some mESC lines, and either serum or serum replacement. However, as neuronal differentiation protocols are performed under serum-free conditions (Ying et al., 2003b), it is necessary to add supplements.

The N2 supplement included in most neuronal differentiation protocols at step 3 (see section III.1.3), consists of five molecules: transferrin, insulin, putrescine, progesterone and selenite. These molecules confer a growth advantages to neurons (Skaper et al., 1979) or even affect neural cell fate decisions. Transferrin is essential for both proliferation of neuronal progenitors and survival of mature neurons (Aizenman and de Vellis, 1987). Insulin is essentially required for progenitor proliferation (Aizenman and de Vellis, 1987). Putrescine is crucial for axonal generation or regeneration (Gilad et al., 1996; Cai et al., 2002), and shows neuroprotective properties (Harada and Sugimoto, 1997). Progesterone is present at nanomolar concentrations in N2-containing medium, such concentrations have been shown to confer neuroprotection *in vitro* (Nilsen and Brinton, 2002). Sodium selenite is a trace metal and a component of selenoproteins, which promote neuronal survival in culture (Yan and Barrett, 1998).

Another widely used supplement at step 3 is B27 (Brewer, 1995). B27 contains over 20 components, among which are vitamins, hormones and growth factors (including transferrin and insulin), antioxidants and fatty acids. It is not yet well known if antioxidants are only neuroprotective or might impact on differentiation processes; the same for fatty acids as nutritional elements.

Some studies use the so-called insulin/transferrin/selenium/fibronectin (ITSFn) medium after the re-attachment of EB at step 2 for effective selection of neural lineage cells (Okabe et al., 1996; Lee et al., 2000). The selected cells are positively stained with an antibody against intermediate filament protein Nestin, marker of neuroepithelial cells.

III.2.1.2 Growth factors and hormones

Work done with mESC demonstrated that neuronal differentiation was enhanced in serum-free media (Okabe et al., 1996; Ying et al., 2003b), suggesting the presence of inhibitory signals in the serum such as BMP family members. In monolayer culture, low cell density was also found to be critical, suggesting the presence of paracrine factors inhibiting neuronal differentiation (Ying et al., 2003b). The approach of Dr. Ying and colleagues is based on the elimination of any inductive signals for alternative fates. Under their conditions, bFGF was crucial for proper neuronal differentiation of ESC (step 3). bFGF is widely used and is present in most culture protocols, but it is generally not included for the initiation of neural induction (step 1 to step 2). Noggin, a BMP antagonist, has also been successfully used by several groups to direct hESC towards the neuronal lineage (Tropepe et al., 2001; Itsykson et al., 2005).

Apart from bFGF and Noggin, most other factors that have been used in differentiation also have a strong influence on the type of neuroectoderm induced, and therefore limit the potential of the resulting cells. For example, retinoic acid (RA), in addition to increase the number of neurons compared to other cell types (Bain et al., 1995; Bain et al., 1996), was shown to have a caudalizing effect on neuroectoderm (Zhang, 2006). Therefore, only some subsets of neurons, such as motor neurons (Lee et al., 2007), can be efficiently generated with RA use. On the contrary, RA induction gives only 2% or less of 5-HT neurons (Alenina et al., 2006).

Other growth factors, such as Shh, FGF8, brain-derived neurotrophic factor (BDNF, member of nerve growth factor protein family), GDNF, or AA (Kalir et al., 1991; Lee et al., 2000), have been used more precisely for the specification of DA neuronal subtype (Shh, FGF8) and during later stages of DA differentiation (BDNF, AA, GDNF) (Barberi et al., 2003; Perrier et

al., 2004; Takagi et al., 2005). BDNF was also shown to increase the amount of 5-HT neurons in primary culture from E13 mouse raphe nuclei (Djalali et al., 2005), thus this factor might be useful for ESC differentiation towards 5-HT neurons.

In conclusion, different types of neurons can be generated using specific combinations of soluble molecules: Shh and FGF8 for DA neurons specification; Shh and FGF4 for 5-HT neurons specification (Ye et al., 1998; Kumar et al., 2009) for example. We have discussed in section II of the role of these last factors in DA and 5-HT neuron specifications.

III.2.1.3 Extracellular matrix

It has been shown that the contact of ESC with different components of the extracellular matrix can alter their differentiation program (Philp et al., 2005; Chen et al., 2007). For example, PLO-laminin coating enhances neuronal differentiation from ESC as compared to plastic or other substrates (Goetz et al., 2006). In contrast, gelatin appears to favor glial commitment. In these studies, it appeared that in addition to the binding of specific cell surface receptors, mechanical factors linked to cell attachment could also contribute to cell differentiation: laminin has shown a certain degree of specificity for the attachment of neural cells (Goetz et al., 2006). Interestingly, laminin is the major component of Matrigel, a commercial substrate also widely used in neuronal differentiation protocols.

The most convincing studies showing extracellular matrix-mediated differentiation of ESC used a complex matrix isolated from human amniotic membrane (Ueno et al., 2006). This matrix not only induces neural differentiation but also favors forebrain and midbrain phenotypes by unknown signals. The use of extracellular matrix components to direct neuronal differentiation from ESC remains largely unexplored.

III.2.1.4 Co-culture with feeder cells

Co-culture with stromal cells has been proved efficient not only for the maintenance of the undifferentiated state of mouse and human ESC but also for promoting neuronal differentiation at steps 1-2 (Barberi et al., 2003; Takagi et al., 2005). Several cell lines have been used for this purpose.

The first one to be reported is the PA6 stromal cell line (Kawasaki et al., 2000), isolated from murine skull bone marrow (Kodama et al., 1986). Strong neuronal induction for both mouse (Kawasaki et al., 2000) and primate (Kawasaki et al., 2002; Takagi et al., 2005; Vazin et al., 2008) ESC was reported, with a marked improvement of dopaminergic differentiation. This activity was maintained even if the stromal cells were fixed with paraformaldehyde,

suggesting that the SDIA effect was partially due to interaction with membrane-bound molecules.

The murine marrow stromal cell line MS5 has also been used successfully as feeder cells to promote neuronal differentiation of mouse and human ESC (Barberi et al., 2003; Perrier et al., 2004). Efficient differentiation towards different neuronal subtypes (DA, 5-HT, etc) as well as astrocytes and oligodendrocytes was achieved by combining co-culture with MS5 and the addition of soluble molecules (mentioned in the section III.2.1.2) (Figure 17).
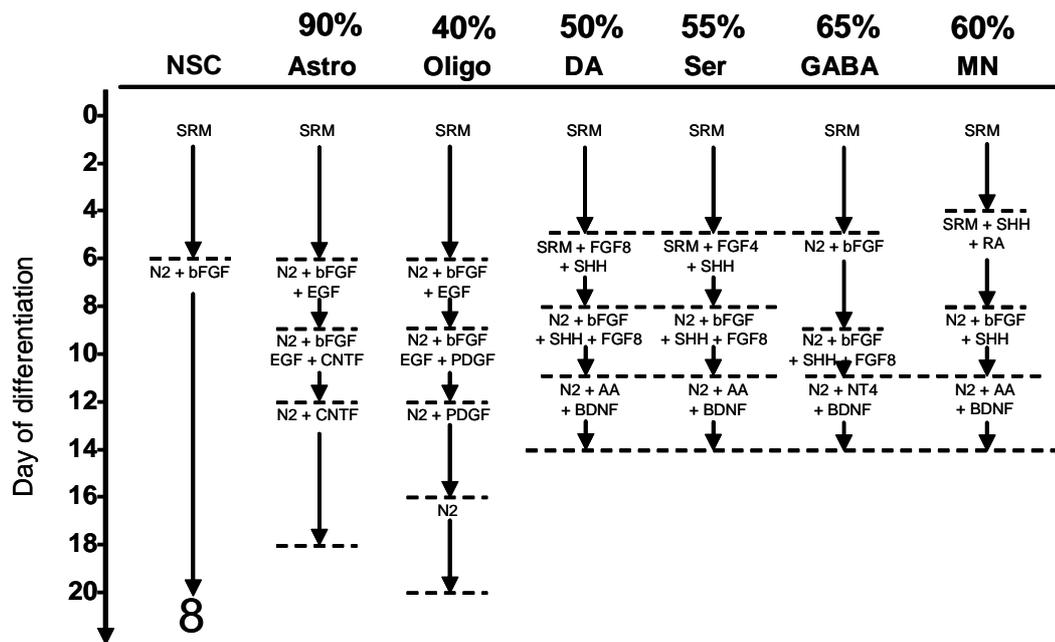
Figure 17: Schematic representation of culture conditions for mESC differentiation

(adapted from Barberi et al., 2003)

The temporal sequence of media and growth factors is listed for the derivation of specific neural subtypes from mESC. Neural induction was done by co-culture of mESC on MS5 stromal cells in serum replacement medium (SRM). Approximate proportions of specific phenotypes obtained with these conditions are indicated above the table (expression of specific markers in percent of the total number of neurons defined by the expression of β–tubulin). In the defined conditions for DA differentiation, 25% of the obtained neurons are serotonergic. Abbreviations: NSC, neural stem cells; Astro, astrocytes; Oligo, oligodendrocytes; DA, dopaminergic neurons; Ser, serotonergic neurons; GABA, GABAergic neurons; MN, motor neurons; SRM, serum replacement medium; CNTF, ciliary neurotrophic factor; PDGF, platelet-derived growth factor.

Co-culture of primate ESC with Sertoli cells, which secrete GDNF normally, has also shown potent neuronal induction, especially towards the DA phenotype (Yue et al., 2006). For DA development *in vivo*, GDNF is thought to protect and to mediate the trophic action of striatal target on SNc neurons (Lin et al., 1993; Tomac et al., 1995).

Another original study was performed by Dr. Roy and colleagues. They reported that culture of hESC derivatives at step 3 with telomerase-immortalized human midbrain astrocytes enriches the final population of neurons in DA neurons (Roy et al., 2006).

Interactions between developing neurons and supportive astrocytes were not elucidated but they are thought to protect the developing neurons, and more importantly to recreate the *in vivo* cellular environment.

Therefore, many cell types seem to have the ability to direct neuronal differentiation from ESC. Some lines appear to promote differentiation towards specific neuronal lineages (Kawasaki et al., 2000; Kawasaki et al., 2002; Park et al., 2005; Yue et al., 2006), whereas others do not by themselves (Barberi et al., 2003).

III.2.1.5 Mechanical and physicochemical factors

Factors such as pH, oxygen tension, osmolarity, temperature and mechanical stress have to be further explored to potentially modulate ESC differentiation. For example, serotonergic neurons from the raphe nuclei have been shown to be sensitive to culture medium pH and carbon dioxide (CO_2) (Wang et al., 2002). Oxygen tension also plays a role in the differentiation of ESC with high oxygen concentrations favoring the maintenance of the undifferentiated state (Kurosawa et al., 2006). Another recent study reported that neuronal differentiation of mESC could be enhanced by electrical stimuli (Yamada et al., 2007).

III.2.2 Intracellular environment

III.2.2.1 Transgenesis and lineage selection

Stable expression of transgenes

Introduction of transgenes in ESC has been a technical challenge for a long time. To generate ESC lines stably expressing a transgene, electroporation of plasmidic DNA is still widely used (Tompers and Labosky, 2004). A more convenient approach is the use of viral vectors to introduce one or several transgene and obtain stable ESC lines. In these respects, lentiviral vector constructions (Ben-Dor et al., 2006; Suter et al., 2006) are now the best option as they demonstrate the ability to carry ubiquitous or tissue-specific expression modules, allowing action on ESC fate at a given stage of differentiation. Retroviral vectors were the first used to achieve stable expression of transgenes in ESC (Grez et al., 1990). However gene silencing during expansion and differentiation of the infected cells, and some promoters' incompatibility, are major limitations of their use (Cherry et al., 2000). These phenomenons are less observed with lentiviral vectors, which are now self-inactivating as well (Zufferey et al., 1998). Bicistronic lentiviral vectors enable to concentrate the successfully

infected cells by coexpression of a gene of interest and a selection marker under the control of the same promoter (Richard et al., 2003).

Different transgenesis strategies can be used to enhance the yield of ESC-derived neurons. First, transgenesis may allow redirection of ESC differentiation through expression of transcription factors involved in cell fate decisions. Dr. Zhao and colleagues (Zhao et al., 2004b) expressed SoxB proteins in mESC and observed the promotion of ectodermal differentiation at the expense of mesodermal and endodermal differentiation. Over-expression of transcription factors can also direct ESC towards a specific neuronal phenotype. Dr. Chung and colleagues (Chung et al., 2002) have shown that over-expression of Nurr1 promotes DA differentiation of mES: four to five times more DA neurons were generated and these results were even greater with addition of Shh and FGF8. The same team also reported that Pitx3 over-expression in mESC facilitates the differentiation towards the DA lineage (Chung et al., 2005).

The limitations of such approaches are that most transcription factors should be present only during a defined developmental window and that infection of differentiating cells are not always successful. One option for infection at the undifferentiated stage is the use of promoters or enhancers active only at a particular stage of differentiation. Stably transformed mESC using a Nestin enhancer-driven expression vector to direct Lmx1a expression to the neural progenitor stage of ESC differentiation have thus been generated (Friling et al., 2009). These Lmx1a-transfected cells gave rise to 75-95% of mDA neurons under permissive culture conditions, with the addition of Shh and FGF8.

<u>Inducible expression of transgenes</u>

Another possibility is to use inducible gene expression systems to allow the temporal control of transgene expression. Such strategies should allow the physiological expression pattern of transcription factors to be mimicked more closely.

The most commonly used system for conditional gene expression in mESC is the tetracycline-dependent (Tet) system (for review see Lewandoski, 2001). This system has also been adapted to primate ESC (Adachi et al., 2006; Zhou et al., 2007). The Tet-Off and Tet-On systems offer a strict temporal on/off regulation and a cell type- or tissue-specific control of expression. The target transgene containing the Tet operator sequence is previously integrated into the genome. For the induction (Tet-On) or suppression (Tet-Off) of transgene expression, the inducer tetracycline or its analog doxycycline (Dox) has to be continuously administered.

Another conditional control of transgene expression is possible through site-specific DNA recombination by the action of the recombinases Cre from bacteriophage P1 and Flp from *Saccharomyces cerevisiae* (Sauer and Henderson, 1989; O'Gorman et al., 1991). The Cre and Flp recombinases catalyse a DNA recombination between two recognition sites, loxP and Flp recombinase target (FRT) respectively. The process is the same for both recombinases and leads to the deletion or inversion, depending on the recognition sites orientation, of a DNA sequence between the two sites in the target transgene. This strategy is used to initiate the transcription of the gene of interest by removing a "stop" cassette for instance.

The Cre/loxP system is the most widely used. There are several methods to deliver or activate the Cre recombinase, among them the activation of the Cre-ERT2, the Cre recombinase fused to a mutated ligand binding domain of the oestrogen receptor, by the addition of Tamoxifen or hydroxy-tamoxifen (OHT). This approach has been successfully used in mouse and human ESC and their differentiated derivatives (Vallier et al., 2001; Vallier et al., 2007).

Selection

A different approach to enrich for a specific cell population is to express a selection marker under the control of cell type-specific promoter. GFP expression controlled by the α–tubulin promoter (Schmandt et al., 2005) allows sorting of early neurons. To select more differentiated cells, the same approach could be used. The Pitx3-eGFP (enhanced GFP) knock-in mES cell line has thus been differentiated and selection of eGFP positive cells by fluorescence activated cell sorting (FACS) has enabled to enrich the DA neuron population to more than 90% (Hedlund et al., 2008).

A major limitation of the previous technique is the poor survival of some cell types after sorting. An alternative approach consists in the expression of an antibiotic resistance using a cell type-specific promoter to allow the selective exclusion of undesired cells. This has been done for the purification of mESC-derived cardiomyocytes by puromycin resistance (Kolossov et al., 2006). This could also allow the purification of neural precursors at a defined stage of differentiation like in the study of Dr. Li and colleagues: non-neural cells were selected out by addition of geneticin (G418) on a population of cells in which geneticin resistance was previously introduced into the *Sox2* gene by homologous recombination (Li et al., 1998).

III.2.2.2 Second messengers

Second messengers are interesting candidates to modulate ESC differentiation. The cyclic adenosine monophosphate (cAMP) analog, dibutyryl cAMP (dbcAMP), enhances *in vitro* DA differentiation from mESC (Branton et al., 1998; Lee et al., 2000). Calcium and reactive oxygen species (ROS) are also thought to play important roles during ESC differentiation, even though they have to be further investigated. As an example in non-neuronal differentiation, a decrease in extracellular calcium impairs differentiation towards cardiomyocytes (Li et al., 2002).

III.2.2.3 Cell-permeant proteins

Recombinant proteins can be engineered to cross the plasma membrane and therefore directly reach the intracellular space. They consist of a protein transduction domain fused to the protein of interest (Becker-Hapak et al., 2001; Dietz and Bahr, 2004). Recently, the human immunodeficiency virus (HIV) transactivator of transcription (TAT) protein transduction domain (PTD) was fused to the Pax6 protein, a determinant of neurogenesis, and added to cell culture in order to promote neuronal differentiation in rodent neural stem cells (NSC) (Spitere et al., 2008). In combination to specific DA growth factors (Shh, FGF8, GDNF), a further increase in DA neuron generation was observed.

Another strategy using a Tat fusion protein is to induce site-specific recombination in ESC and derivatives by direct delivery of biologically active Cre-recombinase. The fusion protein Tat-Cre technique have been successfully demonstrated by Dr. Edenhofer and colleagues in mouse and human ESC and neurons derived from these cells (Nolden et al., 2006; Haupt et al., 2007), and enables an inducible system as already discussed in section III.2.2.1.

This approach can be used to induce the over-expression a gene of interest in a time-specific manner, but this must be preceded by a first genetic modification step to insert the gene of interest. This strategy is attractive to investigate genes involved in neuronal development and to promote specific neuronal differentiation by special, temporal and quantitative control of gene expression. However, the technique efficiency varies a lot with the intracellular delivery of different cell fusion and cell-permeant proteins (Dietz and Bahr, 2004; Lin et al., 2004).

III.2.2.4 Small molecules

Many "small molecules" can modulate intracellular pathways and have already been tested on ESC by high-throughput screening assays to discover differentiation modulators. A

highly specific glycogen synthase kinase 3β (GSK-3β) inhibitor has for example been discovered to promote neuronal differentiation of ESC (Ding et al., 2003a).

III.3 The dopaminergic fate in brief

As described in section III.1, the classic approach of DA differentiation is based on the formation of EB. First encouraging results in DA neurons generation from mESC were reported in 2000. With the use of the midbrain inducing activities of Shh and FGF8 and then the TH promoting effects of AA, the yield of TH-positive neurons reached 35% of the total amount of neurons defined by the expression of β-tubulin (Lee et al., 2000). Further improvements in DA neuron yield to over 60% were obtained in ES lines genetically engineered to over-express Nurr1 (Chung et al., 2002; Kim et al., 2002). Extensive *in vitro* and *in vivo* characterizations demonstrated the functionality of these mESC-derived DA neurons. With the EB approach, best DA neurons yield was obtained recently with the over-expression in neural progenitors of the transcription factor Lmx1a: 75-95% of mESC-derived neurons expressed molecular and physiological properties of mDA neurons (Friling et al., 2009). The second successful approach for DA neurons generation from mESC is based on the SDIA of PA6 or MS5 stromal cell lines (Kawasaki et al., 2000; Barberi et al., 2003). Dopaminergic differentiation occurs even in the absence of patterning factors such as Shh or FGF8, but the yield of DA neurons is greater and reaches 50% with the addition of Shh, FGF8 BDNF and AA (Barberi et al., 2003).

