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Identification of novel pathways in the differentiation of mesencephalic dopaminergic neurons

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… to my family

… to my friends

INDEX

1 - INTRODUCTION

> "*Prior to the advent of brain the entire cosmic process was "a play before empty benches"; there was no color and no sound in the universe, nor was there any flavour or aroma and probably rather little sense and no feeling or emotion. Before brains the universe was also free of pain and anxiety*."
>
> - Roger Wolcott Sperry *Evolution of human brain*, 1964

Brain is one of the most complex entities. It is constituted mainly of glial cells and neurons, but both cell types comprise several sub-populations. There are about fifty different types of neurons that take precise contacts, which other neurons generating a tangled network, sustained by glial cells. This is the biological basis for the accomplishment of elaborate tasks that regulate animal behavior and makes brain the organ of intelligence, consciousness and mind. Indeed evolutionally, it guarantees the survival of individuals and of species by solving complex tasks and handle dangerous situations, choosing rapidly from the *Four F's instincts* (fighting, fleeing, feeding and fucking).

The *Triune Brain Theory* of Paul D MacLean proposes that during the evolution, the brain develops to solve increasingly complex problems, most often associated to the survival and adaptation. Hence the most ancient nuclei of the brain control certain behaviors that are necessary for the survival, such as exploration, feeding, aggression, dominance and sexuality and are therefore conserved among the evolution from reptiles to the human. The aggregate of these nuclei was called *protoreptilian brain*. Subsequently, the *paleomammalian brain* evolved, where the instincts regulated by protoreptilian brain were integrated with the emotions, fear control, memory, constituting the limbic system. The latter includes a large number of structures. At the end, the newest *neomammalian brain* allows mammals to consciousness and the higher cognitive tasks.

This theory highlights the importance of midbrain because its nuclei are involved in primitive tasks as well as in emotional control. The midbrain functions are mediated mainly by dopaminergic neurons that act like a bridge to connect instincts to emotions, although serotonin and norepinephrine play an important role in several physiological functions and behaviors, such as eating, sleep, circadian rhythmicity, neuroendocrine function and *arousal*.

It must be said that dopamine is not a conventional neurotransmitter that excites or simply inhibits the target neurons. Rather, dopamine working through signaling cascades coupled to G-proteins is capable of modulating the gating of ion channels that orchestrate the response to classic neurotransmitters. That is, they change the way neurons respond to the signals from other neurons to which are connected synaptically.

1.1 – Mesencephalic dopaminergic neurons

The catecholamine dopamine (DA) is a neurotransmitter released by an anatomically and functionally heterogeneous class of neurons: the dopaminergic neurons.

In the 1960s the discovery of catecholaminergic nuclei (A) by Carlsson, Falck and Hillarp (Carlsson et al. 1962, Falck et al. 1962) allowed us to understand the variety of action mediated by this catecholamine. In the mammalian central nervous system (CNS) dopaminergic nuclei have a broad distribution and are located in the area A16 of the olfactory bulbs (Gudelsky et al. 1976), area A17 of the retina (Djamgoz et al. 1992), areas A11-A15 of the diencephalon [e.g. hypothalamic arcuate nucleus (A12; Kizer et al. 1976), sub-parafascicular thalamic nucleus (A13; Takada 1993)] and areas **A8-A10** of the ventral mesencephalon, where is located the largest DA population (20000 - 40000 neurons in rodents, 400000 - 60000 in humans; Björklund et al. 2007; Fig. 1.1). Interestingly, in adult mice their number can vary in response to environmental stimuli (Tomas et al. 2015) as the DA neurons in the hypothalamus of adult rats (Dulcis et al. 2013).

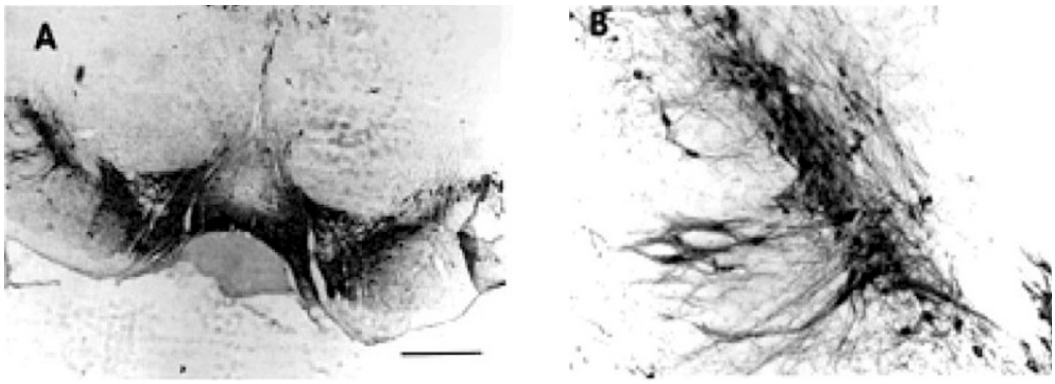

Figure 1.1: Midbrain dopaminergic nulcei.
A) Immunostaining for Tyrosine hydroxilase (TH) on adult mouse ventral midbrain showing Substantia nigra (SN; laterally) and ventral tegmental area (VTA; medially);
B) SN, enlargement. It is possible to distinguish SN *pars compacta* (SNc), where locate DA somata, and SN *pars reticulata* (SNr) with DA dendrites.
(di Porzio et al. 1990).

The mesencephalic dopaminergic neurons (mesDA) are spread in three nuclei: the retrorubral field (RRF, area A8), the *substantia nigra* (SN, area A9) and the ventral tegmental area (VTA, area A10; Fig. 1.2). The retrorubral DA neurons project mainly to SN and VTA and probably coordinate the action of these two nuclei (Arts et al. 1996). Others projections of RRF are involved in the *arousal* (Simmons et al. 2011).

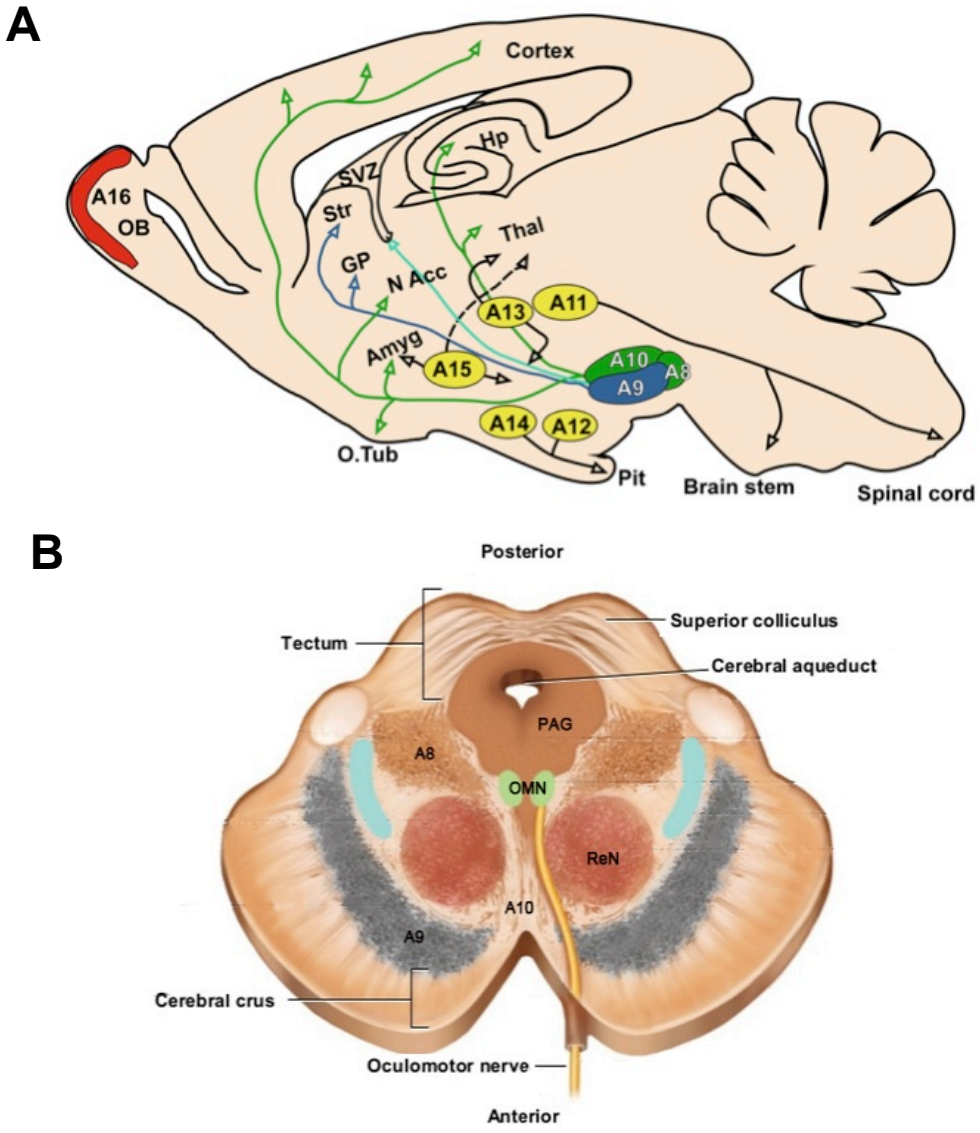

Figure 1.2: Localization of dopaminergic nuclei and their projection in adult rodent and human brain.

A) Dopaminergic neurons are distributed in 10 groups (A8 - A16) from the mesencephalon to the olfactory bulb described by Dahlstrom and Fuxe in 1964 (Dahlstroem et al. 1964). A8, A9 and A10 nuclei are usually indicated as midbrain dopaminergic neurons. Blu arrows represent nigro-striatal pathway; green arrows represents meso-cortico-limbic pathway. A8, Retrorubral field; A9, Substantia Nigra; A10, Ventral Tegmenta Area. Amyg, amygdala; DA, dopaminergic neurons; GP, globus pallidus; Hp, hippocampus; N Acc, nucleus accumbens; OB, olfactory bulb; O. Tub, olfactory tubercle; Pit, pituitary; Str, striatum; SVZ, subventricular zone; Thal, thalamus. Images from Rodríguez-Traver et al. 2015.

B) Schematic planar section of adult human midbrain. PAG, periaqueductal gray; ReN, red nucleus; OMN, oculomotor nucleus.

In rodents, DA fibers depart from SN *pars compacta* (SN_c; that contains DA cell bodies while the dendritic extensions are located in the *pars reticulata* where they connect to the intrinsic GABAergic neurons) and reach the *striatum* (corresponding to nuclei *caudate-putamen* in humans) to form the ascending **nigrostriatal pathway** (NSp). This pathway is involved in the extrapyramidal control of motor functions (Kandel et al. 2000) and integrates multiple aspects of motor behavior such as: the motivational or decisional aspects, the regulation of movement velocity or postural control.

The extrapyramidal pathway is a complex network constituted by different nuclei: the basal ganglia. These ones integrate information from cortex with those of brain stem. Briefly, *striatum* after cortical activation remove the inhibition of internal globus pallidus (GP_i) and SN_r on the **thalamus**, promoting the feedback signals of thalamus to the cortex. The inactivation of GPi and SNr is mediated directly (in the direct pathway) or passing through subthalamic nucleus and external globus pallidus (GP_e) in the indirect pathway (Fig. 1.3). The dopaminergic neurons of SN_c act differently on the two pathways: the direct-pathway neurons are facilitated by DA activation on DA receptors D_1, whereas indirect-pathway neurons are inhibited by activation of D_2 receptors (Ivanova et al. 2012). Therefore SN_c neurons balance the two different pathways and are involved not only in the regulation of movement but also in adaptive behavior (Kandel et al. 2000).

Alteration of the nigrostriatal pathway leads to an over-function of inhibitory GABAergic circuit from the basal ganglia with subsequent decrease of the thalamic excitation of the prefrontal and primary motor cortex. This determines the progressive loss of muscle control causing the pathognomonic symptoms (tremor, rigidity and difficulty to complete simple motor tasks) of Parkinson's disease (PD; Thomas et al. 2007).

On the other hand, mesDA VTA neurons generate the **meso-corticolimbic pathway** (MCLp), which forms the limbic system projecting to the *nucleus accumbens*, *olfactory tubercle*, cortical areas (*prefrontal*, *cingulate* and *perirhinal cortex*) as well as *septum*, *amygdala* and *hippocampus* (Fig 1.2A). Thus VTA mesDA neurons regulate superior cognitive abilities such as reward, attention and emotions that are the basis of social behaviors (sex, sociality and aggression). Dysregulation of MCLp is linked to mood disorders (Zacharko et al. 1991, Martin-Soelch 2009), schizophrenia (Laviolette 2007), attention deficit hyperactivity disorder (ADHD; Ohno 2003), drug addiction and hallucinations (Morales et al. 2012).

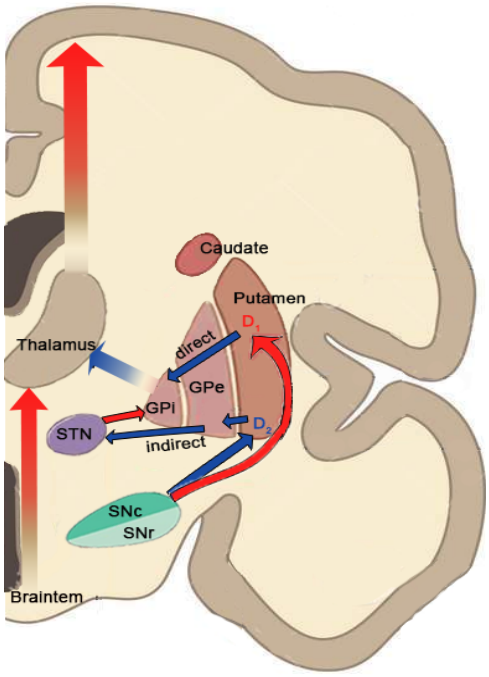

Figure 1.3: Extrapyramidal system neural network. It is shown the modulatory action of *substantia nigra pars compacta* (SNc) dopaminergic neurons on the *striatum*. Excitatory pathways are in red; inhibitory pathways are in blue. SNr, Substantia Nigra *pars reticulata*; GPe, external Globus pallidus; GPi, internal Globus pallidus; STN, Subthalamic nucleus; D_1 and D_2 are different type of DA receptor in the *striatum* (adapted from Kandel et al. 2000).

The two mesDA pathways are not completely separated since their projections partially overlap. Indeed a number of studies have shown that the dysfunction of one can alter the function of the other (Jellinger 1991, Péron et al. 2012). Moreover DA neurons are also involved in the *working memory* formation by connecting basal ganglia with the prefrontal cortex. This specific kind of memory is well described in humans and is necessary to keep "active" in mind the acquired information for several seconds, to allow processes like reasoning, comprehension, problems resolution, planning and other complex cognitive functions (Lieberman 2009).

1.2 – Dopamine metabolism

DA was originally considered simply a precursor in the synthesis of noradrenaline and adrenaline; only at the end of the fifties Von Euler and Lishajko, and subsequently Bertler and Rosengrens, showed that DA is active by itself. Few years later Carlsson, Ehringer and Hornykiewicz identified that the loss of DA in the *striatum* caused PD, proposing the DA precursor L-DOPA (L-3,4-dihydroxyphenylalanine) as potential therapeutic drugs (Carlsson

1959, Ehringer et al. 1960). Then Carlsson and his collaborators identified in the ventral midbrain (Mb) the origins of the DA found in the *striatum* and in the limbic system (Anden et al. 1964).

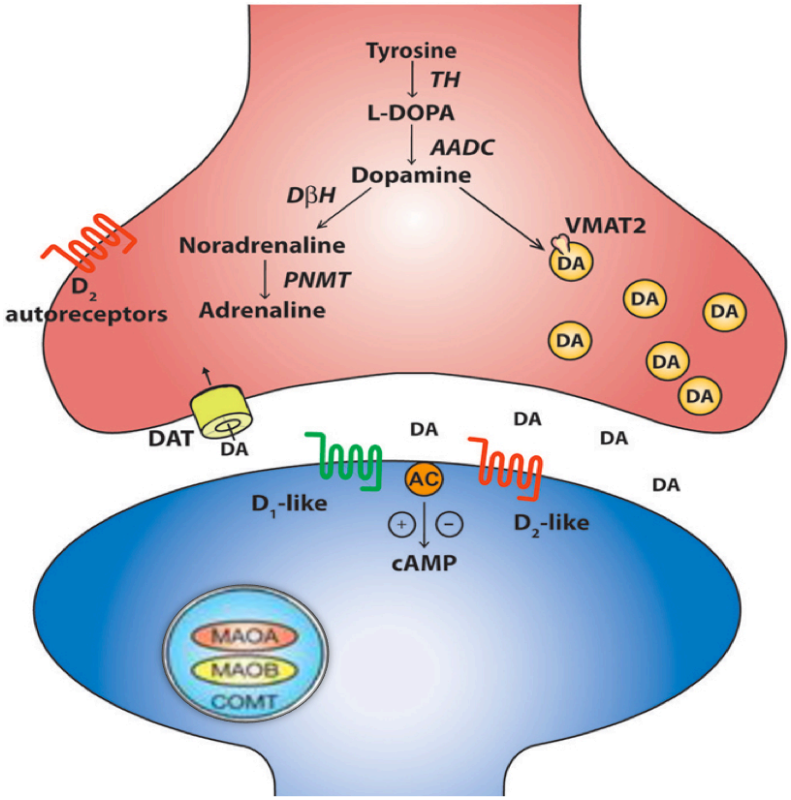

Figure 1.4: Schematic representation of dopaminergic synapse. DA, dopamine; TH, *tyrosine hydroxilase*; L-DOPA, *L–3,4–dihydroxyphenylalanine*; AADC, *Aromatic L-amino acid decarboxylase*; VMAT2, *vesicular monoamine transporter*; MAO, *monoamine oxidase*; COMT, *catechol-O-methyl transferase;* DAT, *dopamine active transporter*; D_2-like dopaminergic inhibitory autoreceptors (DRD2); D_1-like dopaminergic postsynaptic excitatory receptors (DRD1); AC, adenyl cyclase; PNMT, Phenylethanolamine N-methyltransferase; DβH, Dopamine beta-hydroxylase (adapted from Sharples et al. 2014).

The tyrosine hydroxylase (TH) is the limiting enzyme in the catecholamines biosynthesis; it converts tyrosine in the DA precursors L-DOPA (tyrosine itself is derived from phenylalanine by *phenylalanine hydroxylase*, an enzyme related to TH). This reaction takes place mainly in the soma and terminals of neurons. TH activity is modulated by its phosphorylation via the protein kinase cyclic adenosine monophosphate (cAMP)-dependent (PKA). TH requires tetrahydrobiopterin as a cofactor and iron to exert its catalytic activity. In turn, L-DOPA is converted into DA by the ubiquitously distributed Aromatic L-amino acid decarboxylase enzyme (AADC; Fig. 1.4). The

wide expression of the AADC enzyme makes it possible the therapeutic use of L-DOPA, which passes the blood brain barrier, allowing the production of DA in many surrounding cells by administration of the DA precursor.

Following its cytoplasmic synthesis, DA is accumulated in synaptic vesicles through the vesicular monoamine transporter (VMAT2), a twelve transmembrane domain protein. VMAT2 is coupled to the vesicular H^+-ATPases (V-ATPases), which functions as ATP-driven proton pump keeping the internal milieu in synaptic vesicles acidic, since DA oxidizes rapidly. After the activity-induced release, DA can bind to 5 different DA postsynaptic receptors (D_{1-5}). The DRDs are G-protein-coupled, 7 transmembrane domain receptors and can be grouped in two families: the DRD1 (D_1 and D_5) and the DRD2 (D_2 - D_4) that act in an opposite manner, as previously mentioned. The DRD1 type triggers an increase in cAMP, while DRD2 determine cAMP decrease. In general, both families modulate the cAMP/PKA transduction cascade and the intracellular Ca^{2+} levels. These receptors have selective agonists (Vallone et al. 1999) and specific anatomical and cellular distribution that can be pre-synaptic or post-synaptic. The latter types of receptors (e.g. D_2) are also localized on the DA neurons membranes (autoreceptors), regulating DA release as feedback inhibition of DA transmission (Bello et al. 2011).

To turn-off the DA signals, the DA released in the synaptic cleft is mainly recaptured by the dopamine transporter (DAT or SLC6A3) a 12 transmembrane domain Na^+/Cl^- dependent transporter that is target of several drugs (cocaine, amphetamine, etc.) and mice knock-out show hyperactivity and insensibility to treatment by these drugs (Amara et al. 1993).

The DA is also metabolized by catechol-O-methyl transferase (COMT) in homovanillic acid (HVA), or degraded to 3,4-dihydroxyphenylacetic acid (DOPAC) by the extracellular or mitochondrial monoamine oxidases (MAOs).

1.3 – Development of mesDA neurons

In the brain, differences among neuron types is far greater than that observed amongst cells in any other organ of the body. Each neuronal population develops in a distinct anatomical position and establishes specific connections with other cells. This specificity is achieved by a complex process that requires orchestrating a great number of factors that define a sort of informational grid by an accurate space-time sequence. Developing cells are exposed to a sequential

transcriptional activation that follows an accurate genetic program and its interaction with the embryonic environment to determine their phenotype.

The development of mesDA neurons can be broadly divided into 4 stages: early Mb patterning; induction and specification of dopaminergic precursors; differentiation of post-mitotic mesDA neurons; functional maturation of mesDA neurons (Fig. 1.5; Abeliovich and Hammond 2007, Perrone-Capano et al. 2008).

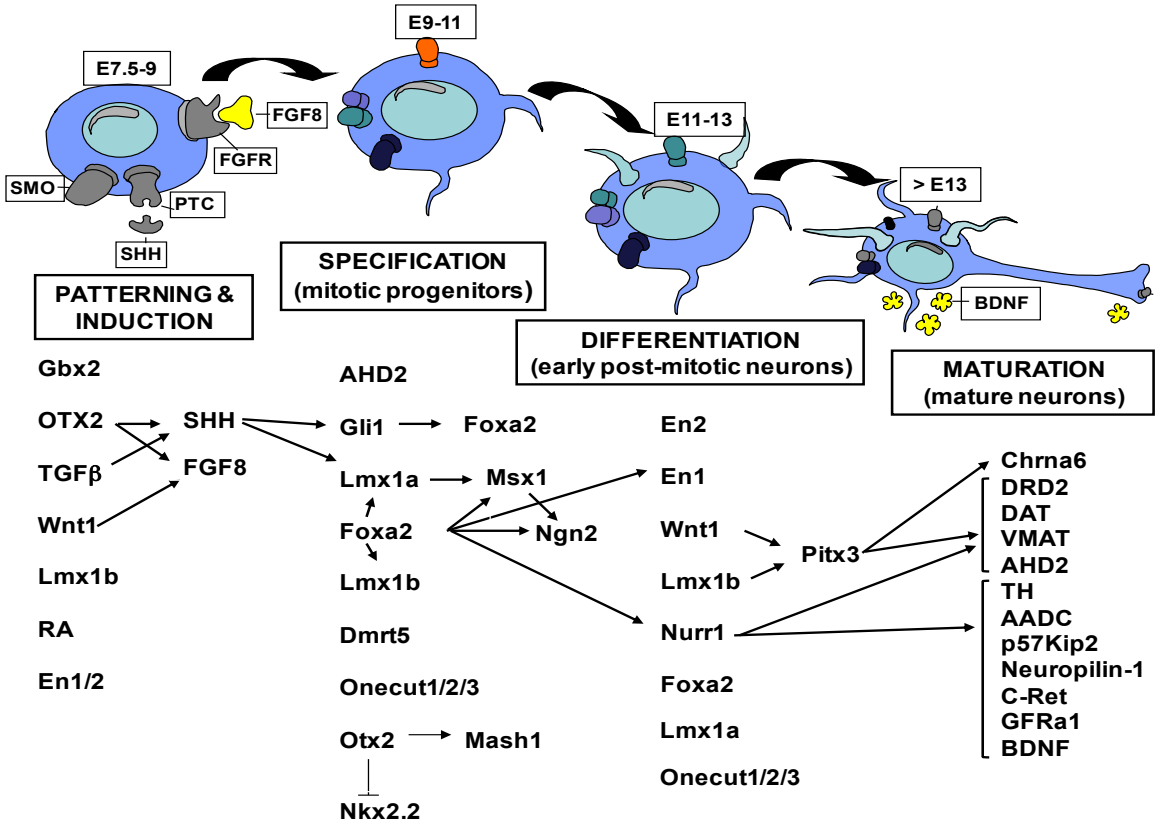

Figure 1.5: Schematic model of mesDA neurons development. Diagram showing the main transcription or inductive factors involved in dopaminergic neurons development, divided in phases of the developmental stage in which they are involved (Perrone-Capano et al. 2008).