Differentiation protocols of primate ESC are of course based on the concepts developed for mESC differentiation. Protocols are very similar but also differ in duration and in differentiation conditions used. Therefore, results vary also. In brief and with a few examples, TH-positive cells obtained from monkey ESC represent from 4% of the total neurons with the EB approach without inducing factors (Kuo et al., 2003) to 35% with the SDIA approach without inducing factors (Kawasaki et al., 2002, Takagi et al., 2005).

The same diversity of protocols and results is for human ESC-derived DA neurons generation. Perrier et al., 2004; Park et al., 2005; Vazin et al., 2008; Friling et al., 2009 are just a few examples of studies on hESC differentiation towards the DA lineage. Consistent with the mESC differentiation knowledge, high yield (up to 80%) of DA neurons was obtained from hESC derived with co-culture on PA6 stromal cells and with subsequent addition of Shh, FGF8, BDNF, AA (Perrier et al., 2004). Another interesting result is the effect of time controlled-Lmx1a over-expression (by infection of early differentiating hESC) on DA

differentiation: up to 50% of TH-positive neurons were generated from the Lmx1a cells previously induced to differentiate by EB formation and subsequently treated with Shh and FGF8 (Friling et al., 2009).

III.4 The serotonergic fate in brief

Most of the conditions and studies described in sections III.1-2 were conducted to optimize the conditions for ESC to become DA neurons, which are of special importance for regenerative medicine interests (treatment of PD for example). In contrast, the ability of ESC to differentiate to the 5-HT fate was much less studied. The first serotonergic differentiation protocols used as a basis optimized dopaminergic differentiation conditions (Barberi et al., 2003). As the pathways of differentiation *in vivo* are quite similar between the two neuronal types (Hynes and Rosenthal, 1999) (see section II), 5-HT neurons are always enriched after dopaminergic differentiation, and the serotonergic fate can be further promoted by the addition of FGF4 (Alenina et al., 2006).

The first *in vitro* differentiations towards the serotonergic lineage were performed on mESC. Exposure of mESC-derived Nestin progenitor population to FGF4 and then to FGF8 and Shh led to a 2,5-fold increase in the number of 5-HT neurons resulting in 25% of 5-HT neurons in the total amount of generated neurons (Kim et al., 2002). In this study, neural differentiation was induced by the EB technique. Another study used stromal cells culture to induce differentiation of mESC, and adding the same combination of factors, was able to obtain 55-60% of 5-HT neurons (Barberi et al., 2003) (Figure 17).

5-HT neurons have also been derived with very high efficiency (>95%) from a rhesus ESC line (EB technique for the induction of differentiation). However, in this study, the addition of factors did not change the efficiency of derivation of 5-HT neurons, suggesting that this line was perhaps committed to a serotonergic lineage by an epigenetic predisposition (Salli et al., 2004).

In contrast, under non-optimized differentiation conditions, less than 1% of 5-HT neurons are obtained from hESC (Reubinoff et al., 2000). Addition of factors that included Shh, FGF8, GDNF and BDNF increased the number of serotonin expressing cells to 5% of the neurons defined by the expression of β-tubulin. A recent study (Kumar et al., 2009) succeeded in generating 40% of 5-HT neurons out of the 20% of neurons obtained from hESC by EB induction as a first step, then selection of Nestin-positive precursors, and finally addition of acidic FGF, Forskolin that increases intracellular cAMP, 5-HT, GDNF and BDNF factors.

We have reviewed above the present knowledge on embryonic stem cells, their specifications *in vivo* and differentiations *in vitro* towards two specific neuronal lineage cell types: dopaminergic and serotonergic neurons. The dopaminergic specification and differentiation have been extensively studied. The serotonergic specification and differentiation are more and more explored in the scope of understanding the role of the serotonergic system in modulating behaviors and in human disorders of mood and mind.

But if we keep in mind the idea of producing specific functional neurons or neural precursors from embryonic stem cells that may be used for cellular therapy purposes, research still faces two big challenges. The first one is to generate clinical grade neurons. The experimental protocols used to generate neurons *in vitro* include not fully characterized materials for example. The second challenge is to obtain a homogeneous population of differentiated cells at the adequate developmental stage. The synchronization and developmental stage of the cell population are crucial for the functional integration into the host tissue.

RESULTS

La vie est un voyageur
qui laisse traîner son manteau derrière lui,
pour effacer ses traces.
Louis Aragon

RESULTS

Forced expression of Lmx1b enhances differentiation of mouse ES cells into serotonergic neurons

Virginie Dolmazon[1,2,3], Natalia Alenina[4], Suzy Markossian[1,2,3], Jimmy Mancip[1,2,3], Yvonne van de Vrede[1,2,3], Emeline Fontaine[1,2,3], Colette Dehay[1,2,3], Henry Kennedy[1,2,3], Michael Bader[4], *Pierre Savatier[1,2,3], *Agnieszka Bernat[1,2,3].

[1]Inserm U846, 18 Avenue Doyen Lepine, 69500 Bron, France. [2]Stem Cell and Brain Research Institute, 69500 Bron, France. [3]Université de Lyon, Université Lyon 1, 69003, Lyon, France. [4]Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, Berlin, Germany.

*Correspondence: Pierre Savatier or Agnieszka Bernat, INSERM U846, 18 avenue Doyen Lépine, 69500 Bron, France. E-mail: pierre.savatier@inserm.fr, agnieszka.bernat@inserm.fr

Running title: Lmx1b enhances differentiation of ES cells into 5-HT neurons

Abstract

The LIM homeodomain transcription factor Lmx1b is a key factor in the specification of the serotonergic neurotransmitter phenotype. Here, we explored the capacity of Lmx1b to direct differentiation of mouse embryonic stem (mES) cells into serotonergic neurons. mES cells stably expressing human Lmx1b were generated by lentiviral vector infection. Clones expressing Lmx1b at a low level showed increased neurogenesis and elevated production of neurons expressing serotonin, serotonin transporter, Tryptophan hydroxylase 2, and transcription factor Pet1, the landmarks of serotonergic differentiation. To explore the role of Lmx1b in the specification of the serotonin neurotransmission phenotype further, a conditional system making use of a floxed inducible vector targeted into the *ROSA26* locus and a hormone-dependent Cre recombinase was engineered. This novel strategy was tested with the reporter gene encoding human placental alkaline phosphatase, and demonstrated its capacity to drive transgene expression in nestin$^+$ neural progenitors and in Tuj1$^+$ neurons. When it was applied to the inducible expression of human Lmx1b, it resulted in elevated expression of serotonergic markers. Treatment of neural precursors with the floor plate signal Sonic hedgehog further enhanced differentiation of Lmx1b-overexpressing neural progenitors into neurons expressing serotonin, serotonin transporter, Tryptophan hydroxylase 2 and Pet1, when compared to Lmx1b-non expressing progenitors. Together, our results demonstrate the capacity of Lmx1b to specify a serotonin neurotransmitter phenotype when overexpressed in mESC-derived neural progenitors.

Introduction

Embryonic stem (ES) cells have the capacity to differentiate into a large variety of cells types *in vitro*, owing to their high self-renewal capacity and intrinsic pluripotentiality (1). ES cells can be used to dissect the molecular mechanisms underlying *in vitro* differentiation into specialized cell types, such as serotonergic neurons. Several protocols to coax differentiation of mouse, rhesus and human ES cells into neural progenitors, then to direct differentiation of those progenitors into serotonergic neurons have been described (2-7). These protocols make use of growth factors and cytokines known to regulate serotonergic differentiation in the mouse central nervous system. Implicit to this experimental paradigm, the developmental programme employed by ES cells to differentiate into serotonergic neurons *in vitro*, largely, if not fully, recapitulates the developmental programme executed during neurogenesis in the developing embryo.

Serotonergic (5-HT) neurons reveal a close ontogenetic relationship to midbrain dopaminergic (DA) neurons as both neuronal sub-types derive from ventral neuroepithelial progenitors located on either side of the midbrain-hindbrain boundary, starting at embryonic day 10.5 in the mouse (8-10). According to the prevailing model, the development of both 5-HT depends critically on sonic hedgehog (Shh) synthesized by the floor plate, FGF8 generated by the midbrain-hindbrain organizer, and FGF4 produced by the primitive streak (11,12). Neural progenitors located more rostrally, thus away from the FGF4 signal, differentiate into dopaminergic neurons.

Several transcription factors are involved in serotonergic differentiation. Mash1 and Nkx2.2 expressed in neural progenitors of the hindbrain activate the transcription

factors Gata2, Gata3, Lmx1b, and Pet1 (13), which together define the serotonergic cell type by activating marker genes such as for Tryptophan hydroxylase 2 (TPH2), aromatic amino acid decarboxylase (AAAD), the serotonin transporter (SERT), and the vesicular monoamine transporter (VMAT) 2 (14). Both Gata2- and Gata3-deficient mice exhibit partial loss of serotonergic neurons (13,15). In mice lacking Pet1, 70% of serotonergic progenitors fail to differentiate, whereas in the remaining Pet1-deficient neurons diminished expression of TPH and SERT was observed (16). Lmx1b (LIM homeodomain transcription factor 1b) is crucially involved in the formation of the entire serotonin system in the hindbrain, because its deletion in mice leads to the absence of 5-HT neurons in the brain (17-19). It is expressed in developing 5-HT neurons together with Pet1 starting around E11 in the rostral cluster of serotonergic differentiation and one day after in the caudal one, consistent with the delayed appearance of serotonergic cells in the latter region (18). Lmx1b ablation does not affect the expression of Nkx2.2, Gata3, and Shh and only partly the one of Pet1, putting these factors upstream or in parallel to Lmx1b (17,18). Together with Nkx2.2 and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube, a function that Nkx2.2 and Pet1 cannot support on their own (18). Lmx1b is also involved in the maintenance of the dopaminergic neurotransmitter phenotype in collaboration with the orphan nuclear receptor Nurr1 (20). Therefore, Lmx1b plays a central role in the differentiation of 5-HT neurons, and an ancillary role in the differentiation of midbrain DA neurons.

Differentiation of murine ES (mES) cells into serotonergic neurons is highly efficient. In a pioneer study, sequential exposure of mES cell-derived nestin[+] neural progenitors to FGF4 and then to FGF8 and Shh led to a 2.5-fold increase in the number of serotonergic neurons resulting in ~25% of the neurons being 5-HT positive (21).

Using stromal cell cultures to induce differentiation of mES cells and adding the same combination of factors, Barberi *et al.* were able to define conditions in which the mouse ES cells were differentiated into 60% 5-HT expressing neurons (3). Similar results were obtained with human ES (hES) cells. Under non-optimized differentiation conditions, less than 1% of the hES cell-derived neurons obtained are serotonin positive (22,23). Addition of factors that included Shh, FGF4, FGF8, Glial-Derived Neurotrophic Factor (GDNF), and Brain-Derived Neurotrophic Factor (BDNF) increased the number of 5-HT expressing cells to 25% of the β-III tubulin–positive cell population. In a recent study, 40% of neural progenitors could be converted to serotonergic neurons expressing serotonin, Tph2, and the transcription factors Mash1, Pet1 and Lmx1b (6).

Herein, we asked if overexpression of Lmx1b expression is sufficient to drive differentiation of mES cell-derived neural progenitors to the serotonergic neuron pathway. We developed two experimental approaches, the first one based on lentiviral vector-based stable expression, the second one on conditional expression induced by 4'hydroxytamoxifen (4'OHT) in neural progenitors. In both experimental systems, Lmx1b was found to promote serotonergic differentiation, either alone, or in cooperation with dorsoventralizing factors.

Material and Methods

Plasmid construction

To engineer *pR4SA-EFS-CreER^{T2}-W*, the Cre-ER^{T2} coding sequence from *pCre-ER^{T2}* (24) was subcloned between the *EcoRV* and *BamHI* sites of plasmid *pR4SA-EFS-GFP-W* (25).

To engineer *p2K7-HAhLmx1b*, the human *Lmx1b* cDNA from *pcDNA3.1hLmx1b* (a kind gift of S. Dreyer) was first HA-tagged at its N-terminus by PCR amplification. It was subsequently subcloned between *BamHI* and *EcoRV* sites in plasmid *pGAE-CAG-eGFP-WPRE* (26). In a second step, the CAG promoter (CMV early enhancer/chicken β-actin) from *pGAE-CAG-eGFP-WPRE* was cloned into *pDONRP4-P1R* (Invitrogen), and the *HAhLmx1b* cDNA from *pGAE-CAG-HAhLmx1b-WPRE* was cloned into *pDONR221* (Invitrogen), both using Gateway BP clonase enzyme mix (Invitrogen). The resulting two entry vectors were then recombined into *2K7neo* lentivector (kind gift of D. Suter) using Gateway LR plus clonase enzyme mix (Invitrogen) (27).

To engineer *pIGTE2-R26-hPLAP*, a 48-mer oligonucleotide containing a *PacI* site (5'-att<u>ttaattaa</u>gaagttcctattctctagaaagtataggaacttcgat-3'), and a 54-mer oligonucleotide containing an AscI site (5'-ctagagctagcgaagttcctattcttcaaatagtataggaactt c<u>ggcgcgcc</u>a-3'), were first subcloned into the *Ssp1* and *AscI* sites of plasmid *pIGTE2-hAP* (28), respectively. The resulting plasmid was digested with *PacI* and *AscI*, and the 3.8kb fragment was subcloned between *PacI* and *AscI* in *pRosa26PA* (29) to generate *pIGTE2-R26-hPLAP*. To engineer *pIGTE2-R26-hLmx1b*, a 1.2 kb *NheI/XbaI* fragment encompassing human *Lmx1b* cDNA was prepared from *pcDNA3.1hLmx1b* (a kind gift of S. Dreyer), and subcloned in *pCMV-Ires-pA* to generate *pCMVhLmx1b*. The *pCMVhLmx1b* plasmid was next digested with *NheI*, and the resulting 2.9-kb insert was subcloned between both *NheI* sites into *pIGTE2* to generate *pIGTE2-hLmx1b*. *PacI* and *AscI* sites were added as described above for *pIGTE2-hAP*, and the resulting *PacI/AscI* fragment was subcloned between *PacI* and *AscI* in *pRosa26PA* to generate *pIGTE2-R26-hLmx1b*.

mES cell culture and electroporation

mES cells (CGR8) were maintained on 0.1% gelatinised (Sigma) tissue culture dishes in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% fetal calf serum (Biowest), 1000 U/ml of LIF, 1 mM sodium pyruvate, 2 mM L-glutamine, 100U/ml penicillin -100µg/ml streptomycin, 100µM β-mercaptoethanol (all from Invitrogen). 5×10^6 CGR8 cells were electroporated at 0.26 kV and 960 µF with 40 µg of *pIGTE2-R26-hPLAP* or *pIGTE2-R26-hLmx1b* plasmid linearized with *SwaI*. Stably transfected cells were selected with 80µg/ml hygromycine B (Roche Applied Science) for 8 days. Drug-resistant colonies were expanded before analysis and frozen.

Neuronal differentiation

Neural induction and neuronal differentiation were performed by means of co-culture of mES cells with the MS5 stromal cell line as previously described (3). In brief, mES cells were seeded at a density of 10-50 cells/cm^2 on the layer of mitomycin-inactivated MS5 cells, and maintained in Knockout DMEM (KO DMEM) medium supplemented with 15% (vol/vol) Knockout Serum Replacement (KSR), 2mM L-glutamine, and 10µM β-mercaptoethanol (all from Invitrogen), for eight days to allow neural induction. Neuroepithelial colonies were harvested with a fire-pulled Pasteur pipette, dissociated by gentle trypsinization, and replated on matrigel (BD Biosciences)-coated dishes in Neurobasal medium without phenol red, supplemented with 200 mM Glutamine, 5 µg/ml bovine fibronectin, N2 and B27 supplements (all from Invitrogen), and 20 ng/ml FGF2 (AbCys). To induce neuronal differentiation, FGF2 was withdrawn from the medium at day 11, and replaced with 20 ng/ml Brain-Derived Neurotrophic

Factor (BDNF, R&D Systems), 200 µM ascorbic acid (Sigma), and 20 ng/ml Neurotrophin 3 (NT3, R&D Systems). Final differentiation was allowed for 5 days. 4'OHT (Calbiochem) was added to culture medium for the time and concentration indicated.

Neural differentiation was also induced by formation of embryoid bodies (EBs) as previously described (5,30,31). In brief, 6×10^6 mES cells were cultured in suspension in 10 ml of medium [GMEM supplemented with KSR] in a 10cm dish. After eight days, EBs were dissociated and replated at a density of 2.5×10^4 cells/cm^2 on poly-D-lysine/laminin (Sigma) in N2B27 medium (Invitrogen). After replating, neural precursor cells were enriched for two days by the addition of 10 ng/ml FGF2. Withdrawal of FGF2 in N2B27 medium resulted in production of post-mitotic neurons (from day 11 to day 18).

Production of lentiviral vectors and infection

The method for Simian Immunodeficiency Virus (SIV)-based vector production in 293T cells is fully described elsewhere (25,32). In brief, 293T cells were transfected by the calcium phosphate method with a mixture of DNAs containing *pGRev* plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, *pSIV3+* plasmid encoding the gag, pol, tat, and rev proteins, and *pR4SA-EFS-CreER^{T2}-W*. The following day, cells were refed with fresh medium and further cultured for 48 hours. The supernatant was then collected, cleared by centrifugation (3000 RPM, 15 minutes) and passed through a 0.8-µM filter to remove cell and debris. For infection, 10^5 freshly trypsinized mES cells were resuspended into 1 ml of 293T cell supernatant producing the desired virus, plated on a 10 cm in diameter dish, and cultured for 48 hours before

cloning by limiting dilution. The production of lentiviral particles from plasmid *p2K7neo-HAhLmx1b* was perfomed as decribed (27,33).

DNA and RNA extraction, Southern blot and real-time polymerase chain reaction (PCR)

For genomic DNA extraction, cells were lysed in 10mM Tris-HCl pH 8, 1mM EDTA pH 8, 100mM NaCl, 0.5% SDS. The resulting lysate was treated with 0.2mg/ml RNase for 1h at 37°C, followed by proteinase K treatment (0.2mg/ml, 55°C, overnight). DNA was separated from proteins by phenol-chloroform extraction, precipitated with isopropanol and resuspended in 1mM Tris-HCl, 0.1mM EDTA pH 8. Ten μg of EcoRV digested genomic DNA was separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N[+] Amersham), and hybridized with ^{32}P-labelled (Ready-to-Go Labeling Kit, Amersham) probe. RNA was extracted using RNAeasy kits with on-column DNAse digestion and reverse transcription carried out with MuMLV-RT (Promega), according to the manufacturer's recommendations. Quantitative PCR was performed using the LightCyclerTM 1.5 system and the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science) according to the manufacturer's instructions. Reactions were carried out in a total volume of 10 μl, comprising 0.4 μM of each primer, 0.75 μl SYBR Green, 2.5 μl of diluted cDNA, and 2-4 mM $MgCl_2$ according to primers. Amplification and online monitoring was performed using the LightCyclerTM 1.5 system. Following 40 amplification cycles, melt-curve analyses were performed to verify that only the desired PCR product had been amplified. PCR efficiency of both the target and reference genes was calculated from the derived slopes of standard curves by the LightCycler software (Roche Molecular Biochemicals

LightCycler Software, Version 3.0). These PCR efficiency values were used to calculate the relative quantification values for calibrator-normalized target gene expression by the LightCycler relative quantification software (Version 1.0). All normalizations were carried out with β-actin. Semi-quantitative PCR was performed using Euroblue Taq polymerase according to supplier (Eurobio) instructions. Sequences of primers are given in Table I.

Histochemical detection and immunolabelling.