1.3.1 – Early midbrain patterning: regionalization

Early in development during the formation of neural tube, the different cell proliferation and the different cell responses to inductive factors released from specific zones, called organizers, determine the formation of several structures (that became the three brain vesicles forebrain, midbrain and hindbrain, which will subsequently divide forming five vesicles, telencephalon,

diencephalon, mesencephalon, metencephalon and myelencephalon). In this processes transcription factors (TFs) are the promoters of structures development and molecules release. For the Mb induction, these factors are mainly growth factors released by the floor plate and the mid-hindbrain boundary (MHB or IsO, *isthmic organizer*) and TFs differently expressed. Otx2 and Gbx2 are two TFs expressed in a mutually exclusive manner (Hidalgo- Sánchez et al. 1999). The former determines the specification and regionalization of telencephalon and mesencephalon (Simeone et al. 2002) while Gbx2 is expressed more caudally and it is essential for the correct development of hindbrain and cerebellum. Indeed *Otx2* null mice fail to generate mesDA that are replaced by serotonergic cells (Puelles et al. 2004), probably because Otx2 inhibits the homodimeric protein Nkx2.2, a negative regulator for the ventral Mb fate (Prakash et al. 2006). Otx2 is also involved in the regulation of some proneural genes like ***Ascl1*** and *Neurogenin2* (*Ngn2*), involved as well in the proliferation phases of the mesencephalic progenitors (Vernay et al. 2005).

Following the proper formation of the MHB (E8 in mice), other factors are secreted. The MHB, that expresses the engrailed transcription factors (En1/2), releases the fibroblast growth factor 8 (**FGF8**) and guides the correct regionalization along the antero-posterior axis of the developing CNS (Fig. 1.6). By gene expression analysis and *in vitro* studies, several other factors needed for the correct positioning of the MHB have been identified. Among these the transforming growth factor β (TGFβ; Farkas et al. 2003), the LIM-homeodomain factor **Lmx1b** (Smidt, Asbreuk et al. 2000) as well as the morphogenetic factor **Wnt1** (Schulte, Bryja et al. 2005) are involved in these steps.

Wnt protein family (associated to wingless phenotype in *Drosophila*) is a class of secreted glycoprotein that binds the transmembrane G-protein-coupled receptors *frizzled* (Fz) determining the activation of the cytoplasmic protein *dishevelled* (Dsh) that regulates transcription of Wnt target genes through its intracellular transducer β-catenin. Numerous members of the Wnt/β-catenin pathway seem to be involved in specification, proliferation and neurogenesis in the ventral Mb (Prakash et al. 2006). For instance, null mutations for *frizzled3* (*Fz3*) and *frizzled6* (*Fz6*) result in a reduction of mesDA neurons (Sousa et al. 2010, Stuebner et al. 2010). Importantly, the activation of Wnt pathway is stronger in the hindbrain.

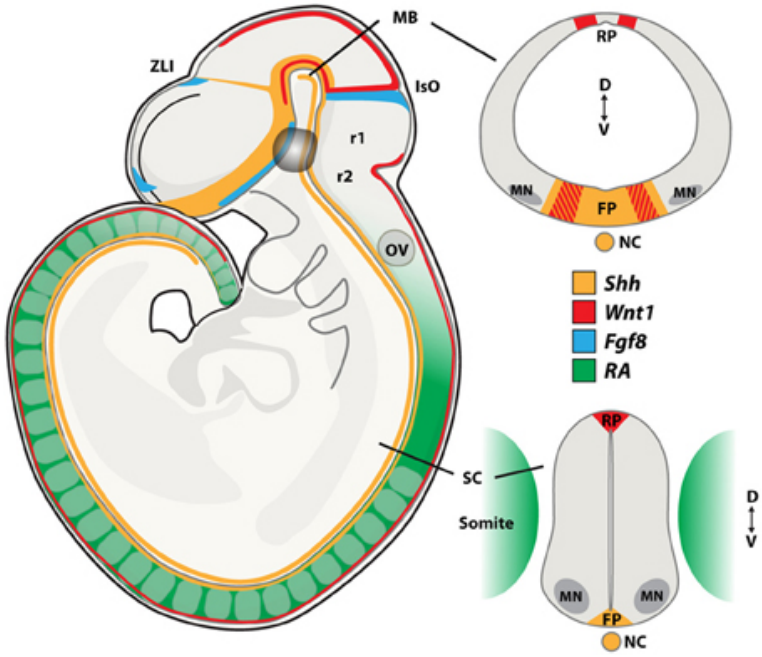

Figure 1.6: Morphogen signaling during neural tube development. Schematic representation of sagittal (left side) and coronal (right side) views of the midbrain (top) and spinal cord (bottom) with the expression pattern for the morphogenes SHH, Wnt1, FGF8 and Retinoic Acid (RA) at E9.5. FP, floor plate; IsO, isthmic organizer; MB, midbrain; NC, notochord; OV, otic vesicle; RP, roof plate; MN, Spinal motor neurons; SC, spinal cord; ZLI, zona limitans intermedia; r1, rhombomer 1; r2, rhombomer 2; D, dorsal; V, ventral. Image from Allodi et al. 2014.

During the early phases of mouse development (E7.5) Lmx1b is expressed in the MHB (Adams et al. 2000, Guo et al. 2007); only at E10.5 it is detectable in the mesDA precursors (Smidt et al. 2000). The loss of *Lmx1b* determines alteration in MHB regulatory genes such as *Fgf8*, *Engrailed 1* and *2* (*En1/2*), *Pax2*, *Gbx2* and *Wnt1*. So Lmx1b has a role in the formation of the MHB and therefore in the development of mesDA neurons (Guo et al. 2007). Indeed specific inactivation of Lmx1b in mesDA progenitors and not in the MHB does not alter the differentiation of these neurons (Yan et al. 2011). Around E11.5 its expression disappears until at E16 when it is expressed in post-mitotic mesDA progenitors and is maintained until the adulthood in co-expression with Pitx3 and TH (Dai et al. 2008). Although loss of Lmx1b leads to a reduction of mDA neurons (Smidt et al. 2000), in *Lmx1b* null mice neural precursors express Nurr1 (Nuclear Receptor Related 1 protein or *Nr4a2*) and TH dopaminergic markers but fail to express Pitx3. These TH^+ neurons lacking Pitx3 expression are lost around birth suggesting a role

for Lmx1b in *Pitx3* regulation and in mesDA neurons survival. Interestingly, a similar phenotype is observed for the *Wnt1* null mutant. In this case, the few TH^+ neurons generated lack Pitx3 expression and are lost by E12.5 (Prakash et al. 2006).

The similar effects caused by loss of these two factors suggest the existence of a regulatory loop between Wnt1 and Lmx1b. Lmx1b and Lmx1a seem to be necessary in inducing and/or maintaining Wnt1 expression around the MHB (Adams et al. 2000, Guo et al. 2007). Lmx1b and Lmx1a together have been shown to regulate proliferation of mesDA progenitors in part through the regulation of *Wnt1* (Panhuysen et al. 2004).

1.3.2 – Induction and specification of DA precursors

As yet described, MHB releases **FGF8** to determine the antero-posterior axis. In parallel the notochord and then the floor plate (FP) release the morphogen *sonic hedgehog* (**SHH**) that is involved in the determination of dorso-ventral axis. SHH binds patched 1 receptor (Ptc1) preventing its inhibition on Smoothened protein (Smo) and triggering the activation of the Gli family transcription factors (Gli-1/3; Stone et al. 1996, Taipale et al. 2002).

The released SHH and FGF8 generate a Cartesian grid in which a gradient of the two molecules is established. Cells located at different points in this grid are exposed to specific concentrations of morphogens, thus establishing the proper inductive signals for the correct differentiation (Hynes et al. 1999). SHH and FGF8 are involved in induction and proliferation of mesDA precursors. The ectopic expression of SHH and FGF8 generate mesDA neurons and for that reason they are usually used for the *in vitro* strategies of mesDA differentiation (Lee et al. 2000).

Lmx1a, like its homologous Lmx1b, is a LIM homeobox conserved transcription factors. LIM TFs have two zinc finger motifs specialized in the interaction with cofactors in order to form transcriptional-regulator complexes (Doucet-Beaupré et al. 2015). In mice brain, Lmx1a is firstly expressed at embryonic days 8.5 (E8.5) in the dorsal midline (roof plate) of the neural tube and then in progenitor zone of ventral midbrain and in otic vesicles (Failli et al. 2002, Millen et al. 2004, Andersson et al. 2006). Studies on *Lmx1a* mutant mice *dreher* show that it is involved in many aspect of CNS development such as the formation of roof plate (Millonig et al. 2000), the specification of dopaminergic neurons (Andersson et al. 2006) and retina formation in *Drosophila*

(Wang et al. 2015). Its expression is induced by Wnt1/SHH and Foxa1/Foxa2 pathways; the former are regulated by Lmx1a generating an autoregulatory loop (Chung et al. 2012). The proliferative action of Lmx1a is mediated by Msx1 that in turn positively regulate the expression of *Ngn2* (Hong et al. 2014) and negatively that of *Nkx6.1*. Thus Lmx1a mediates the general transition from the proliferative zone to the intermediate zone (Andersson et al. 2006, Gale et al. 2008).

Lmx1a is co-expressed with Lmx1b in the neural precursor stage and during the post-mitotic maturation (Zou et al. 2009). The loss of one can be compensated from the other one as confirmed by single KO mice (Ono et al. 2007). Both can ectopically generate mesDA neurons (Nakatani et al. 2010) and their structures are homologues at 80%. In a recent work, it has been hypothesized that during the specification phase, Lmx1b is required and influences the differentiation of several neuronal subtypes in the Mb, including ocular motor and red nucleus neurons, while Lmx1a functions seem to be more restricted to the mesDA fate (Deng et al. 2011).

FoxA1 and FoxA2 are two additional TFs involved in embryonic development and in tissue specification of mesDA by regulating *Ngn2* and later *Nurr1* and *TH* (Ferri et al. 2007, Lin et al. 2009). Interestingly, *FoxA2* heterozygous mice show progressive loss of mesDA after birth and consequent motor deficits, similarly to the PD associated symptomatology (Kittappa et al. 2007). Recent data suggest a role for FoxA2 in the negative regulation of SHH signaling by modulating levels and duration, thus further promoting maturation of developing neurons (Metzakopian et al. 2012).

A recently discovered zinc-finger TF, the *doublesex and mab-3-related transcription factor 5* (***Dmrt5***) has been shown to be crucial in mesDA fate specification (Gennet et al. 2011). This TF was identified from a differential expression screening within the ventral Mb cell populations. At E10.5 in mouse embryo Dmrt5 expression partially overlaps with Lmx1a and FoxA2 expression domain. Since at E12.5 Dmrt5 expression become more restricted to the subventricular zone, where TH expression is not detectable, it can be considered a DA neuronal progenitors marker. Interestingly, several experiments showed that Dmrt5 over-expression is able to trigger an increase in the levels of FoxA2, Lmx1a and Msx1 and a decrease in more laterally expressed markers, confirming a role specifically in the mesDA fate induction (Gennet et al. 2011).

1.3.3 – Post-mitotic maturation of mesDA neurons

As shown by ^{3}H-thymidine incorporation studies, the transition of mesDA progenitors from the proliferative state to post-mitotic immature neurons takes place, in mice, around E9.5 - E13.5 as they exit the cell cycle and migrate radially from the ventricular surface (Bayer et al. 1995, Abeliovich et al. 2007). In this stage, radially migrating cells start to express TH, an event that occurs early (E9.5 in mouse), as suggested by the observation of the first TH^{+} cells and fibers close to the ventricular ependymal layer (di Porzio et al. 1990).