Histochemical analysis for hAP expression in cultured cells and quantification of hAP activity in protein lysates were carried out as described previously (28). For immunolabelling, cells were washed in Phosphate Buffer Saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 30 minutes. After the rinse with PBS, cells were permeabilized in 0.2% Triton X100 in PBS, for 15 minutes. Non-specific binding was blocked with non-immune normal goat serum (Zymed) for 30 minutes at room temperature. Primary antibodies were applied in 1% normal goat serum (NGS) in PBS. The following primary antibodies were used: hPLAP (Sigma), ß-III-Tubulin (Chemicon and Covance), PSA-NCAM (Chemicon), 5-HT (Sigma). After incubation at 37°C for 1 hour, followed by several rinses in PBS, appropriate fluorochrome-conjugated secondary antibodies were added (Molecular Probes). The cells were incubated at 37°C for 30 minutes and, after extensive washes in PBS, mounted on glass microscope slides using Vectashield HARD mounting medium (Vector Labs Inc.) containing DAPI for DNA counterstaining.

ELISA assay

To evaluate the 5-HT level in post-mitotic neuronal culture, cells were lysed in PBS containing 0,1% ascorbic acid (Sigma) by repeated freezing (-80°C) and thawing. The extracts were sonicated and separated from debris by centrifugation (30min 14000 rpm). The protein concentration was estimated using Coomassie Blue staining. Enzymatic immunoassay for 5-HT was performed with nano-detection kit "Serotonin EIA BA 10-0900" (Labor Diagnostika Nord) following manufacturer's instructions. Serotonin concentration measured with the EIA kit was normalized to total protein content.

Results

Neuronal differentiation of mES cells overexpressing Lmx1b.

CGR8 mES cells were infected with *p2K7-HAhLmx1b*, a lentiviral vector expressing human *Lmx1b* cDNA driven by the ubiquitous CAG promoter. Clones expressing *hLmx1b* at low (L1, L2, L3, L4, L9, L10), intermediate (L5, L6, L7, L8, L12), and high (L11) levels were isolated (**Figure 1A**). They were cultured onto MS5 stromal cells for eight days to form neuroepithelial colonies, which were subsequently dissociated and replated on matrigel-coated dishes in the presence of FGF2 to amplify a morphologically homogenous population of neural progenitors (NPs), which is uniformly immunoreactive for NP-specific antigens such as nestin and PSA–NCAM (3) (hereafter called "MS5 protocol"). NPs were induced to differentiate into Tuj1$^+$ neurons by withdrawal of FGF2 for 4-6 days (data not shown). We observed that *hLmx1b* expression dramatically influenced both the yield of neuroepithelial colonies at day 8 and the yield of NPs after colony dissociation and replating at day 12. More specifically, clone L11 (high expressor) produced significantly smaller neuroepithelial colonies in comparison, either with control mES cells (56% reduction in size), or with low expressor L1 (43% reduction) (**Figure 1B,C**). Of note, the number of neuroepithelial colonies did not vary significantly between clones, indicating that *hLmx1b* expression did not interfere with clonigenicity and survival of dissociated mES cells in these culture conditions (data not shown). We thus hypothesized that *hLmx1b* overexpression altered proliferation and/or survival of transient amplifying NPs. This hypothesis was confirmed after counting NPs upon dissociation of neuroepithelial colonies at day 8, and subsequent culture for four days. Clone L11 (high expressor)

exhibited a 50% reduction in the yield of nestin$^+$ NPs at day 12 compared with wild-type ES cells (**Figure 1D**). Moreover, after withdrawal of FGF2, most L11-derived NPs failed to differentiate into mature neurons and degenerated (data not shown). In sharp contrast, clone L1 (low expressor) produced a 63% increase in nestin$^+$ NP number at day 12 (**Figure 1D**) and, at day 15 (*i.e.* three days after mitogen withdrawal), a 12-fold increase in pan-neuronal marker Map2ab expression. Comparable results were obtained with low expressors L2 (**Figure 1E**).

Similar data were obtained after neuronal differentiation induced by formation of embryoid bodies (EBs) for 8 days, followed by replating for 2 days in the presence of FGF2, and subsequent mitogen withdrawal for 7 days ("EB protocol"). Compared to control cells, the low expressors L1 and L3 showed a 2.7- and 4-fold increase in pan-neuronal marker Tuj1 expression, respectively (**Figure 1F**). Of note, *hLmx1b* was expressed at constant levels throughout differentiation (**Figure 1G**). Together, these results suggest that moderate overexpression of Lmx1b significantly increases the yield of neural/neuronal differentiation of mES cells.

ES cells overexpressing Lmx1b show preferential differentiation toward serotonergic lineage *in vitro*.

We next wanted to determine if Lmx1b overexpression influences differentiation of neural progenitors into serotonergic, dopaminergic and motoneurons. The low expressors L1 and L3 were used in this study as they both exhibited vastly elevated neuronal differentiation compared to high expressors. Expression of serotonergic-, dopaminergic- and motoneuron- specific markers, after differentiation induced by EB formation, was measured by real-time PCR. Since both L1 and L3 clones showed

differences in the yield of neuronal differentiation compared with control cells (see **Figure 1**), the expression level of each marker was normalized to *Tuj1* (in addition to *β-actin*) in order to eliminate the bias resulting from overproduction of neurons by *hLmx1b*-expressing clones. E12.5 *dpc* brain extract was used as a reference throughout these experiments. Clones L1 and L3 exhibited increased expression of serotonergic markers, 9- and 3-fold for brain-specific serotonin producing enzyme *Tph2*, 7.7- and 2.5-fold for *serotonin transporter* (*SERT*), and 2.2-fold for serotonergic neuron-specific transcription factor *Pet1* (only L1) (**Figure 2A**). Of note, an increase in dopaminergic markers *tyrosine hydroxylase* (*TH*) and *Lmx1a* was also evidenced, whereas expression of *Isl1*, a marker of motoneuron differentiation, was significantly decreased (**Figure 2C**). The enrichment in serotonergic neurons at day 17 of differentiation in Lmx1b-overexpressing clones was confirmed by immunostaining against serotonin (**Figure 2B**). Quantification of serotonin level by enzymatic immunoassay showed a 20-fold increase in 5-HT content in both L1 and L3 clones compared with control cells (**Figure 2D**). These results indicate that neural precursors overexpressing *hLmx1b* at a low level show increased propensity for differentiation into 5-HT neurons.

Lmx1b cooperates with Shh, FGF4 and FGF8 to induce serotonergic differentiation.

In the next step, serotonergic marker expression was examined in clone L1 after differentiation induced by co-culture with MS5 stromal cells. No change in serotonergic marker expression was observed when compared to control cells at day 18 of differentiation (**Figure 3A**). However, when clone L1 and control cells were treated

either with Shh, or with Shh, FGF4, and FGF8 – this latter condition was described to promote serotonergic differentiation (3,6) – clone L1 exhibited significantly higher expression of *Tph2* (Shh: 1.6-fold; Shh+FGF4+FGF8: 3.5-fold), *SERT* (Shh: 3.5-fold; Shh+FGF4+FGF8: 7.7-fold) and *Pet1* (Shh: 2.1-fold; Shh+FGF4+FGF8: 1.6-fold) compared to control cells (**Figure 3B,C**). Together, these results indicate that Lmx1b cooperates with dorsoventralizing factors to induce serotonergic differentiation.

Generation of an inducible expression vector and ES cells suitable for conditional gene expression in NPs and post-mitotic neurons.

Since Lmx1b expression appeared detrimental to neural differentiation when overexpressed at high levels in mES cells, we generated an inducible expression system to drive conditional expression of hLmx1b in the NP population. To this aim, we made use of a hormone-dependant Cre-ER^{T2} recombinase and of an expression vector in which transgene transcription is blocked by three floxed transcription termination signals (**Figure 4A**). This system displays two significant improvements with respect to our previously published system (28): (*i*) the Cre-ER^{T2} recombinase is expressed from a lentiviral vector in which the Cre-ER^{T2} coding sequence is driven by the ubiquituously active truncated version of the EF1α promoter (25); (*ii)* the expression vector contains 7 Kb of genomic sequences which allow targeted integration into the *ROSA26* locus and subsequent transgene expression under the ubiquitously active regulatory elements of *ROSA26* (29). A mES cell line expressing Cre-ER^{T2} was produced by infection with pR4SA-EFS-CreER^{T2}-W. This line, hereafter called WTC15, expresses CreER^{T2} at high level in ES cells and in their differentiated derivatives (data not shown). Next, an inducible vector called *pIGTE2-R26-hPLAP* that harbors *hPLAP*, the gene encoding

human placental alkaline phosphatase (hPLAP), was introduced by electroporation into WTC15 cells. Ten individual hygromycin-resistant clones were analysed, of which three displayed homologous recombination into the *ROSA26* locus (**Figure 4B**). All three clones showed a 10- to 14-fold increase in hPLAP activity upon treatment with 4'OHT (100 nM, 48 h) (**Figure 4C**). One of them, hereafter called WTC-hPLAP-7, was selected for further analysis. Treatment with 4'OHT resulted in excision of the *hygro-polyA* cassette (**Figure 4D**). Immunofluorescence staining showed hPLAP expression in most cells after treatment with 100 nM 4'OHT for 48 h (**Figure 4E**). Virtually no hPLAP-positive cells could be detected in the untreated cell population, indicating that transgene expression is repressed completely in the absence of hormone.

WTC-hPLAP-7 cells were induced to differentiate into neuroepithelial colonies by means of co-culture with MS5 stromal cells for eight days, followed by amplification of the NP population by FGF2 for three days. To assess transgene induction in the neuroepithelial colonies, day 5 cultures were treated with 100 nM 4'OHT for 48 hours. Expression of hPLAP was subsequently observed in >95% of the neuroepithelial colonies (**Figure 5A, panels b,c**). Day 11 NPs, derived from neuroepithelial colonies in which expression of the transgene was induced at day 5, expressed hPLAP in virtually all cells (**Figure 5A, panels e,f**). Most neurons generated at day 15 also expressed it (**Figure 5A, panels h,i**). Analysis of *hPLAP* mRNA level by real-time PCR showed a 49-fold increase in day 11 NPs and a 9-fold increase in post-mitotic neurons after induction with 4'OHT at the ES cell stage (day 0). When induction was performed at the neuroepithelial colony stage (day 5), a 51-fold increase in day 11 NPs and a 22-fold increase in post-mitotic neurons, were observed (**Figure 5B**). Together these results show that once induced, either in the undifferentiated state or during neural

differentiation, hPLAP expression level is maintained throughout neuronal differentiation.

Inducible expression of Lmx1b in ES-derived NPs increases expression of serotonergic markers.

A mES cell line conditionally expressing *hLmx1b* was engineered by electroporating *pIGTE2-R26-hLmx1b* plasmid into WTC15 mES cells. After selection, nine hygromycin-resistant clone in 12 displayed targeted integration into the *ROSA26* locus (**Figure 6A,B**). Two homologous recombinant clones were tested, showing excision of the transcription termination cassette after 4'OHT treatment (100 nM, 48 hours) (**Figure 6C**). Clone 6, hereafter called WTC-hLmx1b-6, was chosen for all subsequent experiments. Real-time PCR analysis of *hLmx1b* expression in WTC-hLmx1b-6 showed a 55-fold increase in mRNA level upon 4'OHT induction at the ES cell stage (day 0), and a 9- and 14-fold increase in post-mitotic neurons after induction at day 0 and at the neuroepithelial colony stage (day 5), respectively (**Figure 6D**). Of note, *hLmx1b* expression levels in neurons produced from WTC-hLmx1b-6 ES cells after induction at day 5 were similar to expression levels measured in *hLmx1b*-stably expressing clones L1 and L2 (**Figure 6E**). We also confirmed that the induction of *hLmx1b* expression in WTC-hLmx1b-6 ES cell-derived NPs increased Map2ab expression at day 18 (**Figure 6F**), as it was observed with *hLmx1b*-stably clones L1, L2, and L3 (see Figure 1E,F).

We next asked whether the induction of *hLmx1b* expression in NPs promotes the expression of serotonergic markers. The expression of *hLmx1b* was induced in the neuroepithelial colonies (day 5). After amplification for four days, NPs were induced to

differentiate into post-mitotic neurons by mitogen withdrawal and the expression of serotonergic markers analyzed by real-time PCR. mRNA levels measured in the WTC-hLmx1b-6-derived neurons were normalized, first to pan-neuronal *Map2ab* marker level to eliminate variations in the yield of neuronal differentiation, second to mRNA levels measured in the WTC-hPLAP-7-derived neurons to eliminate non-specific effects of 4'OHT. We then observed a 1.4-, 1.6- and 2.5-fold increase of *Pet1*, *Tph2* and *SERT* mRNA levels, respectively (**Figure 7A**).

Neuroepithelial colonies and amplifying NPs derived from both WTC-hPLAP-7 and WTC-hLmx1b-6 were exposed, either to Shh alone, or to combinations of Shh and FGF4 (day 5 to day 8) followed by Shh and FGF8 (day 8 to day 12). Expression of *hLmx1b* and *hPLAP* transgenes was induced by 4'OHT in the developing neuroepithelial colonies at day 5. In the presence of Shh, induction of *hLmx1b* expression- when it was compared with induction of *hPLAP* – resulted in a 10-, 30-, and 55-fold increase in *Pet1*, *Tph2*, and *SERT* expression levels in post-mitotic neurons at day 18, respectively (**Figure 7B**). Treatment with Shh and FGF4, followed by Shh and FGF8, did not further increase the expression of *Tph2* (31-fold) and *SERT* (57-fold), and moderately increased the expression of *Pet1* (16-fold) (**Figure 7D**). Treatment with Shh combined with induced expression of *hLmx1b* also increased the expression of dopaminergic markers, *En* (2.8-fold), *TH* (18.5-fold); and *Nurr1* (6.6-fold), when compared to expression levels in Shh-treated control cells (**Figure 7C**). Furthermore, addition of FGF4 and FGF8 to the differentiation cocktail reduced the expression of *TH* and *Nurr1* by 2-fold in comparison with Shh alone (**Figure 7E**), which is likely to reflect the capacity of FGF4 to inhibit dopaminergic differentiation (11).

Taken together, these results show that enforced expression of Lmx1b, combined with Shh, robustly stimulates expression of serotonergic markers in the differentiating NPs derived from mES cells.

Discussion

The focus of this study was to demonstrate the role of Lmx1b in promoting differentiation of mES cell-derived neural progenitors into serotonergic neurons. Lmx1b is essential to the development of the entire serotonergic system (17-19), as well as to the maintenance of the dopaminergic neurotransmitter phenotype in the mouse (20). We thus asked if overexpression of Lmx1b in mES cells is sufficient to specify serotonergic and dopaminergic phenotypes.

Forced expression of a transgene that drives differentiation often results in inability to form undifferentiated mES colonies and consequent recovery only of low expressing or deviant cells. We successfully generated mES cell lines that express hLmx1b ectopically, but only the lines expressing it at low level retained the capacity to differentiate into neural precursors and post-mitotic neurons harboring the growth characteristics of control cells. In contrast, mES cells expressing Lmx1b at high levels exhibited reduced capacity to form neural progenitors, and those progenitors to differentiate into post-mitotic neurons. Failure to differentiate could result, either from the detrimental effect of Lmx1b when overexpressed at elevated levels, or from the selection of mutant mES cells that resisted to the differentiation-promoting effect of Lmx1b. To overcome this difficulty, we have engineered an inducible gene expression system that makes it possible to overexpress differentiation-promoting genes in mES

cell-derived neural progenitors and to maintain their expression in differentiating neurons. Activation of *Lmx1b* in mES cell-derived neural progenitors and its subsequent expression in post-mitotic neurons, as revealed by the *hPLAP* reporter gene, reflects the expression pattern of *hLmx1b* in the mouse, starting in neural progenitors of the hindbrain and being maintained in 5-HT neurons (17).

In both stably- and 4'OHT induced-Lmx1b expressing mES cells, we observed that Lmx1b increases the expression of *Tph2*, *SERT*, and *Pet1*, the landmarks of serotonergic neurotransmitter phenotype. The effect of Lmx1b was observed using two different differentiation protocols, the first one based on co-culture of ES cells with a stromal feeder cell line (3), the second one based on EB formation (2), both of them including selection and amplification of nestin$^+$ neural progenitors. These results indicate that Lmx1b expression is rate limiting for specifying the 5-HT phenotype, in accordance with the genetic data in the mouse (17-19). The inductive effect of Lmx1b on serotonergic differentiation is strongly enhanced when neural progenitors are treated with the floor plate signal Shh. This result can be explained by the capacity of Shh to activate the expression of ancillary factors acting in concert with Lmx1b to promote serotonergic differentiation. One critical factor is Pet1 whose expression, together with the expression of Lmx1b, is induced by Nkx2.2 in response to Shh signaling (17,18). *Pet1* contributes to serotonergic differentiation since 70% of serotonergic neurons fail to differentiate in Pet1-deficient mice (14,16), and it was shown that both *Lmx1b* and *Pet1* are necessary and sufficient to specify 5-HT transmitter phenotype when overexpressed in the chick ventral spinal cord (18). Whether *Lmx1b* and *Pet1* act strictly in parallel to specify 5-HT neurotransmitter phenotype (18) or whether Pet1 is also a target gene of Lmx1b (17) is still unclear. We observed that *Pet1* expression is activated in neurons

overexpressing *hLmx1b*, but that this activation is moderate compared to the activation of *SERT* and *Tph2* expression. These data support the prevailing model that Lmx1b and Pet1 transcription factors act in parallel to specify the 5-HT phenotype, and that Lmx1b reinforces the transcriptional activity of *Pet1* induced by Shh *via* Nkx2.2 (17,18).

The development of 5-HT neurons depends critically on FGF8 generated at the midbrain-hindbrain boundary and on FGF4 produced by the primitive streak. *In vivo*, FGF4 inhibits the development of midbrain DA neurons and promotes the development of 5-HT neurons (11). In clone L1 that stably expresses Lmx1b, addition of Shh, FGF8 and FGF4 to neural progenitors confers a less than 2-fold increase in the expression of serotonergic markers, when compared to clone L1 treated with Shh alone. Furthermore, in mES cell-derived neural progenitors treated with 4'OHT to activate Lmx1b expression, addition of Shh, FGF4 and FGF8 has no effect on the yield of serotonergic differentiation, when compared to a sister culture treated with Shh alone. Thus, when Lmx1b is overexpressed, FGF8 and FGF4 become dispensable. This finding suggests that activation of Lmx1b expression is the main consequence of FGF8 and FGF4 induction in the developing neural progenitors acquiring a 5-HT phenotype.

Our experiments show that overexpression of Lmx1b, either alone or in combination with Shh, has little or no effect on the yield of dopaminergic neuron differentiation. This is in accordance with a previous report (34) showing that transduction of mouse and human ES cells with *Lmx1b* does not induce maturation to the midbrain DA neuron phenotype unless *Nurr1* is co-transduced in the same cell. Noteworthy, addition of FGF8 and FGF4 slightly reduced the expression of midbrain DA neuron markers in accordance with the function of FGF4 *in vivo* (11).

To summarize, our results underline the need for proper extrinsic signals combined with intrinsic clues when engineering and manipulating ES cells for differentiation purposes. The vectors and cell lines described might be of great value for studying the gene function in serotonergic neuron development, disease pathology and in trying to understand how drugs that affect the serotonergic system alter neurotransmitter release.

Acknowledgements

References

1.   Smith AG. (2001). Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol 17:435-62.

2.   Lee SH, N Lumelsky, L Studer, JM Auerbach and RD McKay. (2000). Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 18:675-9.

3.   Barberi T, P Klivenyi, NY Calingasan, H Lee, H Kawamata, K Loonam, AL Perrier, J Bruses, ME Rubio, N Topf, V Tabar, NL Harrison, MF Beal, MA Moore and L Studer. (2003). Neural subtype specification of fertilization and

nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol 21:1200-7.