A network of key transcription factors, such as En1/2, Nurr1, Pitx3 and Lmx1a, is involved in the development of post-mitotic mesDA neurons. Here the expression of these factors is restricted to the early mesDA neurons, suggesting that the factors function cell-autonomously. However, none of these transcription factors appear sufficient individually to instruct the mesDA phenotype, suggesting a network model (Abeliovich et al. 2007).

The TF **Nurr1** together with Nur77 (Nr4a1) and Nor-1 (Nr4a3), constitute the orphan receptor subfamily of the steroid nuclear hormone receptors (Zhao et al. 2010). It is considered an orphan nuclear receptor in the sense that no ligands have been identified (Mangelsdorf et al. 1995). The current hypothesis proposes that Nurr1 can regulate transcription in absence of any ligand, and other factors can modulate its activity binding to Nurr1 ligand-binding domain (Perlmann et al. 2004). This hypothesis has been supported by structural data showing that side chains of hydrophobic residues occupy the ligand-binding domain (Wang et al. 2003). Previous data also showed Nurr1 capacity to act as a monomer, homodimer or as heterodimer with the retinoic acid receptor (RXR; Law et al. 1992, Perlmann et al. 1995) to transactivate target genes. *Nurr1* gene encodes for a protein of 598 aminoacidic residues identical in human and mouse. Its main *trans*activation domain (AF1, aa. 52-84) is located at its N-terminus, while at carboxylic terminal domain (C-ter) there is the putative ligand-binding domain (LBD, aa. 361-598). A central DNA binding domain (DBD, aa. 261-335) mediates the binding to specific NGF-responsive elements (NBRE; Castro et al. 1999, Alvarez-Castelano et al. 2013, Volakakis et al 2006, Flaig et al. 2005, Wang et al. 2003).

Phosphorylation and sumoylation are proposed as regulatory events of Nurr1 activation (Jacobsen et al. 2008, Sacchetti et al. 2006, Arredondo et al. 2013), but also sub-cellular localization of the TF influences its activity (Kadkhodaei et al. 2013). Its sub-cellular localization is regulated by two nuclear localization signals (NLS1/2, aa. 287-314 and 338-350) and two

nuclear exporting signals (NES1/2, aa. 443-452 and 568-577), located in its structure (García-Yagüe et al. 2013).

Nurr1 is predominantly expressed in the SN, VTA, limbic system and olfactory bulbs (Zetterström et al. 1997). In mouse its expression starts at E10.5, one day before TH appearance (Volpicelli et al. 2004, Jankovic et al. 2005), and persists in the adult brain. The highest increase in Nurr1 levels is observable between E13 and E15 in rat, a crucial phase for mesDA maturation and for the organization of SN and VTA (Volpicelli et al. 2004). Null mice for *Nurr1* show agenesis of TH^+ cells in the Mb, as proven by the absence of several DA markers and consequent loss of striatal innervations (Zetterström et al. 1997). These mutant mice are born with a normal frequency but they die within two days and they show a complete loss of mesDA, both in the SN and in the VTA, but not in the other CNS DA nuclei. The over-expression of Nurr1 in different cell types induces the DA phenotype (Wagner et al. 1999, Chung et al. 2002). Nurr1 has been found decreased in PD patients and mutations in its gene are associated to rare form of familiar PD (Le et al. 2003, Sleiman et al. 2009, Decressac et al. 2013).

Nurr1 regulates the expression of several DA markers, such as *TH* (Zhou et al. 1995), *Dat* (Giros et al. 1996), *Vmat2* (Colebrooke et al. 2006), $p57^{Kip2}$ (Joseph et al. 2003) and *c-Ret* (Jain et al. 2006, Kramer et al. 2007). Nurr1 has also a role in the maintenance of DA population by regulating the expression of *Bdnf* (Volpicelli et al. 2007). However many other genes involved in the normal mesDA differentiation are normally expressed during the early phases of embryonic development in *Nurr1* null mice. Most likely, the explanation could be that more than one regulatory pathway act for the realization of the correct phenotypic maturation of mesDA (Saucedo-Cardenas et al. 1998, Wallén et al. 1999, Smidt et al. 2004).

During embryonic development **Pitx3** (Pituitary homeobox 3, since the first member of this family was found to be necessary in the pituitary gland development) is expressed in several tissues but after birth it is detectable only in mesDA where it is present also during adulthood (Smidt et al. 1997). *Aphakia* mutant mice (*ak*), harbouring a spontaneous mutation in the gene coding for Pitx3, have motor impairment due to specific loss of mesDA neurons in SN while VTA appears unaffected (Smidt et al. 2007). Pitx3 expression in SN precedes that of TH while in VTA it occurs simultaneously (Maxwell et al. 2005). These data strongly suggest that specific differentiation programs must take place in the two DA subpopulations, thus explaining their different susceptibility to the loss of Pitx3.

Targets of Pitx3 are the aldehyde dehydrogenase *Ahd2*, involved in the retinoic acid (RA) synthesis (Jacobs et al. 2007), *Vmat2* and *Dat* (Hwang et al. 2009). As yet described also Nurr1 regulates these genes and both the TFs bind the co-repressor PSF (splicing factor PTB-associated; Jacobs et al. 2009). These evidences suggest a relationship between the two TF (Jacobs et al. 2009). Indeed, in a recent work conducted in our laboratory, we demonstrated that Nurr1 directly binds and activates a non-canonical NBRE element on the *Pitx3* promoter (Volpicelli et al. 2012).

The yet described Lmx1a has recently been correlated also to the maturation of mesDA neurons, since it regulates *Dat, Vmat2* and *Pitx3* (Chung et al. 2012). This hypothesis is supported also by the maintained expression of this TF in post-mitotic cells $TH^+/Nurr1^+$ (Andersson et al. 2006). Lmx1a inhibits non-dopaminergic destinies (Deng et al. 2011) and regulates axonal guidance promoting *Slit2* expression (Yan et al. 2011).

Like Lmx1a, also Lmx1b is involved in the maturation of post-mitotic cells by regulation of *Pitx3*. At the same time, *Lmx1b* KO mice show specific loss of mature mesDA neurons. (Simeone 2005)

TFs En1 and En2, like Lmx1b, are important during the first phase of MHB formation (Liu et al. 2001) but their expression is not detectable anymore till E11.5 when En1 and En2 start to be expressed again in ventral mesDA differentiating neurons. This expression is maintained into and throughout the adulthood (Simon et al. 2001, Albéri et al. 2004). En1 and En2 are required to prevent apoptosis suggesting a role in maintenance and survival of ventral mesDA (Albéri et al. 2004).

1.3.4 – Functional maturation and survival of dopaminergic neurons

The functional maturation of mesDA neurons starts after they reach of the *striatum*. In mouse the *Dat* gene is expressed only at E15.5 after the connection to *striatum* (Fiszman et al. 1991). Furthermore *in vitro* maturation of mesDA neurons is enhanced co-culturing striatal cells (Prochiant et al. 1979, di Porzio et al 1980, Perrone-Capano et al. 2000).

Numerous molecules mediate the guidance of neural projection to their targets. EphrinB2, involved in axonal guidance, and its receptor EphB1 have been shown to facilitate the SN DA striatal innervation, where the EphB1 is expressed by mesDA neurons, mainly in SN, and

EphrinB2 is expressed in the *striatum* (Yue et al. 1999). Furthermore EphrinB2, added in mesencephalic primary cultures, results in an upregulation of *Nurr1* transcript (Calò et al. 2005). Also semaphorins and netrin signals have been associated in the formation of mesDA circuits (Torre et al. 2010, Xu et al. 2010).

Other factors are Slit-2 and its receptor Robo. Slit-2 seems to be regulated by Lmx1a (Smidt at al. 2007) and acts as chemorepellant for mesDA projections inhibiting *in vitro* their axonal growth (Lin et al. 2005, Dugan et al. 2011).

After the reaching of their target, mature neurons receive trophic signals released from post-synaptic cells. This process ensures that unnecessary or non-functional neurons dye. During post-natal development there are two moments, around post-natal day 2 (P2) and around P14, when mesDA neurons undergo towards naturally-occurring cell-death (Burke 2003). During these phases functional neurons receive numerous surviving factors like the glial cell line-derived neurotrophic factor (GDNF; Lin et al. 1993, Beck et al. 1995, Akerud et al. 1999), the brain-derived neurotrophic factor (BDNF; Hyman et al. 1991), and the more recently identified dopamine neurotrophic factor (CDNF; Krieglstein 2004, Lindholm et al. 2007). Both GDNF and BDNF show a protective role on mesDA neurons, following a number of experimental lesions. They also promote neuronal survival and differentiation *in vitro* (Hyman et al. 1991, Feng et al. 1999, Consales et al. 2007).

The absence of neurotrophic signals can be the molecular basis of neurodegeneration and, from the other hand these molecules could be therapeutic drugs for treatment of DA-associated neurological diseases.

1.4 – *In vitro* generation of mesDA neurons

In vitro generation of mesDA neurons is an essential step in research since it could unveil mechanisms associated to the physio/pathological conditions of nigrostriatal and meso-cortico-limbic pathways. To this purpose novel knowledge on the molecular mechanisms underlying mesDA development as well as advances in stem cell biology could allow to generate unlimited numbers of DA cells and overcome the technical limitations nowadays still related to neurons transplants in PD patients.

Stem cells are the "carte blanche" on which every cell phenotype can be drawn. These are undifferentiated or not fully differentiated cells and therefore able to self-renew and to give rise to one or more specific cell type. Based on their capacity in differentiating in all or just few cell types there are more stem cell types. The most powerful are the embryonic stem cells (ESC), due to the lowest differentiating rate that allow generating roughly every cell type. In contrast multipotent and pluripotent stem cell lines can generate less cell types, but can be derived also from adult tissues, as the adipose-derived adult stem cells (ADAS; Gimble et al. 2003) and for these reasons can be useful to generate cell phenotypes from disease affected patients.

From the brain it is possible to isolate neural stem cells (NSC) that are stem cells able to give rise to every glial and neuronal subtype. NSCs were obtained the first time in 1992 for *in vitro* cultures (Reynolds et al. 1992) and later in 1997 they proved to be a good tool for neural developmental studies (McKay 1997).

Several studies have shown that ESC and NSC cultures can generate TH^+ cells expressing mesDA phenotypic markers and that the *in vitro* developmental program appears to recapitulate the temporal course of normal mesDA development (Kim et al. 2002, Barberi et al. 2003, Martinat et al. 2004, Sonntag et al. 2004, Andersson et al. 2006). To direct the lineage-specific neuronal differentiation, including the mesDA phenotype, instructive factors are required. Soluble factors implicated in the specification of mesDA *in vivo* (morphogens, trophic and growth factors) have been proved to enhance the differentiation and survival of the DA phenotype *in vitro* (Krieglstein 2004, Volpicelli et al. 2004). In 2003 Ying and Smith showed that the addition of SHH and FGF8 to the medium was sufficient to strongly increase the number of TH^+ neurons obtained *in vitro* (Ying et al. 2003).

Over-expression of cell-intrinsic transcription factors such as Nurr1 (Chung et al. 2002, Kim et al. 2002, Sonntag et al. 2004) and Pitx3 (Chung et al. 2005) resulted to be another useful approach to drive mesDA phenotype. Over-expression of Lmx1a in ESC also promotes mesDA differentiation, but only in the presence of SHH (Andersson et al. 2006).

The induced pluripotent stem cells (iPSC) are derived from de-differentiation of yet committed cells by over-expression of specific transcription factors (Takahashi et al. 2006) thus to potentially derive patient-specific stem cells to differentiate in the damned phenotype. DA neurons have been also obtained from PD patients' derived-hiPSC as a suitable *in vitro* model for

the pathology (Soldner et al. 2009). Upon transplantation, iPSC-derived DA neurons were able to improve the altered motor behavior in a rat model of Parkinson's disease (Wernig et al. 2008).