4.   Salli U, AP Reddy, N Salli, NZ Lu, HC Kuo, FK Pau, DP Wolf and CL Bethea. (2004). Serotonin neurons derived from rhesus monkey embryonic stem cells: similarities to CNS serotonin neurons. Exp Neurol 188:351-64.

5.   Alenina N, S Bashammakh and M Bader. (2006). Specification and differentiation of serotonergic neurons. Stem Cell Rev 2:5-10.

6.   Kumar M, SK Kaushalya, P Gressens, S Maiti and S Mani. (2009). Optimized derivation and functional characterization of 5-HT neurons from human embryonic stem cells. Stem Cells Dev 18:615-27.

7.   Tokuyama Y, SL Ingram, JS Woodward and CL Bethea. (2010). Functional characterization of rhesus embryonic stem cell-derived serotonin neurons. Exp Biol Med (Maywood) 235:649-57.

8.   Millet S, K Campbell, DJ Epstein, K Losos, E Harris and AL Joyner. (1999). A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature 401:161-4.

9.   Joyner AL, A Liu and S Millet. (2000). Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. Curr Opin Cell Biol 12:736-41.

10.   Brodski C, DM Weisenhorn, M Signore, I Sillaber, M Oesterheld, V Broccoli, D Acampora, A Simeone and W Wurst. (2003). Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain-hindbrain organizer. J Neurosci 23:4199-207.

11. Ye W, K Shimamura, JL Rubenstein, MA Hynes and A Rosenthal. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 93:755-66.

12. Ye W, M Bouchard, D Stone, X Liu, F Vella, J Lee, H Nakamura, SL Ang, M Busslinger and A Rosenthal. (2001). Distinct regulators control the expression of the mid-hindbrain organizer signal FGF8. Nat Neurosci 4:1175-81.

13. Pattyn A, N Simplicio, JH van Doorninck, C Goridis, F Guillemot and JF Brunet. (2004). Ascl1/Mash1 is required for the development of central serotonergic neurons. Nat Neurosci 7:589-95.

14. Hendricks T, N Francis, D Fyodorov and ES Deneris. (1999). The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. J Neurosci 19:10348-56.

15. van Doorninck JH, J van Der Wees, A Karis, E Goedknegt, JD Engel, M Coesmans, M Rutteman, F Grosveld and CI De Zeeuw. (1999). GATA-3 is involved in the development of serotonergic neurons in the caudal raphe nuclei. J Neurosci 19:RC12.

16. Hendricks TJ, DV Fyodorov, LJ Wegman, NB Lelutiu, EA Pehek, B Yamamoto, J Silver, EJ Weeber, JD Sweatt and ES Deneris. (2003). Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37:233-47.

17. Ding YQ, U Marklund, W Yuan, J Yin, L Wegman, J Ericson, E Deneris, RL Johnson and ZF Chen. (2003). Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 6:933-8.

18. Cheng L, CL Chen, P Luo, M Tan, M Qiu, R Johnson and Q Ma. (2003). Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. J Neurosci 23:9961-7.

19. Zhao ZQ, M Scott, S Chiechio, JS Wang, KJ Renner, RWt Gereau, RL Johnson, ES Deneris and ZF Chen. (2006). Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. J Neurosci 26:12781-8.

20. Smidt MP, CH Asbreuk, JJ Cox, H Chen, RL Johnson and JP Burbach. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3:337-41.

21. Kim JH, JM Auerbach, JA Rodriguez-Gomez, I Velasco, D Gavin, N Lumelsky, SH Lee, J Nguyen, R Sanchez-Pernaute, K Bankiewicz and R McKay. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50-6.

22. Reubinoff BE, MF Pera, CY Fong, A Trounson and A Bongso. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399-404.

23. Ben-Hur T, M Idelson, H Khaner, M Pera, E Reinhartz, A Itzik and BE Reubinoff. (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells 22:1246-55.

24. Feil R, J Wagner, D Metzger and P Chambon. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun 237:752-7.

25. Mangeot PE, D Negre, B Dubois, AJ Winter, P Leissner, M Mehtali, D Kaiserlian, FL Cosset and JL Darlix. (2000). Development of minimal lentivirus vectors derived from simian immunodeficiency virus (SIVmac251) and their use for gene transfer into human dendritic cells. J Virol 74:8307-15.

26. Wianny F, A Bernat, G Marcy, C Huissoud, S Markossian, V Leviel, H Kennedy, P Savatier and C Dehay. (2008). Derivation and cloning of a novel Rhesus ES cell line stably expressing tau-GFP. Stem Cells 26:1444-1453.

27. Suter DM, L Cartier, E Bettiol, D Tirefort, ME Jaconi, M Dubois-Dauphin and KH Krause. (2006). Rapid generation of stable transgenic embryonic stem cell lines using modular lentivectors. Stem Cells 24:615-23.

28. Vallier L, J Mancip, S Markossian, A Lukaszewicz, C Dehay, D Metzger, P Chambon, J Samarut and P Savatier. (2001). An efficient system for conditional gene expression in embryonic stem cells and in their in vitro and in vivo differentiated derivatives. Proc Natl Acad Sci U S A 98:2467-72.

29. Srinivas S, T Watanabe, CS Lin, CM William, Y Tanabe, TM Jessell and F Costantini. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4.

30. Wiles MV and BM Johansson. (1999). Embryonic stem cell development in a chemically defined medium. Exp Cell Res 247:241-8.

31. Tropepe V, S Hitoshi, C Sirard, TW Mak, J Rossant and D van der Kooy. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 30:65-78.

32.   Negre D, PE Mangeot, G Duisit, S Blanchard, PO Vidalain, P Leissner, AJ Winter, C Rabourdin-Combe, M Mehtali, P Moullier, JL Darlix and FL Cosset. (2000). Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. Gene Ther 7:1613-23.

33.   Dull T, R Zufferey, M Kelly, RJ Mandel, M Nguyen, D Trono and L Naldini. (1998). A third-generation lentivirus vector with a conditional packaging system. J Virol 72:8463-71.