Another milestone in cell reprogramming studies was achieved by *trans*-differentiating adult somatic cells directly to other defined cell types. Vierbuchen and colleagues have been able to directly convert post-natal fibroblasts into functional neurons *in vitro*, called induced neuronal cells (iN), over-expressing by viral infections a combination of only three factors, Ascl1, Brn2 and Myt1l (Vierbuchen et al. 2010). Subsequently, functional mesDA, called induced dopaminergic cells (iDA) have been obtained *in vitro* over-expressing a minimal set of TFs in mouse and human PD patient-derived adult fibroblast: Ascl1, Nurr1 and Lmx1a (Caiazzo et al. 2011), Ascl1, Brn2, Mytl1, Lmx1a and FoxA1 (Pfisterer et al. 2011), Ascl1, Ngn2, Sox2, Nurr1 and Pitx3 (Liu et al. 2012) or in mouse cortical astrocytes using Ascl1, Nurr1 and Lmx1b (Addis et al. 2011).

It is particularly significant in the context of this thesis the possibility to obtain iN from human fibroblast also by over-expressing **microRNAs** (miRNAs) and not TFs, and recent data confirm this potential direction (Ambasudhan et al. 2011, Yoo et al. 2011, Xue et al. 2013).

miRNAs are a class of evolutionary conserved small non-coding single-strand RNAs (~22nucleotides) processed from pri-pre-miRNA precursors in nucleus and cytosol (Lee at al. 2003, Grishok et al. 2001). They are involved in embryonic development, cell differentiation and many others biological processes (He at al. 2004). miRNAs act as post-transcriptional regulators of gene expression, by targeting partially complementary sequences in the 3' untranslated regions (UTRs) of the target messenger RNA (mRNAs) that are in turn directed to degradation or translational repressed (Bartel et al. 2004). Some works shows DA impairment after alteration of DA-specific miRNA processing deprivation (Kim et al. 2007, Huang et al. 2010). Regarding more specifically the dopaminergic neurons, nowadays very few miRNAs have been identified as involved in their development or function. miR-133b is enriched in human Mb, where it is thought to regulate the maturation and function of mesDA through a negative feedback circuit involving the transcription factor Pitx3 (Kim et al. 2007). **miR-218** has been found to be expressed in the ventral midbrain of E12.5 mouse embryos, and specifically lost in the *Wnt1-Cre* conditional knock out (Huang et al. 2010). miR-132 is expressed in ESC-derived TH^+ cells and regulates Nurr1 mRNA (Yang et al. 2012). Studies on miRNA expression in neuronal diseases suggest a role of these molecules in neurodegeneration (Kim et al. 2007, Hébert et al. 2008, Doxakis et al. 2010).

The increasing knowledge about the role played by miRNAs in the pathogenesis of this kind of diseases will provide important insights into molecular mechanisms of the disease, and could eventually generate novel targets for therapeutic intervention.

1.5 - Aim of the present work

The research project of this PhD thesis is focused on the study of molecular mechanisms involved in the acquisition and maturation of midbrain dopaminergic neurons and on the study of transcription factors essential for mesDA differentiation.

As previously described, in the mammalian brain the circuits formed by DA neurons are the nigrostriatal and meso-cortico-limbic pathways. These pathways are essential for homeostasis, motor control, reward mechanisms and modulation of affective and emotional states. Dysfunctions of these circuits are involved in serious neurological and psychiatric diseases (Parkinson's disease, schizophrenia, ADHD, DOPA-responsive dystonia, etc.). The mesDA neurons are relatively few, therefore it is useful to generate *in vitro* model systems enriched in DA neurons to dissect the molecular events involved in mesDA differentiation and to possibly use these mesDA neurons in disease animal models and in regenerative medicine.

Thus, the first aim of my PhD thesis was to generate *in vitro* a large amount of DA neurons from different cell types and dissect the overlapping and distinct roles of the transcription factors Nurr1 and Lmx1a. As described in the Introduction, Nurr1 is an orphan nuclear receptor with no known ligands. It is involved in the mesDA specification by direct regulation of many DA genes (such as *Th*, *Vmat2* and *Dat*) and its loss determines agenesis of mesDA neurons (Zetterström et al. 1997, Saucedo-Cardenas et al. 1998). Lmx1a has been recently described as positive regulator of *Th*, *Vmat2* and *Dat* (Chung et al. 2012). Considering the importance and the role of these two transcription factors *in vivo*, my first step was to analyze whether Nurr1 and Lmx1a could have a role during *in vitro* DA differentiation and consequently to set-up a new protocol for mesDA differentiation useful to apply on different cell lines.

After the generation of this *in vitro* model enriched in DA neurons, the second step was to verify if it could exist a possible interaction between Nurr1 and Lmx1a during mesDA differentiation and maturation.

As afore mentioned, in the last few years several experimental results have shown the importance of miRNAs in brain development and function. miRNAs dysregulation has also been suggested to be involved in specific dopaminergic (DA) neurons-affecting pathologies, such as Parkinson's disease (PD) or schizophrenia. However miRNAs exclusively related to DA neurons development and function, have not yet been identified. Thus, the second aim of my PhD thesis was to identify miRNAs involved in DA development and differentiation and if there was a synergy between miRNAs and the transcription factors Nurr1 and Lmx1a to promote DA differentiation.

This work contributes to add new and interesting information on the molecular mechanisms underlying the mesDA differentiation and maintenance, leading to the identification of new molecular targets for potential therapeutic strategies to cure pathological disorders affecting the DA system.

2 - MATERIALS AND METHODS

2.1 – Lentiviral production

Last generation lentiviruses have been constructed in order to contain less than 10% of the original viral genome. They express only the sequences needed for reverse transcription and integration of the gene expression cassette into the host genome. To further increase the security level, these genes are cloned in three different vectors to minimize the risk of recombination event. To further minimize the possibility to generate functional autoreplicant viruses, the Psi (ψ) sequence, responsible for the viral genome packaging, is localized exclusively on the *exogenous gene*-containing transfer vector. The gene expression is under a control of a doxycycline inducible promoter (Tet-ON). The used vectors for the lentiviral particles production were: a Gag/Pol containing pMDL vector, a pRev vector, a pVSV-G vector and the exogenous gene-containing transfer vector Tet-O-FUW. Moreover, a prtTA vector, expressing the reverse tetracycline transactivator (rtTA) protein, in combination with pMDL, pRev and pVSV-G was necessary to induce gene expression in the presence of the antibiotic tetracycline or one of its derivatives (e.g. doxycycline; rtTA vector was supplied by Dr. Caiazzo, IGB, Naples). For all these vectors Gigapreps were made using the EndoFree Plasmid Gigaprep (Qiagen) following the manufacturer instructions.

2.1.1 – Cloning exogenous gene vectors

The genes to over-express are cloned into Tet-O-FUW transfer vector. Briefly genomic DNA from E14 embryonic mesencephalon was used as template. Oligos were designed in a way to amplify specific gene containing specific restriction sites (Tab. 2.1). Amplicons were directly, without the restriction passage, cloned into a pCR®2.1-TOPO® TA (Invitrogen) vector following the TOPO® TA Cloning Kit (Invitrogen) subcloning protocol. In order to isolate the insert on each gene-containing TOPO TA plasmid was restricted with a specific enzyme (4 h at 37°C). Insert band was extracted from the agarose using the PureLink® Quick Gel Extraction Kit (Invitrogen) according to the provided instructions. To clone it directly or indirectly in Tet-O-FUW (or Tet-O-FUW_IRES-Cherry or Tet-O-FUW_IRES-GFP) was linearized with EcoRI. The addition of specific tags to Nurr1 (or its truncated forms previously obtained in our

laboratory) and Lmx1a is achieved by subcloning them respectively into pCMV10-3xFlag and pcDNA3.1-V5HIS.

For the ligation step, 50 ng of vector were used. The amount of insert to use for the ligation reaction was calculated using following formula:

$$ng\ insert = \frac{(ng\ vector \times kbp\ insert)}{kbp\ vector} \times 3$$

A control reaction was performed without adding the insert to the reaction. The enzyme I used was a T4 ligase (NEB) and the reaction buffer was the one provided by the company. Ligation was conducted over-night at 16°C in a final volume of 10 µl with 400 units enzyme. 5 µl of this reaction were used to transform 50 µl of competent DH5α cells (Invitrogen) following the standard transformation protocol: 30' (minutes) on ice, heat shock at 42°C for 30'' (seconds), 2' on ice, cells suspension in 250 µl SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), followed by 1 h growth in agitation at 37°C. Finally the cells were seeded on Lysogeny broth (LB)-Agar plates (10 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract, 20 g Bacto-agar) containing ampicillin (50 ng/ml) and growth over-night at 37°C. Single colonies were screened by PCR. Positive colonies were growth in 5 mL LB (10 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract) containing ampicillin (50 ng/ml). Plasmidic DNA was isolated using the PureLink Quick Plasmid Miniprep (Invitrogen) and later sequenced.

2.1.2 – Transfection and lentiviral production

For the viral particles production (Tiscornia et al. 2006), 8.2 millions HEK293T cells were plated in 150 mm × 25 mm dishes in DMEM (Invitrogen) supplied with 10% FBS (Euroclone), 25 mM Hepes (Invitrogen), 100 U/mL Streptomycin and 100 µg/mL Penicillin (Pen/Strep, Sigma-Alrdich). 24 h later an 80% confluence is generally obtained. Medium was replaced by IMDM medium (Invitrogen), 10% FBS, Pen/Strep. 3 h later the 4 vectors was co-transfected following the calcium phosphate transfection protocol.

Amplified cDNA	Oligo sequences with restriction sites		Cloning Enzyme
3xFlagNurr1	Fw	GCGCCAATTGATGGACTACAAAGACCATGA	MfeI
	Rv	GCGCCAATTGTTAGAAAGGTAAGGTGTC	MfeI
3xFlagΔCter-Nurr1	Fw	GCGCCAATTGATGGACTACAAAGACCATGA	MfeI
	Rv	CGCGCAATTGTTACGGCTTCGAGGGT	MfeI
3xFlagΔNter-Nurr1	Fw	GCGCCAATTGATGGACTACAAAGACCATGA	MfeI
	Rv	GCGCCAATTGTTAGAAAGGTAAGGTGTC	MfeI
Lmx1aV5-HIS	Fw	GCGCCAATTGGCTTGAACATGTTGCACGG	MfeI
	Rv	GCGCCAATTGTGATCAGCGGGTTTAAACTCA	MfeI
mmu-miR-27a-3p (687 bps)	Fw	CCGAATTCGTGTTCAGCTATGTGAGACC	EcoRI
	Rv	CCGAATTCCCCATCTATCTGCTTTGGG	EcoRI
mmu-miR-29a-3p (336 bps)	Fw	CCGAATTCTAAGCCTTCTCTGGAAGTGG	EcoRI
	Rv	CCGAATTCTTAACCATGCTGTTGCTGG	EcoRI
mmu-miR-34b/c-5p (983 bps)	Fw	CCGAATTCGGCTTGCGGGAAGAAGGAC	EcoRI
	Rv	CCGAATTCTAGCAGCTAAGGGCTAGCGG	EcoRI
mmu-miR-132-3p (562 bps)	Fw	CCGAATTCGCTGGGACATCTTTGACG	EcoRI
	Rv	CCGAATTCCTCTTGCTCTGTATCTGCC	EcoRI
mmu-miR-148a-3p (294 bps)	Fw	CCGAATTCTCTTCTTTGCCTTCACTGG	EcoRI
	Rv	CCGAATTCTCAGGTTCTTCACAAAGCC	EcoRI
mmu-miR-204-5p (309 bps)	Fw	CCGAATTCCCGGAGAATCAAGATGAGC	EcoRI
	Rv	CCGAATTCGTTATGGGCTCAATGATGG	EcoRI
mmu-miR-210-3p (306 bps)	Fw	CCGAATTCAGGGGGATATGGGTATTGG	EcoRI
	Rv	CCGAATTCCACCCTGTCTATCTGAATCC	EcoRI
mmu-miR-218-1-5p (374 bps)	Fw	CCGAATTCGATCATACACAATCTGCGGGAAG	EcoRI
	Rv	CCGAATTCGGACATTTGTTATTCTCCCCTC	EcoRI
mmu-miR-219-1-5p (358 bps)	Fw	CCGAATTCCATTCACTCGTGTGCTCC	EcoRI
	Rv	CCGAATTCCCCAACTTCTCTCAAGCC	EcoRI
mmu-miR-370-3p (313 bps)	Fw	CCGAATTCGTGGGTGTGGCTTTGAGG	EcoRI
	Rv	CCGAATTCCCCTTTCACAAATCTTTGCCC	EcoRI
mmu-miR-375-3p (346 bps)	Fw	CCGAATTCCGCCACTGCCGCCGACGTG	EcoRI
	Rv	CCGAATTCGGCGGGGCCTGATGGGAACC	EcoRI
mmu-miR-494-3p (322 bps)	Fw	CCGAATTCGTCTCAGGCAATTCTGTGG	EcoRI
	Rv	CCGAATTCATGCCATACTCCCATGTCC	EcoRI

Table 2.1: Oligos for lentiviral construction.