34.   Martinat C, JJ Bacci, T Leete, J Kim, WB Vanti, AH Newman, JH Cha, U Gether, H Wang and A Abeliovich. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103:2874-9.
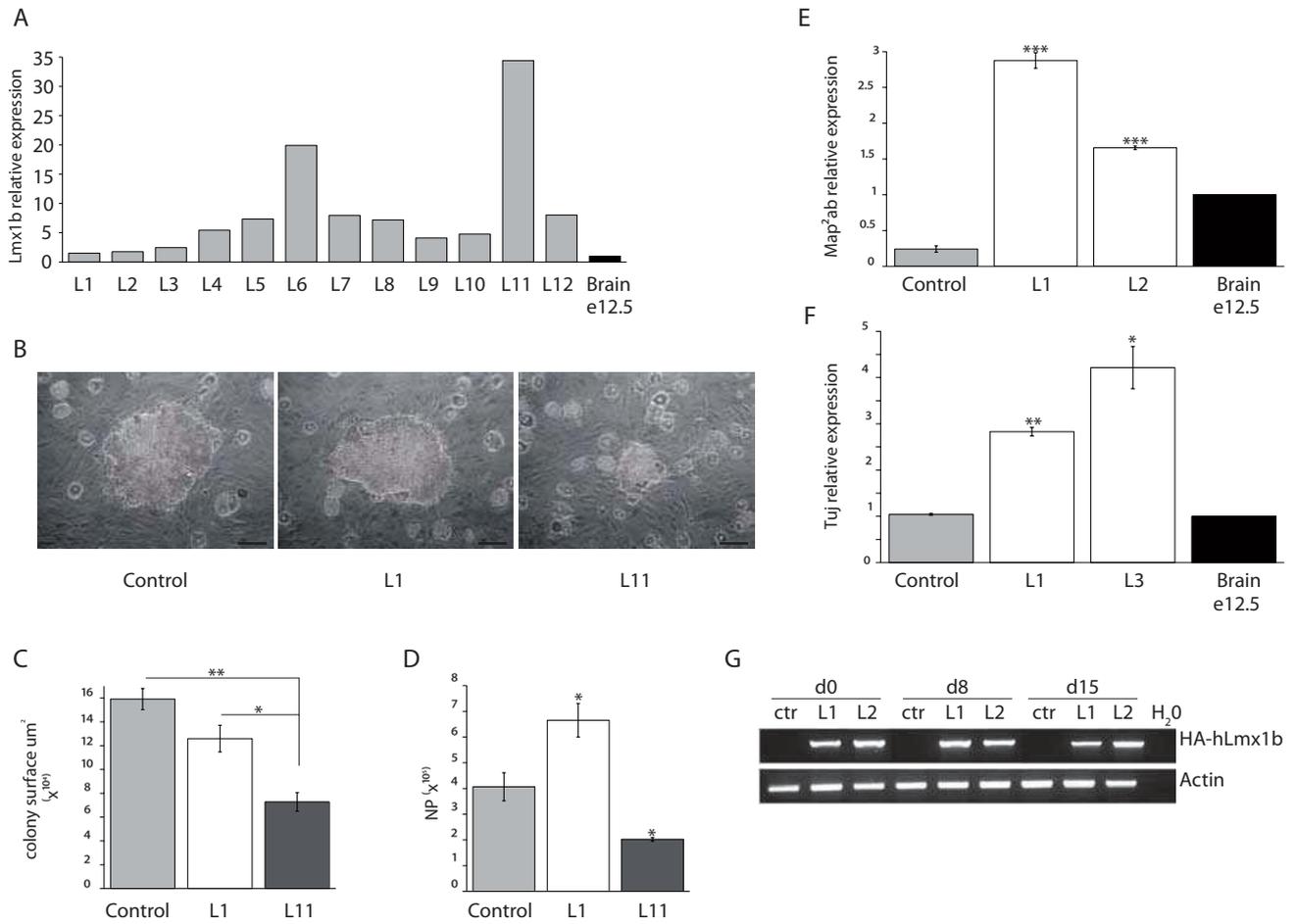
Figure 1: Neuronal differentiation of mES cells overexpressing hLmx1b.
(A) Real-time PCR analysis of hLmx1b mRNA level in mES cell clones (L1 to L12) infected with p2K7-HAhLmx1b.
(B) Neuroepithelial colonies at day 8 of differentiation formed by clones L1, L11, and control cells ("MS5 protocol"). Scale bar, 100µm.
(C) Surface of neuroepithelial colonies at day 8 of differentiation in clones L1 and L11, and with control cells ("MS5 protocol").
(D) Counting of neural progenitors (NPs) at day 12 of differentiation obtained with clones L1 and L11, and in control cells ("MS5 protocol").
(E) Real-time PCR analysis of Map2ab expression levels at day 15 of differentiation with clones L1 and L2, and with control cells ("MS5 protocol").
(F) Real-time PCR analysis of Tuj1 expression levels at day 17 of differentiation in clones L1 and L3, and with control cells ("EB protocol").
(G) Semi-quantitative RT-PCR analysis of HA-hLmx1b expression in clones L1 and L2, and in control cells, at day 0 (mES cells), day 8 (neuroepithelial colonies), and day 15 (post-mitotic neurons) ("MS5 protocol").
(C-F) Histograms represent means and standard errors calculated in three replicates (statistical significance was determined with the unpaired two-sided t test ($p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***). (A, E, F) Expression levels are normalized to the level measured in e12.5 total brain.
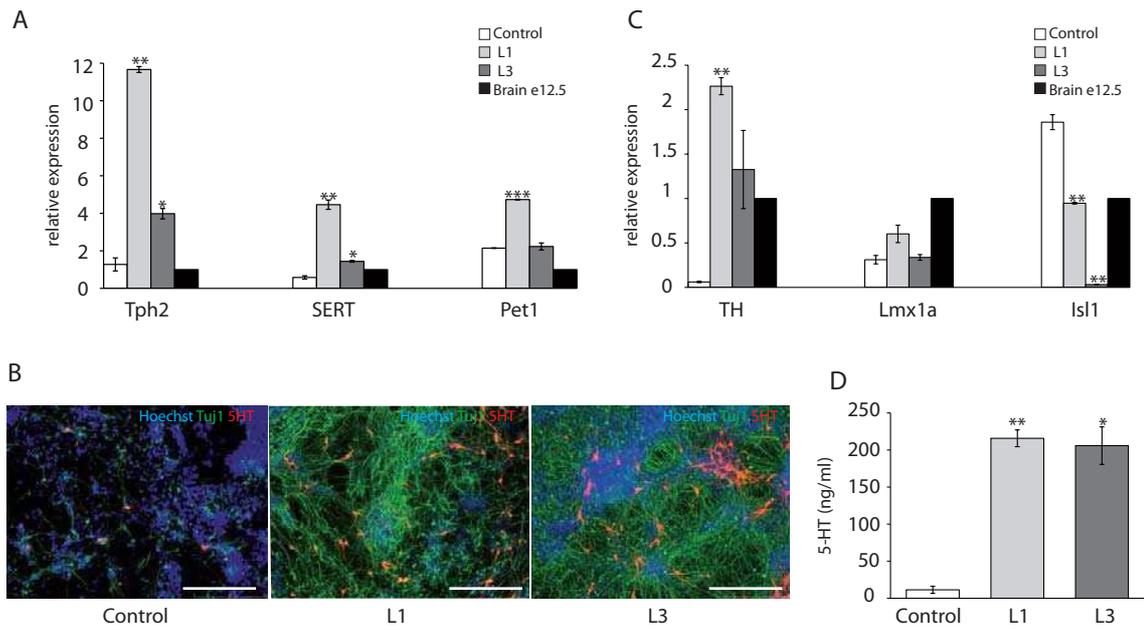
Figure 2: Serotonergic and dopaminergic differentiation of mES cells overexpressing hLmx1b ("EB protocol").
(A,C) Real-time PCR analysis of Tph2, SERT and Pet1 (A), TH, Lmx1a and Isl1 (C), mRNA levels in clones L1 and L3, and in control cells, at day 17 of differentiation. Expression levels are normalized to the level measured in e12.5 total brain.
(B) Double immunofluorescence analysis of Tuj1 (green)- and 5-HT (red)-positive neurons, performed at differentiation day 17 of clones L1 and L3, and control cells. Nuclei are labeled with Hoechst. Scale bar, 250 μm.
(D) Quantification of 5-HT in cell extracts of neuron cultures at day 17 of differentiation by serotonin Elisa.
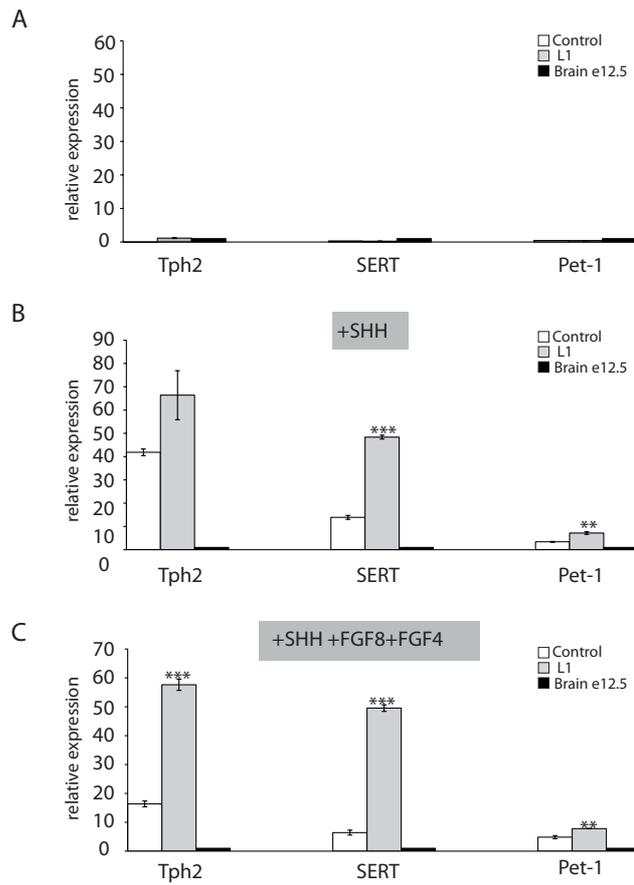
Figure 3: Real-time PCR analysis of Tph2, SERT and Pet1 expression in clone L1 and in control cells after differentiation induced by co-culture with MS5 stromal cells.
(A) Differentiation without dorsoventralizing factors.
(B) Differentiation in the presence of Shh.
(C) Differentiation with Shh + FGF4 + FGF8.
(A-C) Histograms represent means and standard errors calculated in three replicates (statistical significance was determined with the unpaired two-sided t test
($p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***).
Expression levels are normalized to the level measured in e12.5 total brain.
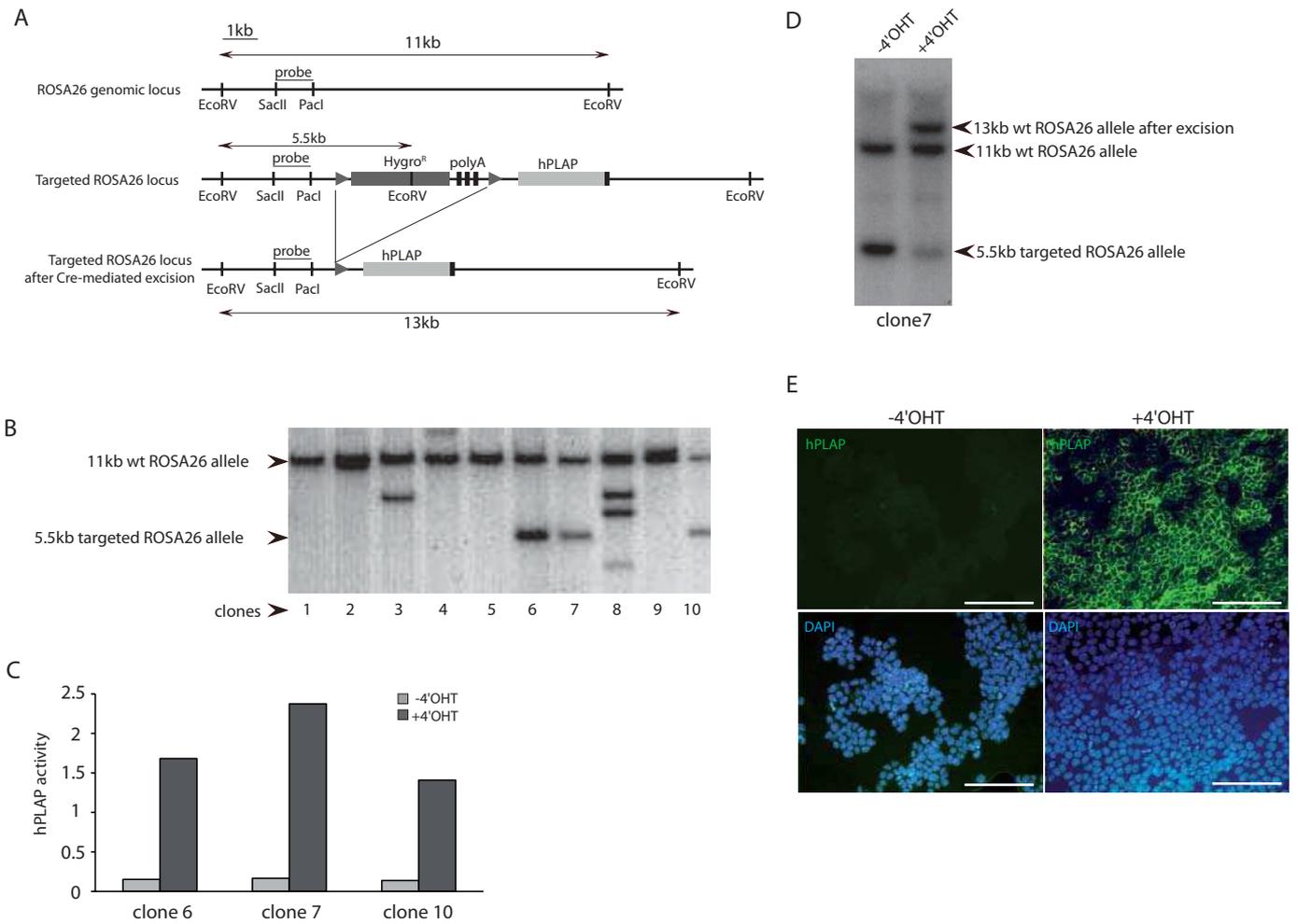
Figure 4: Generation of an inducible expression vector and ES cell line for conditional gene expression.
(A) Schematic representation of the ROSA26 locus after targeted integration of pIGTE2-R26-hPLAP plasmid, prior to,
and after Cre-mediated excision of the hygro-polyA cassette. The position of the SacII-PacI probe used for Southern blot analysis is indicated.
HygroR, gene conferring resistance to hygromycin B; PolyA, transcription termination and polyadenylation signals.
(B) Southern blot analysis the ROSA26 locus after digestion with EcoRV and hybridization with a radioactive probe.
(C) hPLAP activity measured in three independent undifferentiated WTC15-R26-hPLAP clones before and after treatment
with 100 nM 4'OHT for 48 hours.
(D) Southern blot analysis the ROSA26 locus in WTC15-R26-hPLAP-7 clone after digestion with EcoRV,
before and after treatment with 4'OHT (100 nM, 48 hours).
(E) Immunofluorescence analysis of hPLAP expression in neural progenitors and post-mitotic neurons,
before and after treatment with 4'OHT (100 nM, 48 hours).
Low panels show Hoechst staining of DNA. Scale bar: 100 μm.
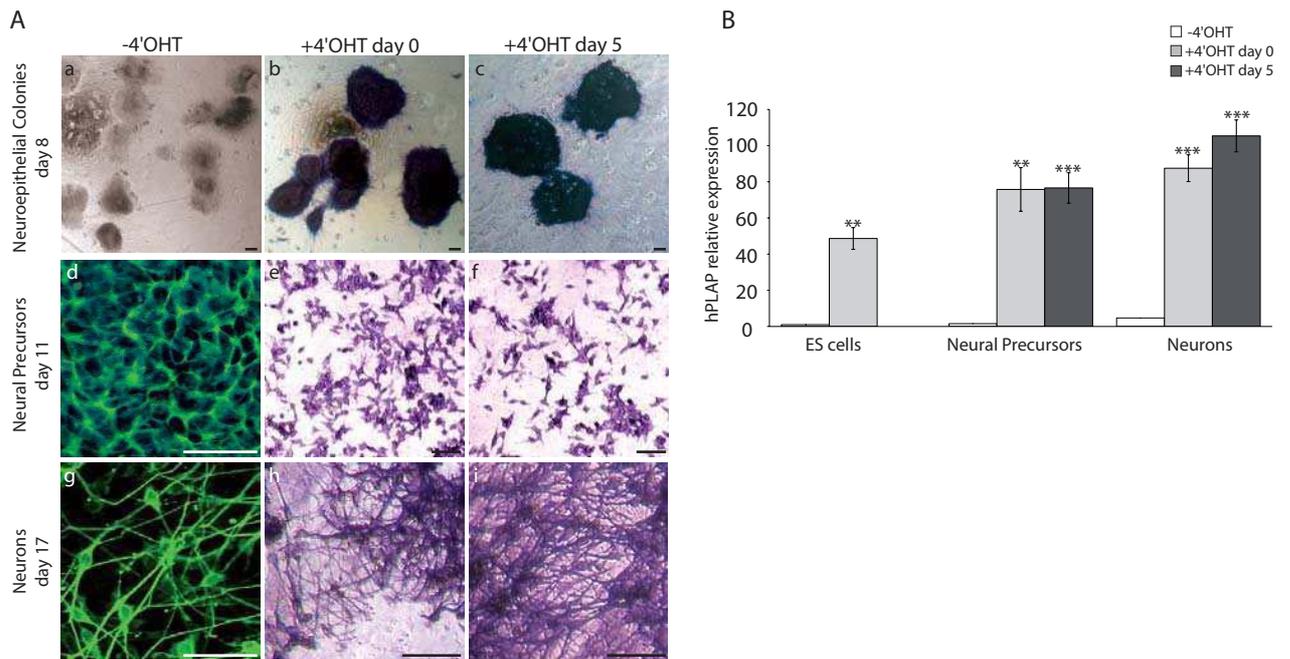
Figure 5: Conditional expression of hPLAP in neuroepthelial colonies, neural progenitors (NPs) and post-mitotic neurons.
(A) Immunohistochemical analysis of hPLAP expression in neuroepithelial colonies at day 8 (b,c), in neural progenitors (NPs)
at day 11 (e,f), and in neurons at day 15, following induction with 4'OHT (100 nM, 48 hours), either in undifferentiated mES cells (b,e,h)
or in neuroepithelial colonies at day 5 (c,f,i). Left panels show unstained neuroepithelial colonies (a),
NPs after immunolabelling for PSA-NCAM (green) (d), and neurons after immunolabelling for Tuj1 (green) (g).
DNA is counterstained with Hoechst. Scale bar, 100 μm.
(B) Real-time PCR analysis of hPLAP expression in ES cells after induction at day 0,
in day 11 NPs after induction both at day 0 and at day 5, and in day 15 neurons after induction both at day 0 and at day 5.
Expression levels are normalized to the level measured in mES cells prior to induction.
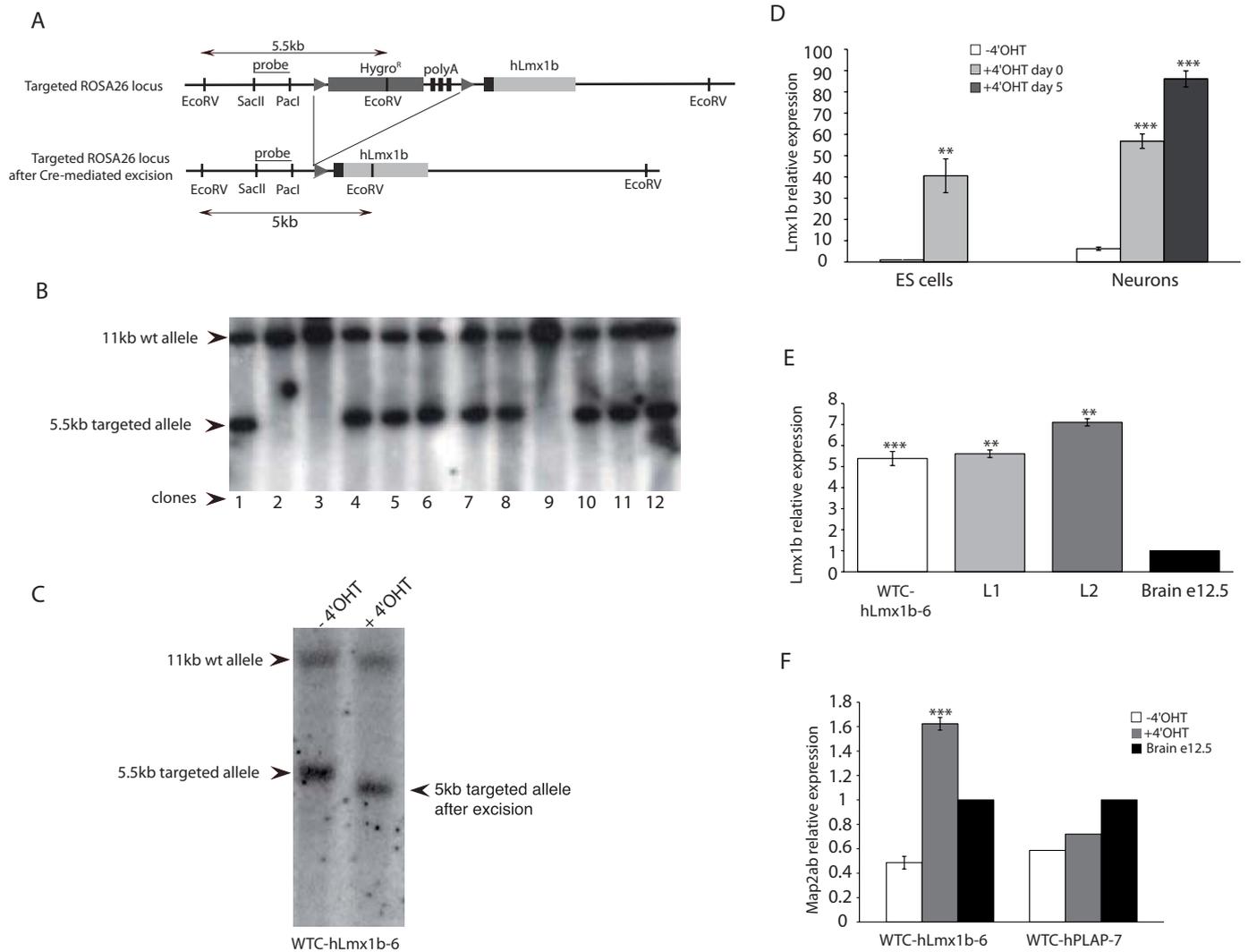
Figure 6: Conditional expression of hLmx1b in neurons.
(A) Schematic representation of the ROSA26 locus after targeted integration of pIGTE2-R26-hLmx1b plasmid,
prior to, and after Cre-mediated excision of the hygro-polyA cassette.
The position of the SacII-PacI probe used for Southern blot analysis is indicated.
HygroR, gene conferring resistance to hygromycin B; PolyA, transcription termination and polyadenylation signals.
(B) Southern blot analysis the ROSA26 locus after digestion with EcoRV and hybridization with a radioactive probe.
(C) Southern blot analysis the ROSA26 locus in WTC15-R26-hLmx1b-6 clone after digestion with EcoRV,
before and after treatment with 4'OHT (100 nM, 48 hours).
(D) Real-time PCR analysis of hLmx1b expression in neurons derived from WTC-Lmx1b-6 clone after induction
with 4'OHT either at day 0 (undifferentiated ES cells) or at day 5 (neuroepithelial colonies).
(E) Real-time PCR analysis of hLmx1b expression in neurons derived from WTC-Lmx1b-6 clone (after induction with 4'OHT at day 5)
and in neurons derived from hLmx1b-stably expressing clones L1 and L2.
(F) Real-time PCR analysis of Map2ab expression in neurons derived from WTC-hPLAP-7 and WTC-hLmx1b-6 clone,
after induction with 4'OHT at day 5.
(E,F) Expression levels are normalized to the level measured in e12.5 total brain.
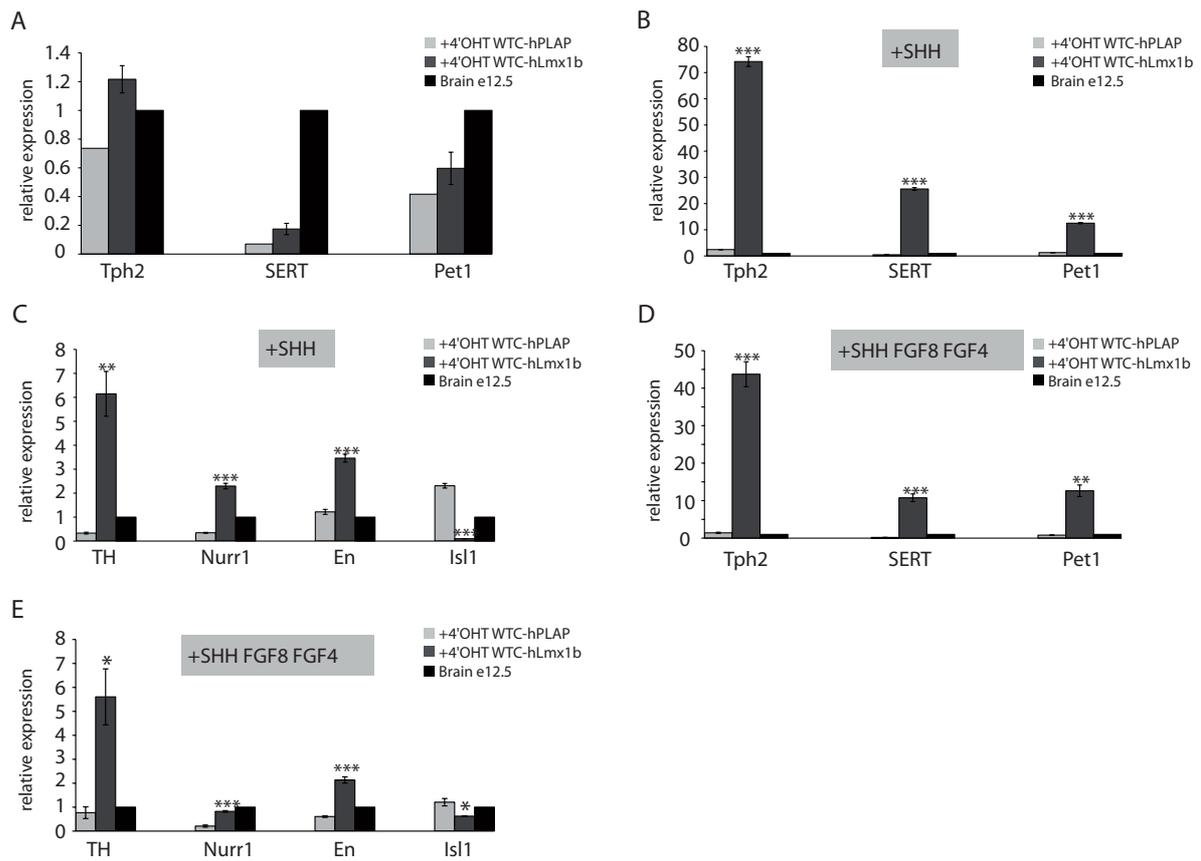
Figure 7: Inducible expression of hLmx1b in ES-derived NPs increases expression of serotonergic markers.
(A,B,D) Real-time PCR analysis of Pet1, Tph2 and SERT mRNA levels in day 18 neurons derived from WTC-HLmx1b-6 clone after induction with 4'OHT at day 5 (neuroepithelial colonies), either in the absence of dorsoventralizing factors (A), or in the presence of Shh (B), or in the presence of Shh, FGF4 and FGF8 (D).
(C,E) Real-time PCR analysis of En, TH, Nurr1 and Isl1 mRNA levels in day 18 neurons derived from WTC-HLmx1b-6 clone after induction with 4'OHT at day 5 (neuroepithelial colonies), either in the absence of dorsoventralizing factors (C), or in the presence of Shh, FGF4 and FGF8 (E).
(A-E) Expression levels are normalized to the level measured in e12.5 total brain.

Gene	Forward Primer	Reverse primer
hPLAP	GGTGAACCGCAACTGGTACT	CATGAGATGGGTCACAGACG
HA-hLmx1b	CCATACGACGTCCCAGACTAC	CTGCCAGTGTCTCTCGGACCTT
hLmx1b	GCCTCGATCCTCCCTTTATC	GACTCGTTGACTCGCATCAG
Lmx1b (mouse+human)	TCCTGATGCGAGTCAACGAGTC	CTGCCAGTGTCTCTCGGACCTT
b-actin	TGAAACAACATACAATTCCATCATGAAGTGTGA	AGGAGCGATAATCTTGATCTTCATGGTGCT
En1	CCTACTCATGGGTTCGGCTA	GATATAGCGGTTTGCCTGGA
Isl1	AGCTGTACGTGCTTTGTTAGGGAT	TCTTCTCGGGCTGTTTGTGGA
Lmx1a	GCAGAGGAGAGCATTCAGG	CTTCTGAGGTTGCCAGGAAG
Map2ab	AGCCGCAACGCCAATGGATT	TTTGTTCCGAGGCTGGCGAT
Nurr1	CCTTGTGTTCAGGCGCAGTATG	TGGCTGTGTTGCTGGTAGTTGTG
Pet1	CCAGTTGTGGCAGTTTCTCC	CTGGAAGTCAAAGCGGTAGG
SERT	CTGGGTTTGGATAGCACGTT	TATTGGAAAAGCCGGAGTTG
TH	AGGGATGGGAATGCTGTTCTCA	ACCAGGTGGTGACACTTGTCCAA
Tph2	CCATCGGAGAATTGAAGCAT	TTCAATGCTCTGCTGGTAGG
Tuj1	AACTATGTAGGGGACTCAGACCTGC	TCTCACACTCTTTCCGCACGAC

Table I: Primers used for PCR

DISCUSSION

La science consiste à oublier ce qu'on croit savoir,
et la sagesse à ne pas s'en soucier.
Charles Nodier

DISCUSSION

I   Stable expression of transgenes in ESC and clonal variability

The study of genetic programmes underlying differentiation of ESC into specialized cell types often rely on the use of the "gain-of-function" experimental paradigm. This paradigm is based on the forced, ectopic, expression of one or several transgenes in undifferentiated ESC, and the study of the resulting phenotype in a large variety of experimental conditions *in vitro* and *in vivo*. Lentiviral vectors are commonly used to introduce transgenes into ESC and express them in a stable manner. Of note, transgene expression is influenced by a number of factors, including local chromatin status and proviral copy number. One limitation of this technology is the capacity of ESC to sustain transgene expression while remaining undifferentiated. Forced expression of a transgene that drives differentiation can result in the inability to form undifferentiated mES colonies and consequent recovery only of low expressing cells. Another risk is the recovery of mutant clones that exhibit stronger capacity to overcome differentiation signals owing to mutations harnessing self-renewal. Here, we successfully generated mESC lines that express Lmx1b ectopically, but only the lines expressing it at low level retained the capacity to differentiate into neural precursors and post-mitotic neurons harboring the growth characteristics of control cells. In contrast, mESC expressing Lmx1b at high levels exhibited reduced capacity to form neural progenitors, and those progenitors to differentiate into post-mitotic neurons. As explained above, failure to differentiate could result, either from the detrimental effect of Lmx1b when over-expressed at elevated levels, or from the selection of mutant mESC that resisted to the differentiation-promoting effect of Lmx1b.

II   Generation of an inducible expression vector and ESC line for conditional gene expression in neural precursors and post-mitotic neurons

Inducible modification of gene expression provides a powerful tool for studying gene function both *in vitro* and *in vivo*. Inducible gain-of-function is a powerful strategy to assess single gene function in regulated and cell-specific manner. Many systems for controlled gene expression in ESC have been designed (O'Gorman et al., 1991; Lewandoski, 2001), however few of them have been proven to be appropriate for transgene expression after differentiation (Vallier et al., 2007). Furthermore, few of them have been characterized beyond the proof of principle (Masui et al., 2005; Vallier et al., 2007). We demonstrated our system useful for

controlling the expression of lineage-commitment genes during neural differentiation of mouse ESC, allowing the engineering of a specific neuronal phenotype.

Targeted integration of a transgene or an expression vector into the *ROSA26* locus so as to place the coding sequence under the regularory elements of *ROSA26* is widely used in mouse transgenesis to achieve stable and ubiquitous expression of the transgene (Zambrowicz et al., 1997; Srinivas et al., 2001). The *ROSA26* locus can be efficiently targeted by homologous recombination both in mouse and in human ESC, even though at rather low efficiency in the latter ones (Irion et al., 2007). In our work, targeted integration of *hPLAP* (human placental alkaline phosphatase) and *hLmx1b* (human Lmx1b) into *ROSA26* is achieved with an efficiency of approximately 30%. After Cre-mediated recombination, the *hPLAP* transgene is expressed in virtually all cells with negligible background. We showed that integration of the inducible expression vectors into the ROSA26 locus is efficient and results in stable expression of the transgene in ESC as well as during neuronal differentiation.

Cre/loxP site-specific recombination system is a commonly used technique for controlling the expression of target genes flanked by loxP sites. It gives the possibility of genetically engineering the mouse genome and of creating conditional mutants in both cultured cells and animals. Yet, frequently, the recombination of loxP-modified alleles requires the introduction of foreign DNA vector into the cells for expression of Cre recombinase. The drawback of such an approach is that continuous expression of Cre recombinase from the foreign vector may yield genotoxicity in various cells (Adams and van der Weyden, 2001; Silver and Livingston, 2001; Baba et al., 2005). Cre-ER^{T2}, a fusion protein between the Cre recombinase and the ligand-binding domain of the mutated estrogen receptor, binding only artificial estrogens, allows tight regulation of Cre recombinase activity as it strictly depends on the addition of synthetic compound to the culture medium. Several studies have confirmed its successful application for conditional activation of gene expression *in vitro* in mouse ESC and *in vivo* (Vallier et al., 2001) during various stages of mouse development, as well as in human ESC (Vallier et al., 2007). Recently, the Tat technology was used for intracellular delivery of Cre by protein transduction, thus enabling rapid and highly efficient conditional gene expression in ESC and their somatic ES derivatives (Nolden et al., 2007). In a recent study (Haupt et al., 2007), Tat-Cre was used to induce the expression of reporter gene in neural progeny derived from ESC with high efficiency. Neural precursor cells exhibited a normal phenotype and were able to differentiate into neurons and/or glial cells, indicating that Cre treatment had no obvious side effect on cell proliferation and neural differentiation.

In our work, the stable expression of hPLAP after induction in undifferentiated cells and during neural differentiation proved that the designed system can be easily applied to the expression of any chosen gene during and after neural induction. We did not observe any deleterious effect of prolonged expression of CreER[T2] on ESC proliferation and differentiation.

III ESC overexpressing Lmx1b show preferential differentiation toward serotonergic lineage *in vitro*

The presence of cells at different developmental stages in the culture during differentiation limits their use as a tool for various applications ranging from drug testing to cell therapy and regenerative medicine. We assumed that the expression of lineage-commitment gene involved in serotonergic development would produce cell cultures enriched into serotonergic neurons. The Lmx1b gene has been chosen for this purpose. This is an essential factor controlling inductive activity of isthmic organizer and consequently midbrain-hindbrain development (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). It has also been shown essential to the differentiation and survival of the entire serotonergic system as Lmx1b knock-out mice lack all central 5-HT neurons (Cheng et al., 2003; Ding et al., 2003b; Zhao et al., 2006). An equally important goal of our experiments was to further explore the role of Lmx1b in serotonergic differentiation.

The effect of Lmx1b on serotonergic differentiation was explored using two different neuronal differentiation protocols. The first one relies on the co-culture of ES cells with the stromal cell line MS5, which releases the so-called "stromal derived-inducing activity" or SDIA (Kawasaki et al., 2000). The second protocol is based on EB formation (Tropepe et al., 2001; Alenina et al., 2006). For the sake of clarity, these protocols will hereafter be referred to as MS5 and EB protocols, respectively. Both protocols include a selection and amplification step of neural progenitor cells. Both have been used to study the capacity of mESC to support 5-HT neuron differentiation (Kim et al., 2002; Barberi et al., 2003; Alenina et al., 2006). The MS5 protocol leads to 7% of neurons adopting a serotonergic phenotype, whereas the EB protocol leads to 10% of 5-HT neurons, in the absence of any dorsoventralizing factors. In both protocols, addition of dorsoventralizing factors to the culture of neural/neuronal progenitors increase the yield of 5-HT neurons by 2,5- to 8-fold, depending on studies (Kawasaki et al., 2000; Kim et al., 2002; Barberi et al., 2003; Alenina et al., 2006). Thus, both protocols seemed appropriate to explore the capacity of Lmx1b to support serotonergic

differentiation of mouse ESC, both in the absence and in the presence of dorsoventralizing factors.

With both stably (L1 cells) and 4'hydroxytamoxifen (4'OHT)-induced (WTC15-hlmx1b) Lmx1b expressing mESC, we observed that Lmx1b increases the yield of neurons expressing Tph2, SERT, and Pet1, the landmarks of serotonergic neurons. With 4'OHT-induced Lmx1b expressing cells the effect is moderate, albeit significant, using either differentiation protocols. In contrast, with mESC which stably express Lmx1b at low level (low expressors), only the EB protocol allows Lmx1b to promote serotonergic differentiation in the absence of dorsoventralizing factors. It can be argued that the MS5 protocol generates fewer neural precursors permissive to Lmx1b action and, therefore, requires stronger Lmx1b transgene expression to trigger serotonergic differentiation. This hypothesis remains to be experimentally validated. Immunofluorescence analysis shows an increase in the number of 5-HT neurons produced with the Lmx1b-expressing clone L1 compared to control cells. Together, these results indicate that overexpression of Lmx1b in mES-derived neural progenitors increases the yield of 5'HT neurons. However, it is not sufficient to impose a serotonergic phenotype on the whole neuron population, which indicates that Lmx1b must act cooperatively with other factors to trigger the serotonergic differentiation programme.

IV Lmx1b cooperates with Shh to induce serotonergic differentiation.

According to the prevailing model, the development of 5-HT neurons depends critically on the floor plate signal sonic hedgehog (Shh), fibroblast growth factor 8 (FGF8) from the midbrain-hindbrain boundary, and on the early pre-patterning signal FGF4 (Ye et al., 1998). When added sequentially to mES cell-derived neural progenitors, they promote 5-HT differentiation with strong efficiency (Barberi et al., 2003; Alenina et al., 2006).

We observed that the effect of 4'OHT-induced Lmx1b expression on serotonergic differentiation is strongly enhanced when neural progenitors are concomitantly treated with Shh. The combined actions of Shh and Lmx1b results in 30-, 55-, and 10-fold increase in *Tph2*, *SERT*, and *Pet1* expression, respectively. By contrast, overexpression of Lmx1b alone results in 1,6-, 2,5-, and 1,4-fold increase in *Tph2*, *SERT*, and *Pet1* expression, respectively. These results demonstrate that Lmx1b and Shh act in synergy to activate the serotonergic differentiation programme.

Such a synergy between Shh and Lmx1b may be explained by the capacity of Shh to activate the expression of ancillary factors acting in concert with Lmx1b to promote serotonergic differentiation. One critical factor is Pet1 whose expression, together with the expression of Lmx1b, is induced by Nkx2.2 in response to Shh signaling (Cheng et al., 2003; Ding et al., 2003b). *Pet1* contributes to serotonergic differentiation since 70% of serotonergic neurons fail to differentiate in Pet1-deficient mice (Hendricks et al., 1999;Hendricks et al., 2003), and it was shown that both *Lmx1b* and *Pet1* are necessary and sufficient to specify 5-HT transmitter phenotype when overexpressed in the chick ventral spinal cord (Cheng et al., 2003). Whether *Lmx1b* and *Pet1* act strictly in parallel to specify 5-HT neurotransmitter phenotype (Cheng et al., 2003) or whether *Pet1* is also a target gene of Lmx1b (Ding et al., 2003b) is still unclear. We observed that *Pet1* expression is activated in neurons overexpressing *hLmx1b*, but that this activation is moderate compared to the activation *SERT* and *Tph2* expression. These data support the prevailing model that Lmx1b and Pet1 transcription factors act in parallel to specify the 5-HT phenotype, and that Lmx1b reinforces the transcriptional activity of *Pet1* induced by Shh *via* Nkx2.2 (Cheng et al., 2003; Ding et al., 2003b).

The development of 5-HT neurons depends critically on FGF8 generated at the midbrain-hindbrain boundary and on FGF4 produced by the primitive streak. *In vivo*, FGF4 inhibits the development of midbrain DA neurons and promotes the development of 5-HT neurons (Ye et al., 1998). In clone L1 that stably expresses Lmx1b, addition of Shh, FGF8 and FGF4 to neural progenitors confers a less than 2-fold increase in the expression of serotonergic markers, when compared to clone L1 treated with Shh alone. Furthermore, in mES cell-derived neural progenitors treated with 4'OHT to activate Lmx1b expression, addition of Shh, FGF4 and FGF8 has no effect on the yield of serotonergic differentiation, when compared to a sister culture treated with Shh alone. Thus, when Lmx1b is overexpressed, FGF8 and FGF4 become dispensable. This finding suggests that activation of Lmx1b expression is the main consequence of FGF8 and FGF4 induction in the developing neural progenitors acquiring a 5-HT neuron fate.

V   Role of Lmx1b in dopaminergic differentiation

Lmx1b has been shown to play a crucial role in the development of midbrain DA neurons in the mouse. More specifically, Lmx1b is required for the maintenance of the DA neurotransmitter phenotype by Nurr-1 independent pathway (Smidt et al., 2000). Our

experiments show that overexpression of Lmx1b, either alone or in combination with Shh, has little or no effect on the yield of dopaminergic neuron differentiation. This is in accordance with a previous report (Martinat et al., 2006) showing that transduction of mouse and human ESC with *Lmx1b* does not induce maturation to the midbrain DA neuron phenotype unless the orphan nuclear receptor Nurr1 is co-transduced in the same cells. Together, these results indicate that Lmx1b is necessary to midbrain DA neuron differentiation and survival, but it not sufficient to drive uncommitted neural progenitors into a DA neuron pathway.

After Lmx1b induction and treatment with Shh, FGF8 and FGF4, the expression of midbrain DA neuron markers TH and Nurr1 is reduced when compared to their expression in a sister culture treated with Shh alone. FGF8 is required for the development of both midbrain DA and 5'HT neurons (Alexandre et al., 2006). By contrast, FGF4 has been shown to inhibit the development of DA neurons and to promote the development of 5-HT neurons (Ye et al., 1998).

FUTURE DIRECTIONS

Dans la vie, il n'y a pas de solutions.
Il y a des forces en marche.
Antoine de Saint-Exupéry

FUTURE DIRECTIONS

WTC15-hLmx1b cells carry the hLmx1b cDNA knocked into the 1st allele of ROSA26. Lmx1b expression is induced after treatment with 4'OHT to excise out the floxed STOP of transcription inserted between the ROSA26 promoter and the hLmx1b cDNA. Since both alleles of ROSA26 can be disrupted without any detrimental effect on development (Srinivas et al., 2001), the 2nd allele of ROSA26 could also be targeted, using a second knockin vector to introduce a 2nd gene of interest. A 2nd knockin vector has been engineered in the laboratory but it has not yet been tested. Such a dual inducible expression system might be instrumental to explore the capacity of Lmx1b, in combination with Pet1, to induce serotonergic phenotype when overexpressed in neural progenitors.

WTC15-hLmx1b cell line could be used to analyze the transcriptome of Lmx1b in neural progenitors and post-mitotic neurons, by comparing gene expression profiles of induced (+4'OHT) and non-induced (-4'OHT) cells in a DNA array experiment. This analysis would help exploring the molecular mechanisms of serotonergic differentiation.

Last, WTC15-hLmx1b cells could be used in cell transplantation experiments to rescue the phenotype of mice deficient in 5-HT neurons such as the *Lmx1b* null mice (Guo et al., 2007), or deficient in serotonin such as the *Tph2* null mice (Alenina et al., 2009). $Tph2^{-/-}$ mice lack serotonin in the central nervous system. Surprisingly, these mice can be born and survive until adulthood. However, depletion of serotonin signaling in the brain leads to growth retardation and 50% lethality in the first four weeks of postnatal life. They exhibit extended daytime sleep, suppressed respiration, altered body temperature control, and decreased blood pressure and heart rate during nighttime. $Tph2^{-/-}$ females also exhibit impaired maternal care leading to poor survival of their pups (Alenina et al., 2009). Should WTC15-hLmx1b-derived neural progenitors (with or without prior treatment with Shh) partly rescue the phenotype of $Tph2^{-/-}$ mice, it would demonstrate their functionality *in vivo*.

REFERENCES

REFERENCES

Abeliovich, A. and R. Hammond (2007). "Midbrain dopamine neuron differentiation: factors and fates." Dev Biol **304**(2): 447-54.

Adachi, K., E. Kawase, et al. (2006). "Establishment of the gene-inducible system in primate embryonic stem cell lines." Stem Cells **24**(11): 2566-72.

Adams, D. J. and L. van der Weyden (2001). "Are we creating problems? Negative effects of Cre recombinase." Genesis **29**(3): 115.

Adams, K. A., J. M. Maida, et al. (2000). "The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer." Development **127**(9): 1857-67.

Aizenman, Y. and J. de Vellis (1987). "Brain neurons develop in a serum and glial free environment: effects of transferrin, insulin, insulin-like growth factor-I and thyroid hormone on neuronal survival, growth and differentiation." Brain Res **406**(1-2): 32-42.

Alenina, N., S. Bashammakh, et al. (2006). "Specification and differentiation of serotonergic neurons." Stem Cell Rev **2**(1): 5-10.

Alenina, N., D. Kikic, et al. (2009). "Growth retardation and altered autonomic control in mice lacking brain serotonin." Proc Natl Acad Sci U S A **106**(25): 10332-7.

Alexandre, P., I. Bachy, et al. (2006). "Positive and negative regulations by FGF8 contribute to midbrain roof plate developmental plasticity." Development **133**(15): 2905-13.

Ali, N. N., A. J. Edgar, et al. (2002). "Derivation of type II alveolar epithelial cells from murine embryonic stem cells." Tissue Eng **8**(4): 541-50.

Alison, M. R., S. Islam, et al. (2009). "Cell therapy for liver disease." Curr Opin Mol Ther **11**(4): 364-74.

Amit, M., M. K. Carpenter, et al. (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." Dev Biol **227**(2): 271-8.

Andersson, E., J. B. Jensen, et al. (2006a). "Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2." Development **133**(3): 507-16.

Andersson, E., U. Tryggvason, et al. (2006b). "Identification of intrinsic determinants of midbrain dopamine neurons." Cell **124**(2): 393-405.

Asano, M. and P. Gruss (1992). "Pax-5 is expressed at the midbrain-hindbrain boundary during mouse development." Mech Dev **39**(1-2): 29-39.

Asbreuk, C. H., C. F. Vogelaar, et al. (2002). "CNS expression pattern of Lmx1b and coexpression with ptx genes suggest functional cooperativity in the development of forebrain motor control systems." Mol Cell Neurosci **21**(3): 410-20.

Avilion, A. A., S. K. Nicolis, et al. (2003). "Multipotent cell lineages in early mouse development depend on SOX2 function." Genes Dev **17**(1): 126-40.

Baba, Y., M. Nakano, et al. (2005). "Practical range of effective dose for Cre recombinase-expressing recombinant adenovirus without cell toxicity in mammalian cells." Microbiol Immunol **49**(6): 559-70.

Bagutti, C., A. M. Wobus, et al. (1996). "Differentiation of embryonal stem cells into keratinocytes: comparison of wild-type and beta 1 integrin-deficient cells." Dev Biol **179**(1):

184-96.

Bain, G., D. Kitchens, et al. (1995). "Embryonic stem cells express neuronal properties in vitro." Dev Biol **168**(2): 342-57.

Bain, G., W. J. Ray, et al. (1996). "Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture." Biochem Biophys Res Commun **223**(3): 691-4.

Barberi, T., P. Klivenyi, et al. (2003). "Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice." Nat Biotechnol **21**(10): 1200-7.

Barzilai, A. and E. Melamed (2003). "Molecular mechanisms of selective dopaminergic neuronal death in Parkinson's disease." Trends Mol Med **9**(3): 126-32.

Bautch, V. L., W. L. Stanford, et al. (1996). "Blood island formation in attached cultures of murine embryonic stem cells." Dev Dyn **205**(1): 1-12.

Becker-Hapak, M., S. S. McAllister, et al. (2001). "TAT-mediated protein transduction into mammalian cells." Methods **24**(3): 247-56.

Ben-Dor, I., P. Itsykson, et al. (2006). "Lentiviral vectors harboring a dual-gene system allow high and homogeneous transgene expression in selected polyclonal human embryonic stem cells." Mol Ther **14**(2): 255-67.

Ben-Hur, T., M. Idelson, et al. (2004). "Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats." Stem Cells **22**(7): 1246-55.

Bertrand, N., D. S. Castro, et al. (2002). "Proneural genes and the specification of neural cell types." Nat Rev Neurosci **3**(7): 517-30.

Bjorklund, A. and S. B. Dunnett (2007). "Dopamine neuron systems in the brain: an update." Trends Neurosci **30**(5): 194-202.

Blakely, R. D., H. E. Berson, et al. (1991). "Cloning and expression of a functional uptake-specific antidepressants, including paroxetine, citalopram and fluoxetine.

Boheler, K. R., J. Czyz, et al. (2002). "Differentiation of pluripotent embryonic stem cells into cardiomyocytes." Circ Res **91**(3): 189-201.

Bradley, A., M. Evans, et al. (1984). "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines." Nature **309**(5965): 255-6.

Bradley, A., P. Hasty, et al. (1992). "Modifying the mouse: design and desire." Biotechnology (N Y) **10**(5): 534-9.

Branton, R. L., R. M. Love, et al. (1998). "cAMP included during cell suspension preparation improves survival of dopaminergic neurons in vitro." Neuroreport **9**(14): 3223-7.

Brevini, T. A., V. Tosetti, et al. (2007). "Derivation and characterization of pluripotent cell lines from pig embryos of different origins." Theriogenology **67**(1): 54-63.

Brewer, G. J. (1995). "Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus." J Neurosci Res **42**(5): 674-83.

Briscoe, J., L. Sussel, et al. (1999). "Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling." Nature **398**(6728): 622-7.

Brodski, C., D. M. Weisenhorn, et al. (2003). "Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain-hindbrain organizer." J Neurosci **23**(10): 4199-207.

Brustle, O., K. N. Jones, et al. (1999). "Embryonic stem cell-derived glial precursors: a source of myelinating transplants." Science **285**(5428): 754-6.

Buehr, M., S. Meek, et al. (2008). "Capture of authentic embryonic stem cells from rat blastocysts." Cell **135**(7): 1287-98.

Burbach, J. P. and M. P. Smidt (2006). "Molecular programming of stem cells into mesodiencephalic dopaminergic neurons." Trends Neurosci **29**(11): 601-3.

Buttery, L. D., S. Bourne, et al. (2001). "Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells." Tissue Eng **7**(1): 89-99.

Cai, D., K. Deng, et al. (2002). "Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro." Neuron **35**(4): 711-9.

Carlsson, T., M. Carta, et al. (2007). "Serotonin neuron transplants exacerbate L-DOPA-induced dyskinesias in a rat model of Parkinson's disease." J Neurosci **27**(30): 8011-22.

Castelo-Branco, G., J. Wagner, et al. (2003). "Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a." Proc Natl Acad Sci U S A **100**(22): 12747-52.

Castillo, S. O., J. S. Baffi, et al. (1998). "Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene." Mol Cell Neurosci **11**(1-2): 36-46.

Chambers, I., D. Colby, et al. (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell **113**(5): 643-55.

Chen, H., Y. Lun, et al. (1998a). "Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome." Nat Genet **19**(1): 51-5.

Chen, H., D. Ovchinnikov, et al. (1998b). "Multiple calvarial defects in lmx1b mutant mice." Dev Genet **22**(4): 314-20.

Chen, N. and M. E. Reith (2000). "Structure and function of the dopamine transporter." Eur J Pharmacol **405**(1-3): 329-39.

Chen, S. S., W. Fitzgerald, et al. (2007). "Cell-cell and cell-extracellular matrix interactions regulate embryonic stem cell differentiation." Stem Cells **25**(3): 553-61.

Cheng, L., C. L. Chen, et al. (2003). "Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype." J Neurosci **23**(31): 9961-7.

Cherry, S. R., D. Biniszkiewicz, et al. (2000). "Retroviral expression in embryonic stem cells and hematopoietic stem cells." Mol Cell Biol **20**(20): 7419-26.