In details, for each dish, a solution was prepared containing 270 mM $CaCl_2$, 6.25 µg pRev, 9 µg pVSVG, 14.6 µg pMDL and 32 µg of insert containing transfer vector. After 5' of incubation at room temperature, drop-by-drop, in low agitation, a 2xHBS pH 7.12 (NaCl 280 mM, Na_2HPO4 1.5 mM, HEPES 50 mM) solution was added to the vectors mix and incubated for 15' at room temperature. Later, the entire solution was spread, drop by drop, in culture medium

and HEK293T cells were incubated at 37°C and 5% CO_2. 12 h later, medium was refreshed and reduced its amount. 30 h later, medium was harvested, filtered (0.22 μm filters) and ultra-centrifuged at 19400 rpm at 20°C for 2 h (Optima L90K; Beckman Coulter). Lentiviruses containing pellet was resuspended in PBS and stored at -80°C.

Lentiviruses infectivity and the *'maximum tolerated dose'* were empirically determined through subsequential infections on HEK293T cells with increasing amounts of virus.

In my experiments, infections were performed just adding both Tet-O-FUW-Insert viruses and rtTA viruses to culture medium. At the same time or later doxycycline (4 μg/ml) was also supplied to the medium.

2.2 - Cell cultures

Materials for tissue culture, like multiwell plates, serological pipettes or pipette tips, were purchased from Corning, BD Biosciences or Nunc. All steps were carried out inside a laminar flow sterile hood (Jupiter) to avoid contamination. All the equipment was sprayed with Ethanol (70%) before use. Cells were incubated at 37°C with 5% CO_2 in a humidified incubator (Thermo Forma). Dissections were performed with horizontal flow hood (Hermes II). All the factors and reagents mentioned in the below described methods are listed in Table 2.2.

2.2.1 - HEK293T

Human embryonic kidney 293T (HEK293T) cells were generated from human embryonic kidney cells obtained from a single apparently healthy foetus legally aborted. In the early 70s, these cells were genetically transformed with Adenovirus 5 DNA to obtain a stable cell line (Graham et al. 1977).

Cells were maintained at 37°C with 5% CO_2 in a humidified incubator, in DMEM with 10% foetal bovine serum (FBS), Pen/Strep and 25 mM Hepes.

2.2.2 - Animals and dissections

Timed pregnant wild type C57BL/6 (Charles-River) or C57BL-6-Tg.pTH-GFP (Dr. Hideyuki Okano) mice were sacrificed in accordance with Society for Neuroscience guidelines

and Italian law. The embryonic age (E) was determined by considering the day of insemination (as confirmed by vaginal plug) as day E0. Embryos from day 12.5 (El2.5) or E14.5 of gestation were quickly removed and placed in phosphate buffered saline (PBS), without calcium and magnesium, and supplemented with 33 mM glucose. The ventral midbrain was carefully dissected under a stereoscope in sterile conditions and processed for cell cultures. Tissues were pooled and triturated with a mechanical dissociation.

Description	Manufacturer	Catalogue number
Fibronectin	Millipore	FC010
Gelatine	Sigma-Aldrich	G1393
Laminin	Sigma-Aldrich	L2020
Poly-D-lysine	Sigma-Aldrich	P7405
DMEM	Invitrogen	11995065
DMEM/F12	Invitrogen	21331-020
F12	Invitrogen	21700-026
GMEM	Sigma-Aldrich	G5154
IMDM	Invitrogen	31980-030
MEM	Invitrogen	61100-087
Neurobasal NBM	Invitrogen	21103-049
FBS	Euroclone	ECS0180L
FCS	Biosera	1810-500
B27	Invitrogen	17504-044
B27 wo vit. A	Invitrogen	12587-010
N2	Invitrogen	17502-048
βmercaptoethanol	Invitrogen	31350-010
HEPES	Invitrogen	15630-106
L-glutamine	Euroclone	ECB3000D
Na-piruvate	Invitrogen	11360-039
NEAA	Invitrogen	11140-035
Pen/Strep	Sigma-Aldrich	P0781
Trypsin	Sigma-Aldrich	T4799
DNase	Sigma-Aldrich	DN25
Activin	R&D	338-AC-025
bFGF	Sigma-Aldrich	F0291
EGF	Sigma-Aldrich	E9644
cAMP analog	Sigma-Aldrich	D0627
Doxycycline	Clontech	631311
FGF8	Sigma-Aldrich	F6926
L-ascorbic acid	Sigma-Aldrich	A4544
LIF	Millipre	ESG1107
SHH	R&D	1845-SH-100
SAG	Sigma-Aldrich	SML1314

Table 2.2: Factors and reagents used for cell cultures.

2.2.3 - Mesencephalic primary cultures (mE12.5-PCs)

Single cells were obtained from embryonic midbrain as previously described (Prochiantz et al. 1979, di Porzio et al. 1980). Briefly, the tissues were transferred into a 15 mL tube and mechanically dissociated with a sterile pipette in a solution containing 0,01% pancreatic DNAse. The cell suspension was centrifuged 5' at 100 g and resuspended in plating medium and counted. For the viable count, cell suspension was diluted 1:10 with 0,1% Trypan blue and loaded into a Burker's counting chamber slide. Cell concentration was determined on the basis of the total cell count, the dilution factor and the trypan blue exclusion.

Dissociated cell were plated at a density of 4×10^4 cells/cm^2 on multiwells previously coated with 15 µg/mL poly-D-lysine for 1 h at 37°C and washed three times with sterile H_2O. Cells were grown in NBM, supplemented with B27, 0.5 mM L-glutamine, Pen/Strep, bFGF (20 ng/mL) FGF8 (10 ng/mL) and SHH (50 ng/mL) to induce mesDA phenotype.

After 3 days in culture (*days in vitro*, DIV) half of the medium was replaced and inducible lentiviruses were added at respective dilutions. At DIV6, proliferative medium was replaced with a differentiating medium, NBM supplemented with B27, 100 mM L-glutamine, Pen/Strep, Ascorbic acid (200 µM), and 1 mM dibutyryl cyclic adenosine 3', 5'-monophosphate (cAMP). From DIV6 the expression of *trans*genes was induced by the addition of doxycycline (4µg/mL) to the medium. At DIV12 cells were fixed or collected for further analyses.

2.2.4 - mes-*c-myc*-A1

mes-*c-myc*-A1 (A1) cells were obtained from embryonic mesencephalon at day 11 and immortalized by the *c-myc* proto-oncogene (Colucci-D'Amato et al. 1999). A1 are cultured in minimal essential medium MEM/F12 (Invitrogen) supplemented with 10% FBS and Pen/Strep. Before seeding the cells, plates were previously coated with 15 µg/mL poly-D-Lysine for 1 h at 37°C and washed three times with sterile water.

For lentiviruses infections and mesDA differentiation, cells were plated at 1×10^4 cells/cm^2 density and infected 24 h later. At the same time, expression of the inserted TF was induced adding doxycycline (4 µg/mL) to the medium. 48 h later the infection, medium containing serum was replaced by MEM/F12 supplemented with N2, 1 mM dibutyryl cyclic adenosine 3', 5'-

monophosphate (cAMP), SHH (50 ng/mL), FGF8 (10 ng/mL) and Ascorbic acid (200 μM). Medium was replaced once every three days. Cells were harvested after 6 days.

2.2.4a – Cell cycle analysis by propidium iodide assay

mes-*c-myc*-A1 cells infected with Nurr1, Lmx1a, Pitx3 or with rtTA virus alone were cultured to perform cell cycle analysis in two different conditions. In basal condition (***B***) cells were cultured for 3 days in a proliferative medium (MEM/F12, 10% FBS, Pen/Strep) and the day after plating doxycycline (4 μg/mL) was added. To synchronize cell cycle cells were starved from serum for 48 h after plating and this condition is indicated as reloaded (***R***). During these days exogenous gene expression was activated by doxycycline administration (4 μg/mL). At 3DIV, FBS was added to re-start cell cycle. As positive control of cell cycle arrest, starved cells (***S***) were harvested at 3DIV from reloaded control samples before the serum reloading.

To perform cell cycle analysis cells were trypsinized and washed in PBS by sequential centrifuged at 400 × g for 5'. Cell pellets were dissolved and fixed for 30' in ice adding 1 mL of dropwise ice-cold 70% ethanol. After centrifugation and wash in PBS, cells were resuspended in 250 μL of RnaseA (100 μg/mL, Roche) in PBS and incubated 30' at 37°C. Then propidium iodide (PI; 20 μg/mL, Sigma-Aldrich) was added 30' before samples processing with FACS BD Canto (Darzynkiewicz et al. 2001).

2.2.5 – Embryonic stem cell (ESC)

$R26^{CreER/+}$ mouse embryonic stem cells (Omodei et al. 2008) were used to generate ESC population line carrying stably exogenous genes 3xFlagNurr1_IRES-Cherry and/or Lmx1a-V5HIS_IRES-GFP ($ESC_{N\text{-}RED}$; $ESC_{L\text{-}GFP}$; $ESC_{NL\text{-}RED/GFP}$). Cells were cultured on gelatinized wells (0.1% Gelatine in PBS) in ESC medium: GMEM supplemented with 10% foetal calf serum (FCS), 1 mM sodium pyruvate, 1x non-essential amino acid (NEAA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 100 U/mL leukaemia inhibitory factor (LIF). ESC were plated at low density (8×10^3 cells/cm^2) and infected after one day. 6 h after infection, exogenous genes were activated by dox administration (4μg/mL). Cells were sorted with FACS ARIA (BD Falcon), 12 h after dox induction and plated in ESC medium dox-free at density of 7.5×10^3 /cm^2. After amplification different population were differentiated to mesDA neurons. At this purpose cells

were plated at low density in *N2B27 medium* (half DMEM/F12 and half Neuro basal medium, supplemented with N2, retinol-free B27, 2 mM L-Glutamine, 0.05 mM β-mercaptoethanol) and cultured for 4 days. At DIV5 cells were splitted to reduce density on wells coated with solution of poly-D-lysine (two hours at 37°C) and then with a solution of Laminin (20 μg/mL in PBS for at least two hours). From DIV5 to DIV9, SHH (100 ng/mL or SAG 0.4 μM) and FGF8 (100 ng/mL) were added to medium, while dox (4 μg/mL) was added only at DIV7. At DIV9, SHH and FGF8 was removed and ascorbic acid was added (200 μM). Cells were harvested at DIV14.

2.2.6 - epiSCs

epiSCs have been derived as described by Guo and colleagues (Guo et al. 2009). They are cultured in *epiSC medium* containing half DMEM/F12 and half Neuralbasal medium, supplemented with N2, retinol-free B27, 2mM L-Glutamine, 0.05 mM β-mercaptoethanol, 10 ng/mL bFGF and 20 ng/mL Activin. Cells were splitted every 2-3 days as epiSC by using mechanical dissociation with 2mL serological pipet and plated in multiwell plates, coated with FCS for 30' at 37°C. For DA differentiation epiSCs were plated one day before the bFGF and Activin withdrawal in 12-wells plate, previously coated with a solution of 15 μg/mL Fibronectin for 30' at 37°C. One day later (DIV1), cells should reach 60 to 80% of confluence and at this point is possible to switch the medium from *epiSC medium* to simple *N2B27 medium*, that is *epiSC medium* but without bFGF and Activin. During these phases cells were infected with lentiviral particles as described in single experiments. At DIV2 cells where splitted and diluted on a new 12-wells plate previously coated with Fibronectin. From DIV5 to DIV9 cells were maintained in *N2B27 medium* supplemented with SHH (100 ng/mL or SAG 0.4 μM) and FGF8 (100 ng/mL). Usually, at DIV9 *N2B27* is supplemented with doxycycline (4 g/mL) and ascorbic acid (200 μM). Cells were cultured until DIV16.