Chizhikov, V. V. and K. J. Millen (2004a). "Control of roof plate development and signaling by Lmx1b in the caudal vertebrate CNS." J Neurosci **24**(25): 5694-703.

Chizhikov, V. V. and K. J. Millen (2004b). "Mechanisms of roof plate formation in the vertebrate CNS." Nat Rev Neurosci **5**(10): 808-12.

Cho, S. K., T. D. Webber, et al. (1999). "Functional characterization of B lymphocytes

generated in vitro from embryonic stem cells." Proc Natl Acad Sci U S A **96**(17): 9797-802.

Chung, S., E. Hedlund, et al. (2005). "The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons." Mol Cell Neurosci **28**(2): 241-52.

Chung, S., A. Leung, et al. (2009). "Wnt1-lmx1a forms a novel autoregulatory loop and controls midbrain dopaminergic differentiation synergistically with the SHH-FoxA2 pathway." Cell Stem Cell **5**(6): 646-58.

Chung, S., K. C. Sonntag, et al. (2002). "Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons." Eur J Neurosci **16**(10): 1829-38.

Coraux, C., C. Hilmi, et al. (2003). "Reconstituted skin from murine embryonic stem cells." Curr Biol **13**(10): 849-53.

Craven, S. E., K. C. Lim, et al. (2004). "Gata2 specifies serotonergic neurons downstream of sonic hedgehog." Development **131**(5): 1165-73.

Curtiss, J. and J. S. Heilig (1998). "DeLIMiting development." Bioessays **20**(1): 58-69.

Daheron, L., S. L. Opitz, et al. (2004). "LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells." Stem Cells **22**(5): 770-8.

Dai, J. X., R. L. Johnson, et al. (2009). "Manifold functions of the Nail-Patella Syndrome gene Lmx1b in vertebrate development." Dev Growth Differ **51**(3): 241-50.

Dani, C., A. G. Smith, et al. (1997). "Differentiation of embryonic stem cells into adipocytes in vitro." J Cell Sci **110 (Pt 11)**: 1279-85.

Danielian, P. S. and A. P. McMahon (1996). "Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development." Nature **383**(6598): 332-4.

Darr, H., Y. Mayshar, et al. (2006). "Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features." Development **133**(6): 1193-201.

Davis, C. A. and A. L. Joyner (1988). "Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development." Genes Dev **2**(12B): 1736-44.

Dawson, T. M. and V. L. Dawson (2003). "Molecular pathways of neurodegeneration in Parkinson's disease." Science **302**(5646): 819-22.

Dietz, G. P. and M. Bahr (2004). "Delivery of bioactive molecules into the cell: the Trojan horse approach." Mol Cell Neurosci **27**(2): 85-131.

Ding, S., T. Y. Wu, et al. (2003a). "Synthetic small molecules that control stem cell fate." Proc Natl Acad Sci U S A **100**(13): 7632-7.

Ding, Y. Q., U. Marklund, et al. (2003b). "Lmx1b is essential for the development of serotonergic neurons." Nat Neurosci **6**(9): 933-8.

Djalali, S., M. Holtje, et al. (2005). "Effects of brain-derived neurotrophic factor (BDNF) on glial cells and serotonergic neurones during development." J Neurochem **92**(3): 616-27.

Doetschman, T. C., H. Eistetter, et al. (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and

myocardium." <u>J Embryol Exp Morphol</u> **87**: 27-45.

Dreyer, S. D., R. Morello, et al. (2000). "LMX1B transactivation and expression in nail-patella syndrome." <u>Hum Mol Genet</u> **9**(7): 1067-74.

Durbec, P., C. V. Marcos-Gutierrez, et al. (1996). "GDNF signalling through the Ret receptor tyrosine kinase." <u>Nature</u> **381**(6585): 789-93.

Elkabetz, Y., G. Panagiotakos, et al. (2008). "Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage." <u>Genes Dev</u> **22**(2): 152-65.

Epstein, D. J., A. P. McMahon, et al. (1999). "Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms." <u>Development</u> **126**(2): 281-92.

Erceg, S., M. Ronaghi, et al. (2009). "Human embryonic stem cell differentiation toward regional specific neural precursors." <u>Stem Cells</u> **27**(1): 78-87.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." <u>Nature</u> **292**(5819): 154-6.

Farkas, L. M., N. Dunker, et al. (2003). "Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo." <u>J Neurosci</u> **23**(12): 5178-86.

Ferri, A. L., W. Lin, et al. (2007). "Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner." <u>Development</u> **134**(15): 2761-9.

Finley, M. F., N. Kulkarni, et al. (1996). "Synapse formation and establishment of neuronal polarity by P19 embryonic carcinoma cells and embryonic stem cells." <u>J Neurosci</u> **16**(3): 1056-65.

Flanders, K. C., G. Ludecke, et al. (1991). "Localization and actions of transforming growth factor-beta s in the embryonic nervous system." <u>Development</u> **113**(1): 183-91.

Fon, E. A., E. N. Pothos, et al. (1997). "Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action." <u>Neuron</u> **19**(6): 1271-83.

Fraichard, A., O. Chassande, et al. (1995). "In vitro differentiation of embryonic stem cells into glial cells and functional neurons." <u>J Cell Sci</u> **108 (Pt 10)**: 3181-8.

Friling, S., E. Andersson, et al. (2009). "Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells." <u>Proc Natl Acad Sci U S A</u> **106**(18): 7613-8.

Gajovic, S., L. St-Onge, et al. (1997). "Retinoic acid mediates Pax6 expression during in vitro differentiation of embryonic stem cells." <u>Differentiation</u> **62**(4): 187-92.

Gale, E. and M. Li (2008). "Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells." <u>Mol Brain</u> **1**(1): 8.

Gaspar, P., O. Cases, et al. (2003). "The developmental role of serotonin: news from mouse molecular genetics." <u>Nat Rev Neurosci</u> **4**(12): 1002-12.

Geijsen, N., M. Horoschak, et al. (2004). "Derivation of embryonic germ cells and male gametes from embryonic stem cells." <u>Nature</u> **427**(6970): 148-54.

Gilad, V. H., W. G. Tetzlaff, et al. (1996). "Accelerated recovery following polyamines and aminoguanidine treatment after facial nerve injury in rats." <u>Brain Res</u> **724**(1): 141-4.

Goetz, A. K., B. Scheffler, et al. (2006). "Temporally restricted substrate interactions direct fate and specification of neural precursors derived from embryonic stem cells." Proc Natl Acad Sci U S A **103**(29): 11063-8.

Goridis, C. and H. Rohrer (2002). "Specification of catecholaminergic and serotonergic neurons." Nat Rev Neurosci **3**(7): 531-41.

Grez, M., E. Akgun, et al. (1990). "Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells." Proc Natl Acad Sci U S A **87**(23): 9202-6.

Guo, C., H. Y. Qiu, et al. (2007). "Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice." Development

Hamazaki, T., Y. Iiboshi, et al. (2001). "Hepatic maturation in differentiating embryonic stem cells in vitro." FEBS Lett **497**(1): 15-9.

Harada, J. and M. Sugimoto (1997). "Polyamines prevent apoptotic cell death in cultured cerebellar granule neurons." Brain Res **753**(2): 251-9.

Harvey, M., E. Shink, et al. (2004). "Support for the involvement of TPH2 gene in affective disorders." Mol Psychiatry **9**(11): 980-1.

Haupt, S., F. Edenhofer, et al. (2007). "Stage-specific conditional mutagenesis in mouse embryonic stem cell-derived neural cells and postmitotic neurons by direct delivery of biologically active Cre recombinase." Stem Cells **25**(1): 181-8.

Hayes, B., S. R. Fagerlie, et al. (2008). "Derivation, characterization, and in vitro differentiation of canine embryonic stem cells." Stem Cells **26**(2): 465-73.

Hedlund, E., J. Pruszak, et al. (2008). "Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease." Stem Cells **26**(6): 1526-36.

Hendricks, T., N. Francis, et al. (1999). "The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes." J Neurosci **19**(23): 10348-56.

Hendricks, T. J., D. V. Fyodorov, et al. (2003). "Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior." Neuron **37**(2): 233-47.

Hobert, O. and H. Westphal (2000). "Functions of LIM-homeobox genes." Trends Genet **16**(2): 75-83.

Hubner, K., G. Fuhrmann, et al. (2003). "Derivation of oocytes from mouse embryonic stem cells." Science **300**(5623): 1251-6.

Hwang, D. Y., P. Ardayfio, et al. (2003). "Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice." Brain Res Mol Brain Res **114**(2): 123-31.

Hynes, M., J. A. Porter, et al. (1995a). "Induction of midbrain dopaminergic neurons by Sonic hedgehog." Neuron **15**(1): 35-44.

Hynes, M., K. Poulsen, et al. (1995b). "Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons." Cell **80**(1): 95-101.

Hynes, M. and A. Rosenthal (1999). "Specification of dopaminergic and serotonergic neurons in the vertebrate CNS." Curr Opin Neurobiol **9**(1): 26-36.

Inamdar, N., D. Arulmozhi, et al. (2007). "Parkinson's disease: genetics and beyond." Curr Neuropharmacol **5**(2): 99-113.

Ingham, P. W. (2008). "Hedgehog signalling." Curr Biol **18**(6): R238-41.

Ingham, P. W. and A. P. McMahon (2001). "Hedgehog signaling in animal development: paradigms and principles." Genes Dev **15**(23): 3059-87.

Irion, S., H. Luche, et al. (2007). "Identification and targeting of the ROSA26 locus in human embryonic stem cells." Nat Biotechnol **25**(12): 1477-82.

Itsykson, P., N. Ilouz, et al. (2005). "Derivation of neural precursors from human embryonic stem cells in the presence of noggin." Mol Cell Neurosci **30**(1): 24-36.

Jacobs, F. M., S. van Erp, et al. (2009). "Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression." Development **136**(4): 531-40.

Jaeger, C. B., D. A. Ruggiero, et al. (1984). "Aromatic L-amino acid decarboxylase in the rat brain: immunocytochemical localization in neurons of the brain stem." Neuroscience **11**(3): 691-713.

Jakob, H., T. Boon, et al. (1973). "[Teratocarcinoma of the mouse: isolation, culture and properties of pluripotential cells]." Ann Microbiol (Paris) **124**(3): 269-82.

Kalir, H. H. and C. Mytilineou (1991). "Ascorbic acid in mesencephalic cultures: effects on dopaminergic neuron development." J Neurochem **57**(2): 458-64.

Kawano, H., K. Ohyama, et al. (1995). "Migration of dopaminergic neurons in the embryonic mesencephalon of mice." Brain Res Dev Brain Res **86**(1-2): 101-13.

Kawasaki, H., K. Mizuseki, et al. (2000). "Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity." Neuron **28**(1): 31-40.

Kawasaki, H., H. Suemori, et al. (2002). "Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity." Proc Natl Acad Sci U S A **99**(3): 1580-5.

Kele, J., N. Simplicio, et al. (2006). "Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons." Development **133**(3): 495-505.

Keller, G. (2005). "Embryonic stem cell differentiation: emergence of a new era in biology and medicine." Genes Dev **19**(10): 1129-55.

Keller, G., M. Kennedy, et al. (1993). "Hematopoietic commitment during embryonic stem cell differentiation in culture." Mol Cell Biol **13**(1): 473-86.

Kiecker, C. and A. Lumsden (2005). "Compartments and their boundaries in vertebrate brain development." Nat Rev Neurosci **6**(7): 553-64.

Kim, J. H., J. M. Auerbach, et al. (2002). "Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease." Nature **418**(6893): 50-6.

Kim, J. J., J. C. Shih, et al. (1997). "Selective enhancement of emotional, but not motor, learning in monoamine oxidase A-deficient mice." Proc Natl Acad Sci U S A **94**(11): 5929-33.

Klug, M. G., M. H. Soonpaa, et al. (1996). "Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts." J Clin Invest **98**(1): 216-24.

Kodama, H., H. Hagiwara, et al. (1986). "MC3T3-G2/PA6 preadipocytes support in vitro proliferation of hemopoietic stem cells through a mechanism different from that of interleukin 3." J Cell Physiol **129**(1): 20-6.

Kolossov, E., T. Bostani, et al. (2006). "Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium." J Exp Med **203**(10): 2315-27.

Kramer, J., C. Hegert, et al. (2000). "Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4." Mech Dev **92**(2): 193-205.

Kumar, M., S. K. Kaushalya, et al. (2009). "Optimized derivation and functional characterization of 5-HT neurons from human embryonic stem cells." Stem Cells Dev **18**(4): 615-27.

Kuo, H. C., K. Y. Pau, et al. (2003). "Differentiation of monkey embryonic stem cells into neural lineages." Biol Reprod **68**(5): 1727-35.

Kurosawa, H., M. Kimura, et al. (2006). "Effect of oxygen on in vitro differentiation of mouse embryonic stem cells." J Biosci Bioeng **101**(1): 26-30.

Lambe, E. K., L. S. Krimer, et al. (2000). "Differential postnatal development of catecholamine and serotonin inputs to identified neurons in prefrontal cortex of rhesus monkey." J Neurosci **20**(23): 8780-7.

Lang, A. E. and A. M. Lozano (1998a). "Parkinson's disease. First of two parts." N Engl J Med **339**(15): 1044-53.

Lang, A. E. and A. M. Lozano (1998b). "Parkinson's disease. Second of two parts." N Engl J Med **339**(16): 1130-43.

Law, S. W., O. M. Conneely, et al. (1992). "Identification of a new brain-specific transcription factor, NURR1." Mol Endocrinol **6**(12): 2129-35.

Lee, H., G. A. Shamy, et al. (2007). "Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons." Stem Cells **25**(8): 1931-9.

Lee, S. H., N. Lumelsky, et al. (2000). "Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells." Nat Biotechnol **18**(6): 675-9.

Lee, S. M., P. S. Danielian, et al. (1997). "Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain." Development **124**(5): 959-69.

Lewandoski, M. (2001). "Conditional control of gene expression in the mouse." Nat Rev Genet **2**(10): 743-55.

Li, J., M. Puceat, et al. (2002). "Calreticulin reveals a critical Ca(2+) checkpoint in cardiac myofibrillogenesis." J Cell Biol **158**(1): 103-13.

Li, M., L. Pevny, et al. (1998). "Generation of purified neural precursors from embryonic stem cells by lineage selection." Curr Biol **8**(17): 971-4.

Lidov, H. G. and M. E. Molliver (1982). "An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields." Brain Res Bull **8**(4): 389-430.

Lin, L. F., D. H. Doherty, et al. (1993). "GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons." Science **260**(5111): 1130-2.

Lin, Q., D. Jo, et al. (2004). "Enhanced cell-permeant Cre protein for site-specific recombination in cultured cells." BMC Biotechnol **4**: 25.

Lin, R. Y., A. Kubo, et al. (2003). "Committing embryonic stem cells to differentiate into thyrocyte-like cells in vitro." Endocrinology **144**(6): 2644-9.

Lin, W., E. Metzakopian, et al. (2009). "Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development." Dev Biol **333**(2): 386-96.

Liu, A. and A. L. Joyner (2001). "Early anterior/posterior patterning of the midbrain and cerebellum." Annu Rev Neurosci **24**: 869-96.

Lucki, I. (1998). "The spectrum of behaviors influenced by serotonin." Biol Psychiatry **44**(3): 151-62.

Lumsden, A. (2004). "Segmentation and compartition in the early avian hindbrain." Mech Dev **121**(9): 1081-8.

Lumsden, A. and R. Krumlauf (1996). "Patterning the vertebrate neuraxis." Science **274**(5290): 1109-15.

Maherali, N. and K. Hochedlinger (2008). "Guidelines and techniques for the generation of induced pluripotent stem cells." Cell Stem Cell **3**(6): 595-605.

Martin, G. R. (1980). "Teratocarcinomas and mammalian embryogenesis." Science **209**(4458): 768-76.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A **78**(12): 7634-8.

Martinat, C., J. J. Bacci, et al. (2006). "Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype." Proc Natl Acad Sci U S A **103**(8): 2874-9.

Masui, S., D. Shimosato, et al. (2005). "An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit." Nucleic Acids Res **33**(4): e43.

Matsunaga, E., T. Katahira, et al. (2002). "Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development." Development **129**(22): 5269-77.

Maxwell, S. L., H. Y. Ho, et al. (2005). "Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development." Dev Biol **282**(2): 467-79.

McCaffery, P. and U. C. Drager (1994). "High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system." Proc Natl Acad Sci U S A **91**(16): 7772-6.

Meyers, E. N., M. Lewandoski, et al. (1998). "An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination." Nat Genet **18**(2): 136-41.

Miller, G. W., R. R. Gainetdinov, et al. (1999). "Dopamine transporters and neuronal injury." Trends Pharmacol Sci **20**(10): 424-9.

Mintz, B. and K. Illmensee (1975). "Normal genetically mosaic mice produced from malignant teratocarcinoma cells." Proc Natl Acad Sci U S A **72**(9): 3585-9.

Muhr, J., E. Andersson, et al. (2001). "Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube." Cell **104**(6): 861-73.

Nakamura, H., T. Katahira, et al. (2005). "Isthmus organizer for midbrain and hindbrain development." Brain Res Brain Res Rev **49**(2): 120-6.

Nakano, T., H. Kodama, et al. (1994). "Generation of lymphohematopoietic cells from embryonic stem cells in culture." Science **265**(5175): 1098-101.

Nilsen, J. and R. D. Brinton (2002). "Impact of progestins on estrogen-induced neuroprotection: synergy by progesterone and 19-norprogesterone and antagonism by medroxyprogesterone acetate." Endocrinology **143**(1): 205-12.

Nishikawa, S. I., S. Nishikawa, et al. (1998). "Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages." Development **125**(9): 1747-57.

Niswander, L. and G. R. Martin (1992). "Fgf-4 expression during gastrulation,

Niwa, H., T. Burdon, et al. (1998). "Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3." Genes Dev **12**(13): 2048-60.

Niwa, H., J. Miyazaki, et al. (2000). "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells." Nat Genet **24**(4): 372-6.

Nolden, L., F. Edenhofer, et al. (2006). "Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase." Nat Methods **3**(6): 461-7.

Nolden, L., F. Edenhofer, et al. (2007). "Stem cell engineering using transducible Cre recombinase." Methods Mol Med **140**: 17-32.

Nunes, I., L. T. Tovmasian, et al. (2003). "Pitx3 is required for development of substantia nigra dopaminergic neurons." Proc Natl Acad Sci U S A **100**(7): 4245-50.

O'Gorman, S., D. T. Fox, et al. (1991). "Recombinase-mediated gene activation and site-specific integration in mammalian cells." Science **251**(4999): 1351-5.

O'Hara, F. P., E. Beck, et al. (2005). "Zebrafish Lmx1b.1 and Lmx1b.2 are required for maintenance of the isthmic organizer." Development **132**(14): 3163-73.

Okabe, S., K. Forsberg-Nilsson, et al. (1996). "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro." Mech Dev **59**(1): 89-102.

Ono, Y., T. Nakatani, et al. (2007). "Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells." Development **134**(17): 3213-25.

Pacey, L., S. Stead, et al. "Neural Stem Cell Culture: Neurosphere generation, microscopical analysis and cryopreservation." Nature protocols.

Pain, B., M. E. Clark, et al. (1996). "Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities." Development **122**(8): 2339-48.

Pankratz, M. T., X. J. Li, et al. (2007). "Directed neural differentiation of human embryonic stem cells via an obligated primitive anterior stage." Stem Cells **25**(6): 1511-20.

Park, C. H., Y. K. Minn, et al. (2005). "In vitro and in vivo analyses of human

embryonic stem cell-derived dopamine neurons." J Neurochem **92**(5): 1265-76.

Patient, R. K. and J. D. McGhee (2002). "The GATA family (vertebrates and invertebrates)." Curr Opin Genet Dev **12**(4): 416-22.