2.2.7 – Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblast was used to *trans*differentiate into DA neurons by over-expression of specific combination of transcription factors and miRNAs. MEFs were isolated from E14.5 wild-type or TH-GFP knock-in mice embryos as previously described (Caiazzo et al. 2011). Briefly from embryos were removed head, vertebral column and all internal organs; the remaining

embryonic tissue was manually dissociated and incubated in 0.25% trypsin for 15'. Cells from each embryo were plated onto a 150 mm × 25 mm tissue culture dish in MEF media (DMEM, 10% FBS, NEAA, sodium pyruvate and Pen/Strep). In all experiments cells were not splitted more than four times. To *trans*differentiate MEFs in DA neurons cells were plated at density of 25×10^3 cells/cm^2 and infected after 24 h. 16 - 20 h after infection cells were switched into fresh MEF media containing doxycycline (4 μg/mL). After 48 h medium was replaced with neuronal inducing media (DMEM/F12, B27 and dox). Cells were harvested at DIV22.

2.2.8 – Mouse adipose derived adult stem cells (ADAS)

Adult mice were sacrificed to remove inguinal, and abdominal fat pads and digested in 1mg/mL collagenase in HBSS for a 1 h at 37°C and 5% CO_2. Tissue fragments were centrifuged two times at 400 g for 5' to remove collagenase. Cells were plated for 1 h at 37°C in DMEM supplemented with 10% FBS, 25 mM HEPES, Pen/Strep, NEAA EGF (10 ng/mL) and bFGF (20 ng/mL). Then supernatant was transferred in new well in the same medium (Safford et al. 2002). Differentiation in DA cells was achieved by infection with lentiviral particles carrying Ascl1, Nurr1 and Lmx1a and culturing cells in DMEM/F12 supplemented with N2 and dox for at least DIV12.

2.3 – Molecular biology methods

2.3.1 – RNA extraction, reverse transcription and *real time* PCR

RNA was extracted from cells using the TRI-REAGENT (Sigma-Aldrich) classic method as described by Chomczynski and Sacchi (Chomczynski et al. 1987). RNA was treated with DNAase-I (New England Biolabs, NEB) to eliminate possible DNA contaminations. The yield and the integrity of RNA were determined by spectrophotometric measurements and by agarose gel electrophoresis, respectively. For the reverse transcription 2 μg of RNA were incubated for 5' at 65°C with 6 μM random hexanucleotides (NEB) as primers in presence of 500 μM dNTP (Euroclone). Reverse transcription is performed with 200 U of Moloney's murine leukemia virus reverse transcriptase (M-MuLV; NEB) in an appropriate buffer (50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT) supplemented with 40 U of human placenta derived RNase inhibitor (NEB). Samples were incubated in a thermal cycler at 25°C for 10', 42°C for 60'

and finally at 90°C for 10' in order to denature RNA-cDNA hybrids. cDNA is diluted 1:5 in ddH_2O prior to use.

Analyses were carried out at least on triplicate samples for each experiment and they were processed separately. RT-PCR was performed on 4 µl of previously diluted cDNA (1:5) template using the Power Sybr Green Master mix (Applied Biosystem), in presence of 0.5 µM specific oligos. All oligos were designed using the Oligo7 software and are listed in Table 2.3 and were synthesized by Eurofins Genomics. The experiments were performed on a 7900 HT Fast Real time PCR system (Applied Biosystem) with a thermal profile consisted of 2' at 50°C, 10' at 95°C and 40 cycles of 15" at 95°C and 1' at 60°C. Gene expression levels were quantified by the comparative threshold cycle (CT) method (Livak et al. 2001, Schmittgen et al. 2008) using hypoxanthine phosphoribosyl-transferase (*HPRT*) as an internal control gene (Pernas-Alonso et al. 1999). The fractional number of PCR cycles CT required to obtain a given amount of amplified product in the exponential phase of amplification was determined for the gene of interest and for *HPRT* in each cDNA sample. The relative expression level of the gene of interest was then expressed as $2^{-\Delta CT}$ or as $2^{-\Delta\Delta CT}$ where $\Delta CT = (CT_{GENE} - CT_{HPRT})$ and $\Delta\Delta CT = (\Delta CT_{SAMPLE} - \Delta CT_{CTRL})$.

2.3.2 - miRVana RNA extraction and TaqMan® MicroRNA Assays

Usual RNA purification strategies rely on organic extraction, followed by alcohol precipitation. These strategies, because of the alcohol precipitation, are inefficient in recovering small RNA forms. The miRVana miRNA isolation (Ambion) strategy uses organic extraction followed by purification on a glass fiber filter (GFF) under specialized binding and wash conditions. With this strategy all RNAs are recovered, from large mRNAs to ribosomal RNAs down to 10-mers small RNAs. 1 volume of cold sterile PBS was added to samples in RNA later. Samples were centrifuged, RNA later/PBS solution was removed and cell pellet was resuspended in the provided lysis buffer. Later, RNA was extracted following the manufacturer instructions. As last step, RNA was eluted in 100 µl of RNAase-free water, previously warmed at 95°C and then quantified by spectrophotometric analysis. For the TaqMan® MicroRNA Assays sample were further diluted to 2 ng/µl. Instead, for miQPCR samples were diluted to 10 ng/µl.

TaqMan® MicroRNA Assays (Applied Biosystem) is the most commonly used strategy to quantify miRNAs. This assay is based on a two-step process. First, a reverse transcription is performed starting from a miRNA specific stem&loop primer. Secondly, target amplification is obtained using a miRNA specific forward primer and a reverse primer able to bind the opened loop. Amplification levels detection is based on a TaqMan® miRNA specific probe conjugated with a fluorescent dye (FAM™ dye) on its 5'end and a non-fluorescent quencher on its 3'end. Moreover a minor groove binder (MGB) is conjugated too on the 3'end. It is a particular tripeptide that allows stabilizing the probe specific binding without increasing probe length. Briefly, reverse transcription (RT) for miR-218-5p, miR-124a, miR-34b/c, miR-204, miR-148 and the reference snoRNA-202 was performed using the TaqMan® microRNA Assay provided RT primers for each of the listed miRNAs. The RT reaction was carried out with the suggested TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem) using reagents and enzymes provided with the kit and following the protocol provided by the company. qPCR was performed using primers and probe provided with the miRNA specific TaqMan® microRNA Assay e with the TaqMan® Universal Master Mix II, no UNG (Applied Biosystem). To perform qPCR I followed the protocol provided by the manufacturer. The instrument was the 7900 HT Fast Real time PCR system (Applied Biosystems).

2.3.3 – Western Blot analysis

To verify the enrichment of TH protein, mE12.5-PCs cells differentiated into mesDA neurons by over-expression of Nurr1, Lmx1a and both, were lysed in Lysis Buffer (150 mM NaCl, 50 mMTris-HCl pH7.4, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 0.2% NP40 and protease inhibitors). Proteins were extracted by centrifugation at 15000 g for 10' 4°C. Protein concentration was measured at spectrophotometer by Bradford assay (Bradford 1976). Proteins were separated on 12% SDS-polyacrilamide gel and transferred to PVDF membranes (Millipore) as yet described (Speranza et al. 2013). Filters were probed for 2 h at room temperature or overnight at 4°C with the primary antibody (Tab. 2.4). After washing, immunoblots were incubated with secondary antibodies (1:10000, Amersham) and the reaction detected with the ECL plus procedure (Amersham).

GENE	Fw oligo sequences (5'→3')	Rv oligo sequences (5'→3')	Aplicon size (base pair)
Bdnf	ACAATGTGACTCCACTGCCG	CACTCTTCTCACCTGGTGGAACT	70 bp
Dat	TCTGGGTATCGACAGTGCCA	GCAGCTGGAACTCATCGACAA	70 bp
DBh	GACCGGCTACTGCACAGACAA	GAGAGGCAAAGATGTGGATTCC	70 bp
Gad2	AGGGTTACTGATGTCCCGGAA	TCCATGTCACAGAGTTGGC	52 bp
Gfap	GAGGGACAACTTTGCACAGGA	CCAGCCTCAGGTTGGTTTCAT	70 bp
Hprt	TGGGAGGCCATCACATTGT	AATCCAGCAGGTCAGCAAAGA	71 bp
Lmx1a	AACCAGCGAGCCAAGATGAA	TGGGTGTTCTGTTGGTCCTGT	70 bp
Nestin	AGCAACTGGCACACCTCAAGA	CTCAGCCTCCAGCAGAGTCC	71 bp
Msx1	CTCCCCGCCCCCAGCCAGAC	GGGCTTGCGGTTGGTCTTGT	53 bp
Ngn2	ATCTGGAGCCGCGTAGGAT	CATCAGTACCTCCTCTTCCTCCTT	70 bp
Nurr1	CAACTACAGCACAGGCTACGA	GCATCTGAATGTCTTCTACCTTAAT	98 bp
Pitx3	GCAACTGGCCGCCCAAGG	AGGCCCCACGTTGACCGA	83 bp
pre-miR-204	AGATTCTCCCAGATTAACAAC	AGAGCATCCACAGAAGGTAG	118 bp
pre-miR-211	CAACCTATCAGGGCCGCACACT	GATCAAGCCGGTCAGTATTCC	108 bp
Pnmt	GTGTATAGTCAGCATGCCTGCC	CGAAGCTGGCGTTCTTTCTC	70 bp
c-Ret	TGAACCTACCCAGGGCCTACT	GACTTTCCCGATCTGGGCAT	70 bp
Sert	GGAACGAAGACGTGTCCGAG	TGCCTCCGCATATGTGATGA	70 bp
Sox2	AGGGCTGGACTGCGAACTG	TTTGCACCCCTCCCAATTC	71 bp
Th	CCTTTGACCCAGACACAGCA	ATACGAGAGGCATAGTTCCTGAG	121 bp
Tph2	GACCACCATTGTGACCCTGAA	ACGGCACATCCTCGAGATCT	70 bp
Trpm1	CTGCCTTGCTCAAAGGAACCAA	GAGGGGCCAGGCGGCCCAG	138 bp
Trpm3	CATGCACTCCCACTTCATCC	TGGAACCCCTTGACCGATT	208 bp
Tubb3	CGTGGGCTCAAAATGTCATC	TGGCTGTGAACTGCTCCGAGAT	66 bp
Vmat2	TTGCTCATCTGTGGCTGGG	TGGCGTTACCCCTCTCTTCAT	91bp

Table 2.3: Oligos for *real time* PCR.

2.4 - Microscopy and quantitative analyses

2.4.1 - Immunocytochemistry

After medium removal, cells were washed twice with PBS and then fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) in PBS for 30' at RT. Cells were permeabilized for 15 min with PBS-0.1%Triton-X-100 (Sigma-Aldrich). Later, fixed cells were incubated in a blocking solution with 5% BSA (Sigma-Aldrich) and 10% of normal goat serum (NGS; Sigma-Aldrich) in PBS for 1 h at RT and then incubated with primary antibody (the dilution was specific for each antibody), usually overnight at 4°C or 2 h at RT. Subsequently, cells were

washed three times in PBST, incubated 1 h at RT with fluorescence-labeled secondary antibody (1:400; Alexa Fluor®, Invitrogen). At the end, cells were washed again in PBS and counterstained with DAPI (1:1000; Invitrogen). As negative controls some cells were processed as described above, but without incubation with primary antibody.

Images were acquired using a Leica DMI6000 inverted microscope at 10× and 20×. In order to compare pictures from the same set of experiments, images were taken keeping the same settings on the acquisition software (Leica Application Suite AF). For cell counting, the number of positive cells was quantified using a custom macro disposable in the ImageJ software.