Pattyn, A., N. Simplicio, et al. (2004). "Ascl1/Mash1 is required for the development of central serotonergic neurons." Nat Neurosci **7**(6): 589-95.

Pattyn, A., A. Vallstedt, et al. (2003). "Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors." Genes Dev **17**(6): 729-37.

Penev, P. D., P. C. Zee, et al. (1994). "Monoamine depletion blocks triazolam-induced phase advances of the circadian clock in hamsters." Brain Res **637**(1-2): 255-61.

Perrier, A. L. and L. Studer (2003). "Making and repairing the mammalian brain--in vitro production of dopaminergic neurons." Semin Cell Dev Biol **14**(3): 181-9.

Perrier, A. L., V. Tabar, et al. (2004). "Derivation of midbrain dopamine neurons from human embryonic stem cells." Proc Natl Acad Sci U S A **101**(34): 12543-8.

Pesce, M. and H. R. Scholer (2001). "Oct-4: gatekeeper in the beginnings of mammalian development." Stem Cells **19**(4): 271-8.

Pevny, L. and M. Placzek (2005). "SOX genes and neural progenitor identity." Curr Opin Neurobiol **15**(1): 7-13.

Philp, D., S. S. Chen, et al. (2005). "Complex extracellular matrices promote tissue-specific stem cell differentiation." Stem Cells **23**(2): 288-96.

Placzek, M. and J. Briscoe (2005). "The floor plate: multiple cells, multiple signals." Nat Rev Neurosci **6**(3): 230-40.

Prakash, N., C. Brodski, et al. (2006). "A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo." Development **133**(1): 89-98.

Pressman, C. L., H. Chen, et al. (2000). "LMX1B, a LIM homeodomain class transcription factor, is necessary for normal development of multiple tissues in the anterior segment of the murine eye." Genesis **26**(1): 15-25.

Puelles, E. (2007). "Genetic control of basal midbrain development." J Neurosci Res **85**(16): 3530-4.

Puelles, E., A. Annino, et al. (2004). "Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain." Development **131**(9): 2037-48.

Puelles, L. (2001). "Brain segmentation and forebrain development in amniotes." Brain Res Bull **55**(6): 695-710.

Puelles, L. and J. L. Rubenstein (2003). "Forebrain gene expression domains and the evolving prosomeric model." Trends Neurosci **26**(9): 469-76.

Rapport, M. M., A. A. Green, et al. (1948). "Serum vasoconstrictor, serotonin; isolation and characterization." J Biol Chem **176**(3): 1243-51.

Reubinoff, B. E., M. F. Pera, et al. (2000). "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." Nat Biotechnol **18**(4): 399-404.

Reynolds, B. A. and S. Weiss (1992). "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." Science **255**(5052): 1707-10.

Richard, E., F. Geronimi, et al. (2003). "A bicistronic SIN-lentiviral vector containing

G156A MGMT allows selection and metabolic correction of hematopoietic protoporphyric cell lines." J Gene Med **5**(9): 737-47.

Rohwedel, J., K. Guan, et al. (1999). "Induction of cellular differentiation by retinoic acid in vitro." Cells Tissues Organs **165**(3-4): 190-202.

Rohwedel, J., V. Maltsev, et al. (1994). "Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents." Dev Biol **164**(1): 87-101.

Rolletschek, A., H. Chang, et al. (2001). "Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors." Mech Dev **105**(1-2): 93-104.

Rowitch, D. H. and A. P. McMahon (1995). "Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1." Mech Dev **52**(1): 3-8.

Roy, N. S., C. Cleren, et al. (2006). "Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes." Nat Med **12**(11): 1259-68.

Salli, U., A. P. Reddy, et al. (2004). "Serotonin neurons derived from rhesus monkey embryonic stem cells: similarities to CNS serotonin neurons." Exp Neurol **188**(2): 351-64.

Samad, O. A., M. J. Geisen, et al. (2004). "Integration of anteroposterior and dorsoventral regulation of Phox2b transcription in cranial motoneuron progenitors by homeodomain proteins." Development **131**(16): 4071-83.

Sandberg, M., M. Kallstrom, et al. (2005). "Sox21 promotes the progression of vertebrate neurogenesis." Nat Neurosci **8**(8): 995-1001.

Sauer, B. and N. Henderson (1989). "Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome." Nucleic Acids Res **17**(1): 147-61.

Schmandt, T., E. Meents, et al. (2005). "High-purity lineage selection of embryonic stem cell-derived neurons." Stem Cells Dev **14**(1): 55-64.

Schmitt, R. M., E. Bruyns, et al. (1991). "Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression." Genes Dev **5**(5): 728-40.

Schwartz, P. H., D. J. Brick, et al. (2008). "Differentiation of neural lineage cells from human pluripotent stem cells." Methods **45**(2): 142-58.

Scott, M. M., C. J. Wylie, et al. (2005). "A genetic approach to access serotonin neurons for in vivo and in vitro studies." Proc Natl Acad Sci U S A **102**(45): 16472-7.

Semina, E. V., R. S. Reiter, et al. (1997). "Isolation of a new homeobox gene belonging to the Pitx/Rieg family: expression during lens development and mapping to the aphakia region on mouse chromosome 19." Hum Mol Genet **6**(12): 2109-16.

Shimamura, K., D. J. Hartigan, et al. (1995). "Longitudinal organization of the anterior neural plate and neural tube." Development **121**(12): 3923-33.

Silver, D. P. and D. M. Livingston (2001). "Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity." Mol Cell **8**(1): 233-43.

Simon, H. H., H. Saueressig, et al. (2001). "Fate of midbrain dopaminergic neurons controlled by the engrailed genes." J Neurosci **21**(9): 3126-34.

Simon, H. H., C. Scholz, et al. (2005). "Engrailed genes control developmental fate of serotonergic and noradrenergic neurons in mid- and hindbrain in a gene dose-dependent manner." Mol Cell Neurosci **28**(1): 96-105.

Simon, H. H., S. Thuret, et al. (2004). "Midbrain dopaminergic neurons: control of their cell fate by the engrailed transcription factors." Cell Tissue Res **318**(1): 53-61.

Skaper, S. D., R. Adler, et al. (1979). "A procedure for purifying neuron-like cells in cultures from central nervous tissue with a defined medium." Dev Neurosci **2**(5): 233-7.

Smidt, M. P., C. H. Asbreuk, et al. (2000). "A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b." Nat Neurosci **3**(4): 337-41.

Smidt, M. P. and J. P. Burbach (2007). "How to make a mesodiencephalic dopaminergic neuron." Nat Rev Neurosci **8**(1): 21-32.

Smidt, M. P., S. M. Smits, et al. (2004). "Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3." Development **131**(5): 1145-55.

Smidt, M. P., H. S. van Schaick, et al. (1997). "A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons." Proc Natl Acad Sci U S A **94**(24): 13305-10.

Smith, A. G. (2001). "Embryo-derived stem cells: of mice and men." Annu Rev Cell Dev Biol **17**: 435-62.

Smith, A. G., J. K. Heath, et al. (1988). "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides." Nature **336**(6200): 688-90.

Smits, S. M., J. P. Burbach, et al. (2006). "Developmental origin and fate of meso-diencephalic dopamine neurons." Prog Neurobiol **78**(1): 1-16.

Solter, D., N. Skreb, et al. (1970). "Extrauterine growth of mouse egg-cylinders results in malignant teratoma." Nature **227**(5257): 503-4.

Spitere, K., A. Toulouse, et al. (2008). "TAT-PAX6 protein transduction in neural progenitor cells: a novel approach for generation of dopaminergic neurones in vitro." Brain Res **1208**: 25-34.

Srinivas, S., T. Watanabe, et al. (2001). "Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus." BMC Dev Biol **1**: 4.

Stevens, L. C. (1970). "The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos." Dev Biol **21**(3): 364-82.

Stoffel, M., L. Vallier, et al. (2004). "Navigating the pathway from embryonic stem cells to beta cells." Semin Cell Dev Biol **15**(3): 327-36.

Strubing, C., G. Ahnert-Hilger, et al. (1995). "Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons." Mech Dev **53**(2): 275-87.

Suemori, H., T. Tada, et al. (2001). "Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI." Dev Dyn **222**(2): 273-9.

Sukoyan, M. A., S. Y. Vatolin, et al. (1993). "Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies." Mol Reprod Dev **36**(2): 148-58.

Sun, X., E. N. Meyers, et al. (1999). "Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo." Genes Dev **13**(14): 1834-46.

Suter, D. M., L. Cartier, et al. (2006). "Rapid generation of stable transgenic embryonic stem cell lines using modular lentivectors." Stem Cells **24**(3): 615-23.

Suter, D. M. and K. H. Krause (2008). "Neural commitment of embryonic stem cells: molecules, pathways and potential for cell therapy." J Pathol **215**(4): 355-68.

Takagi, Y., J. Takahashi, et al. (2005). "Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model." J Clin Invest **115**(1): 102-9.

Tang, F., K. Shang, et al. (2002). "Differentiation of embryonic stem cell to astrocytes visualized by green fluorescent protein." Cell Mol Neurobiol **22**(1): 95-101.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-7.

Thomson, J. A., J. Kalishman, et al. (1995). "Isolation of a primate embryonic stem cell line." Proc Natl Acad Sci U S A **92**(17): 7844-8.

Thomson, J. A., J. Kalishman, et al. (1996). "Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts." Biol Reprod **55**(2): 254-9.

Tomac, A., E. Lindqvist, et al. (1995). "Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo." Nature **373**(6512): 335-9.

Tompers, D. M. and P. A. Labosky (2004). "Electroporation of murine embryonic stem cells: a step-by-step guide." Stem Cells **22**(3): 243-9.

Toyooka, Y., N. Tsunekawa, et al. (2003). "Embryonic stem cells can form germ cells in vitro." Proc Natl Acad Sci U S A **100**(20): 11457-62.

Tropepe, V., S. Hitoshi, et al. (2001). "Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism." Neuron **30**(1): 65-78.

Trupp, M., E. Arenas, et al. (1996). "Functional receptor for GDNF encoded by the c-ret proto-oncogene." Nature **381**(6585): 785-9.

Twarog, B. M. and I. H. Page (1953). "Serotonin content of some mammalian tissues and urine and a method for its determination." Am J Physiol **175**(1): 157-61.

Tzschentke, T. M. and W. J. Schmidt (2000). "Functional relationship among medial prefrontal cortex, nucleus accumbens, and ventral tegmental area in locomotion and reward." Crit Rev Neurobiol **14**(2): 131-42.

Ueno, M., M. Matsumura, et al. (2006). "Neural conversion of ES cells by an inductive activity on human amniotic membrane matrix." Proc Natl Acad Sci U S A **103**(25): 9554-9.

Vallier, L., M. Alexander, et al. (2007). "Conditional gene expression in human embryonic stem cells." Stem Cells **25**(6): 1490-7.

Vallier, L., J. Mancip, et al. (2001). "An efficient system for conditional gene expression in embryonic stem cells and in their in vitro and in vivo differentiated derivatives." Proc Natl Acad

Vallstedt, A., J. Muhr, et al. (2001). "Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification." Neuron **31**(5): 743-55.

Vazin, T., J. Chen, et al. (2008). "Assessment of stromal-derived inducing activity in the generation of dopaminergic neurons from human embryonic stem cells." Stem Cells **26**(6): 1517-25.

Vernay, B., M. Koch, et al. (2005). "Otx2 regulates subtype specification and neurogenesis in the midbrain." J Neurosci **25**(19): 4856-67.

Vitalis, T., O. Cases, et al. (2005). "Development of the dopaminergic neurons in the rodent brainstem." Exp Neurol **191 Suppl 1**: S104-12.

Vogel, A., C. Rodriguez, et al. (1995). "Dorsal cell fate specified by chick Lmx1 during vertebrate limb development." Nature **378**(6558): 716-20.

Vollrath, D., V. L. Jaramillo-Babb, et al. (1998). "Loss-of-function mutations in the LIM-homeodomain gene, LMX1B, in nail-patella syndrome." Hum Mol Genet **7**(7): 1091-8.

Wagner, J., P. Akerud, et al. (1999). "Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes." Nat Biotechnol **17**(7): 653-9.

Wallen, A. and T. Perlmann (2003). "Transcriptional control of dopamine neuron development." Ann N Y Acad Sci **991**: 48-60.

Wallen, A., R. H. Zetterstrom, et al. (1999). "Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice." Exp Cell Res **253**(2): 737-46.

Walther, D. J. and M. Bader (2003). "A unique central tryptophan hydroxylase isoform." Biochem Pharmacol **66**(9): 1673-80.

Walther, D. J., J. U. Peter, et al. (2003). "Synthesis of serotonin by a second tryptophan hydroxylase isoform." Science **299**(5603): 76.

Wang, W., S. R. Bradley, et al. (2002). "Quantification of the response of rat medullary raphe neurones to independent changes in pH(o) and P(CO2)." J Physiol **540**(Pt 3): 951-70.

Williams, R. L., D. J. Hilton, et al. (1988). "Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells." Nature **336**(6200): 684-7.

Wobus, A. M. and K. R. Boheler (2005). "Embryonic stem cells: prospects for developmental biology and cell therapy." Physiol Rev **85**(2): 635-78.

Wobus, A. M., H. Holzhausen, et al. (1984). "Characterization of a pluripotent stem cell line derived from a mouse embryo." Exp Cell Res **152**(1): 212-9.

Wurst, W. and L. Bally-Cuif (2001). "Neural plate patterning: upstream and downstream of the isthmic organizer." Nat Rev Neurosci **2**(2): 99-108.

Xu, C., M. S. Inokuma, et al. (2001). "Feeder-free growth of undifferentiated human embryonic stem cells." Nat Biotechnol **19**(10): 971-4.

Xu, H., X. Fan, et al. (2005). "Neural precursor cells differentiated from mouse embryonic stem cells relieve symptomatic motor behavior in a rat model of Parkinson's disease." Biochem Biophys Res Commun **326**(1): 115-22.

Yamada, M., K. Tanemura, et al. (2007). "Electrical stimulation modulates fate determination of differentiating embryonic stem cells." Stem Cells **25**(3): 562-70.

Yamada, T., M. Placzek, et al. (1991). "Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord." Cell **64**(3): 635-47.

Yamada, T., M. Yoshikawa, et al. (2002). "In vitro functional gut-like organ formation from mouse embryonic stem cells." Stem Cells **20**(1): 41-9.

Yamashita, J., H. Itoh, et al. (2000). "Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors." Nature **408**(6808): 92-6.

Yan, J. and J. N. Barrett (1998). "Purification from bovine serum of a survival-promoting factor for cultured central neurons and its identification as selenoprotein-P." J Neurosci **18**(21): 8682-91.

Yan, Y., D. Yang, et al. (2005). "Directed differentiation of dopaminergic neuronal subtypes

Ye, W., K. Shimamura, et al. (1998). "FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate." Cell **93**(5): 755-66.

Ying, Q. L., J. Nichols, et al. (2003a). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell **115**(3): 281-92.

Ying, Q. L., M. Stavridis, et al. (2003b). "Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture." Nat Biotechnol **21**(2): 183-6.

Yue, F., L. Cui, et al. (2006). "Induction of midbrain dopaminergic neurons from primate embryonic stem cells by coculture with sertoli cells." Stem Cells **24**(7): 1695-706.

Zambrowicz, B. P., A. Imamoto, et al. (1997). "Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells." Proc Natl Acad Sci U S A **94**(8): 3789-94.

Zhang, S. C. (2006). "Neural subtype specification from embryonic stem cells." Brain Pathol **16**(2): 132-42.

Zhao, S., S. Maxwell, et al. (2004a). "Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons." Eur J Neurosci **19**(5): 1133-40.

Zhao, S., J. Nichols, et al. (2004b). "SoxB transcription factors specify neuroectodermal lineage choice in ES cells." Mol Cell Neurosci **27**(3): 332-42.

Zhao, Z. Q., M. Scott, et al. (2006). "Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity." J Neurosci **26**(49): 12781-8.

Zhou, B. Y., Z. Ye, et al. (2007). "Inducible and reversible transgene expression in human stem cells after efficient and stable gene transfer." Stem Cells **25**(3): 779-89.

Zufferey, R., T. Dull, et al. (1998). "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery." J Virol **72**(12): 9873-80.

Résumé

Les cellules souches embryonnaires (cellules ES) sont pluripotentes et ont donc le potentiel de se différencier en cellules des trois feuillets embryonnaires, ainsi qu'en cellules de la lignée germinale. Ces propriétés en font un modèle pour l'étude des mécanismes de prolifération et de différenciation. Le facteur de transcription Lmx1b est impliqué dans la maintenance du phénotype différencié des neurones dopaminergiques mésencéphaliques. Et il a aussi été montré comme un facteur clef dans la différenciation et la maintenance des neurones sérotoninergiques du rhombencéphale générés dans les noyaux du Raphé. Dans ce travail, nous nous sommes intéressés aux capacités de Lmx1b d'influencer la différenciation des cellules ES de souris en neurones sérotoninergiques.

La première stratégie adoptée a résulté en une expression ectopique stable de Lmx1b dans les cellules ES et leurs dérivés. Le niveau d'expression de Lmx1b a fortement influencé les capacités de différenciation neuronale des cellules. Puis, l'analyse de marqueurs de différenciation spécifiques a montré une augmentation de l'expression des marqueurs sérotoninergiques, au contraire des marqueurs dopaminergiques ou de neurones moteur. La seconde stratégie a consistée en une surexpression inductible de Lmx1b dans les précurseurs neuraux dérivés de cellules ES pour mimer l'expression physiologique de Lmx1b. Après induction, Lmx1b était bien exprimé dans les cellules durant toutes les étapes de différenciation neuronale. L'activation de l'expression de Lmx1b au stade des colonies neuroépithéliales a aussi résulté en une amélioration de la différenciation sérotoninergique.

Les résultats de ce travail soulignent les capacités de Lmx1b à diriger la différenciation des précurseurs neuraux dérivés de cellules ES vers la voie sérotoninergique *in vitro*.

Mots clefs

Cellules Souches Embryonnaires (ESC), Lmx1b, facteur de transcription, différenciation neuronale, *in vitro*, expression stable, expression inductible, neurones sérotoninergiques, neurones dopaminergiques, PCR en temps réel.

Abstract

Pluripotent Embryonic Stem Cells (ESC) have the potential to develop into cells of the three germ layers and of the germ line. Therefore, they are used as a model to study the proliferation and differentiation mechanisms. The LIM homeodomain transcription factor Lmx1b is involved in the maintenance of the differentiated phenotype of midbrain dopaminergic neurons. And it has been also demonstrated to be a key factor in differentiation and maintenance of hindbrain serotonergic neurons generated in the Raphe Nuclei. Here, we explored the capacity of Lmx1b to direct differentiation of mouse ESC (mESC) into serotonergic neurons.

In the first approach, stable ectopic expression of Lmx1b was achieved. First, the level of Lmx1b expression was found to strongly influence the capacity of mESC to accomplish neuronal differentiation. Then, analysis of lineage-specific differentiation markers showed an increase in serotonergic markers' expression by contrast to dopaminergic or motor neurons markers. In the second approach, Lmx1b was over-expressed in mESC-derived neural precursors by an inducible system in order to mimic the physiological onset of Lmx1b expression. After induction, Lmx1b was found to be stably expressed throughout neuronal differentiation. Activation of Lmx1b expression in neuroepithelial colonies resulted in enhancement of serotonergic differentiation, consistently with the stable system results.

The results of this work highlight the capacity of Lmx1b to promote the shift of mESC-derived neural precursors toward a serotonergic fate *in vitro*.

Key words

Embryonic Stem Cells (ESC), Lmx1b, transcription factor, neuronal differentiation, *in vitro*, stable expression, inducible expression, serotonergic neurons, dopaminergic neurons, real-time PCR.