Epitopes	Host species	Applications	Working dilution	Manufacturer	Catalogue number
TH	Rabbit	IF	1:500	Chemicon	MAB318
TH	Mouse	WB	1:500	Chemicon	AB152
Lmx1a	Rabbit	WB	1:4000	Millipore	AB10533
V5	Mouse	IF	1:500	Invitrogen	R960CUS
Flag	Rabbit	IF/WB	1:1000	Sigma-Aldrich	F7425
Flag (M2)	Mouse	IF/WB	1:1000	Sigma-Aldrich	F3165
Ascl1	Mouse	IF	1:500	BD Pharmingen	556604
βIII-Tubulin	Mouse	IF	1:500	Covance	MMS-435P
β-Actin	Mouse	WB	1:10000	Sigma-Aldrich	MAB1501
Mouse IgG	Sheep	WB	1:10000	Amersham	NA931
Rabbit IgG	Donkey	WB	1:10000	Amersham	NA934
Mouse IgG	Goat	IF	1:400	Millipore	AP124F
Rabbit IgG	Goat	IF	1:400	Millipore	AP132F
Rabbit IgG	Donkey	IF	1:500	Jackson ImmunoResearch	711-605-152

Table 2.4: List of antibodies.

2.4.2 – High-Content imaging

Screening of miRNAs involved in DA induction was performed plating mE12.5-PCs in an optical 384-wells (Thermo scientific) as described above. The number of TH^+ cells were automatically analyzed by cell-based High Content Screening (HCS) confocal microscope Opera® Phenix (Perkim Elmer). HCS is an analysis tool used to acquire, manage, and search multi-parametric information regarding the composite phenotype of cells. In my experiments I acquired at least 270 areas per condition.

2.5 – Statistical analysis

For all experiments, analysis of variance was carried out, followed by post hoc comparison (ANOVA, Dunnett's or Newmann-Keuls test). A value of $p \leq 0.05$ was considered significant. Data were expressed as mean ± SEM and show p values with special symbols. At least three independent replicates were used for RT-PCR and for cell counts.

Analysis of High-Content data was performed on R software with one-way analysis of variance and Benjamini-Hocheberg's post hoc test, after standardization of value.

3 - RESULTS

Different types of neurons in the brain are interconnected to form the most complex network described in nature. In the brain, although all cell types are interspersed with each other every neuron maintains its individual phenotype. To recapitulate, *in vitro*, the processes that underlie neuronal development is obviously complex and require the identification of the factors involved, their right concentration and their application at a precise window of time. Moreover, often the same molecules can define the specification of different cell types depending by the surrounding trophic niche.

To bypass these problems, over the years, the scientists tried to simplify the scheme that physiologically occurs *in vivo* by plating neurons precursor cells and studying the *in vitro* processes underlying neuronal differentiation. Nevertheless obtaining homogeneous populations, *in vitro,* remains still a difficult procedure.

The aim of this work is to reproduce, *in vitro,* mesencephalic dopaminergic neurons (mesDA) development and to identify new players involved in this process.

The data herein 1) describe a synergy between the two transcription factors Nurr1 and Lmx1a able to improve the differentiation and the maturation of the mesDA phenotype and 2) identify two microRNAs involved in DA development.

3.1- Lmx1a synergizes with Nurr1 in the specification of mesDA phenotype

Lmx1a is reported as transcription factor (TF) involved in early phases of DA induction (Andersson et al. 2006). Only in the last years it has been associated also to differentiation and maturation of mesDA neurons and in promoting *trans*differentiation into DA cells (Chung et al. 2012, Caiazzo et al. 2011). How Lmx1a mediates its function is still unclear. To dissect the role of Lmx1a in the developing midbrain, I over-expressed Lmx1a and Nurr1 in different cell types to generate mesDA neurons and to study the eventual synergy between these two TFs.

3.1.1 – Screening of transcription factors affecting mesDA differentiation

To generate an *in vitro* dopaminergic model system, we isolated neural progenitor cells (NPCs) from midbrains of mouse embryos (E) at 12.5 gestational day. E12.5 is the time window, during midbrain development, where TH^+ cells have been already generated (the process start at E9) but have not yet completed the differentiation phase. Indeed DA neuron maturation starts at around E13 (di Porzio et al. 1990), thus E12.5 is the time point where we expect to obtain the higher number of mesDA precursors not yet committed to differentiation that maintain responsiveness to differentiating factors.

Based on these considerations, I dissected E12.5 midbrains from wild type (WT) and TH-GFP C57 BL/6 mice, a transgenic mouse line carrying the green fluorescent protein (GFP) under the control of TH promoter (Matsushita et al. 2002).

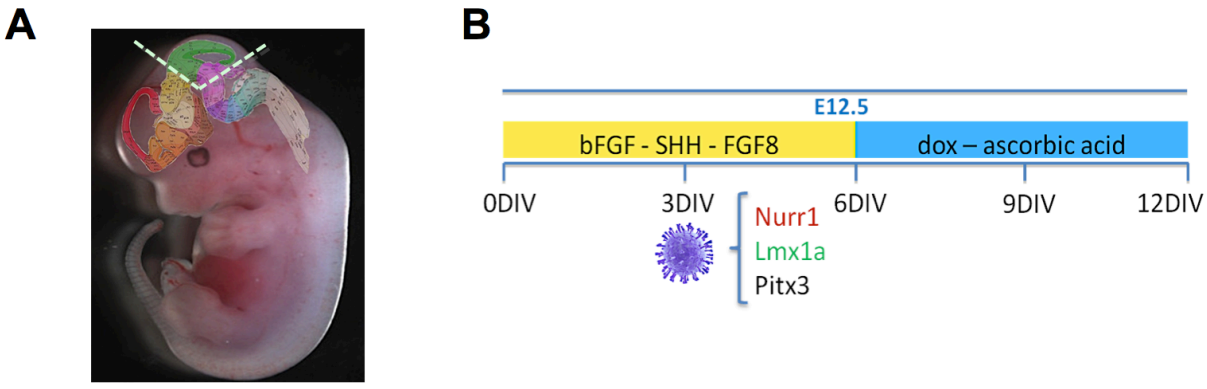

Figure 3.1: *in vitro* differentiation of midbrain neuroblasts.
(A) Image of mouse embryo at E12.5 with schematic diagram of encephalic areas (red, telencephalon; yellow, diencephalon; green, mesencephalon; pink, metencephalon; cyan, myelencephalon; white: spinal cord).
(B) Schematic protocol of *in vitro* mesDA differentiation of mE12.5-PCs cells (view Materials and Methods section for further details).

Midbrain-derived NPCs were plated at low density (≈ 4×10^4 cells/ cm^2) in proliferative medium in order to expand pluripotent cells. During this phase, ventral-midbrain commitment was induced by the addition of the morphogens SHH (50 ng/mL) and FGF8 (10 ng/mL). After six days *in vitro* (DIV6) SHH, FGF8 and bFGF were replaced by ascorbic acid (200 µM) to promote neuronal survival and differentiation. By using this basic protocol we were able to identify in the culture dish TH^+ cells (Volpicelli et al. 2004); however their amount was still too low to perform functional studies. In order to implement the amount of TH^+ cells, I over-

expressed specific pro-dopaminergic transcription factors by using inducible lentiviral vectors as described in Matherial and Methods section (Fig. 3.1).

The reason why I approached the lentiviral system is that, differently from classical transfection protocols, such as Lipofectamine, it allows high efficiency of infection in non-dividing primary cultures, thus generating higher number of cells expressing the gene of interest. To this purpose I used inducible lentiviruses that allow controlling over time the expression of the transfected gene by adding doxycycline to the culture media (see Material and Methods section).

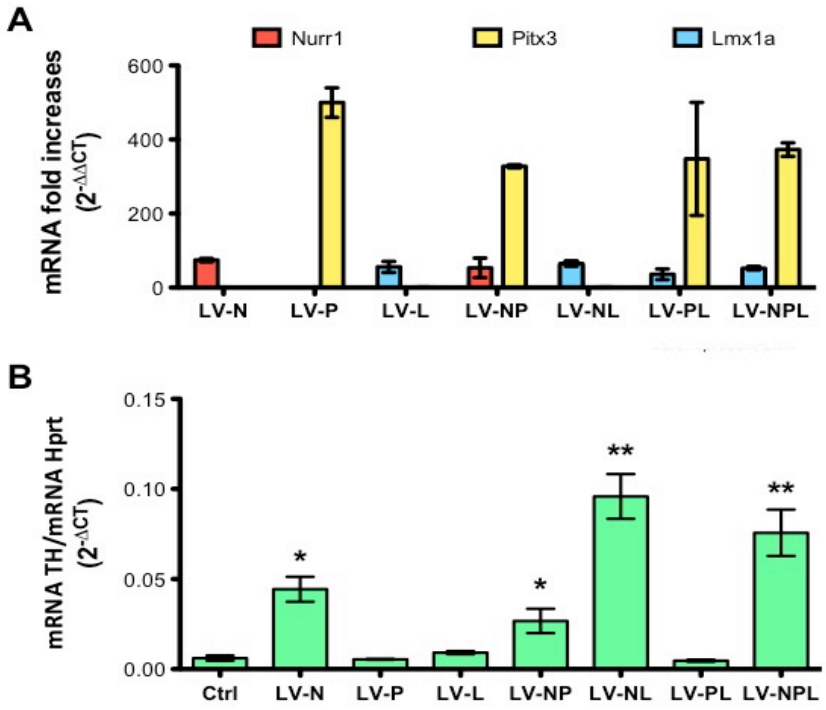

Figure 3.2: Screening of pro-dopaminergic transcription factors.
(A) The diagram shows *real time* PCR evaluation of exogenous genes expression induced by doxycycline in mE12.5-PCs infected with lentiviral particles carrying 3xFlag-Nurr1 (LV-N), Pitx3 (LV-P), Lmx1a-V5HIS (LV-L) and their combinations. The mRNA fold increse of interest genes were normalized on the housekeeping gene level (*Hprt*), and compared to the average of control (Ctrl=1; $2^{-\Delta\Delta CT}$ method). Bars represent mean ± SEM of quadruplicated samples.
(B) The diagram shows the TH mRNA level in mE12.5-PCs infected as previously described. TH mRNA level were normalized on the Hprt mRNA ($2^{-\Delta CT}$ method). Bars represent mean ± SEM of quadruplicated samples. * $p \leq 0.05$ respect Ctrl ; ** $p \leq 0.01$ respect Ctrl.
p values were calculated after one-way analysis of variance and Dunnett's post hoc test to compare all columns to control.

In detail, during the proliferative stages at DIV3, midbrain E12.5 primary cultures (mE12.5-PCs) were infected with inducible lentiviral particles carrying the transcription factors Nurr1 (tagged at N-terminus with 3xFLAG peptide), Lmx1a (tagged at C-terminus with V5-HIS) and

Pitx3. The infected cells were amplified for other 3DIV and the transcription factors expression was induced by the addition of doxycycline (4 μg/mL) at DIV6, when the differentiation was induced. The efficiency of infection was evaluated by *real time* PCR and immunostaining indicating that more than 70% of cultured cells was infected (data not shown) expressing higher levels of exogenous genes if compared to non-infected cells (Fig. 3.2A). To evaluate if our experimental conditions allowed maturation of dopaminergic precursors, I measured by *real time* PCR the expression of TH at DIV12 in different conditions. As shown in figure 3.2B, the over-expression of Nurr1 increases TH mRNA levels compared to control, while Lmx1a and Pitx3 do not affect TH expression. Interestingly, the combination of Nurr1 with Lmx1a improves the yield of TH^+ cells if compared to Nurr1 alone (Fig. 3.2B). Surprisingly, I did not obtain any effect, in term of TH expression, when Pitx3 was combined with Nurr1, suggesting that addition of Pitx3 does not influence DA differentiation but probably acting mainly as a pro-survival factor (see Introduction).

These preliminary observations allowed to set up a model system and to select the transcription factors most appropriate to recapitulate *in vitro* mesDA development.

3.1.2 - Lmx1a synergizes with Nurr1 to regulate TH expression

Starting from these data, I chose to further investigate the interaction of Nurr1 and Lmx1a during *in vitro* mesDA differentiation. Lentiviral over-expression of Nurr1 is able to induce 10 times higher levels of TH mRNA when compared to control (Fig. 3.3A) similarly to what previously described (Zhou et al. 1995). Interestingly, over-expression of Lmx1a alone does not affect TH mRNA levels, but potentiates Nurr1 activity with a 15-fold increase with respect to control, when co-expressed with Nurr1 (Fig. 3.3A).

To verify if similar differences were observed also at protein levels I performed western blot analysis for TH. As shown in figure 3.3B-C, Nurr1 over-expression increases two times TH protein expression while the combination Nurr1-Lmx1a triplicates TH level if compared to control (Fig. 3.3B-C).

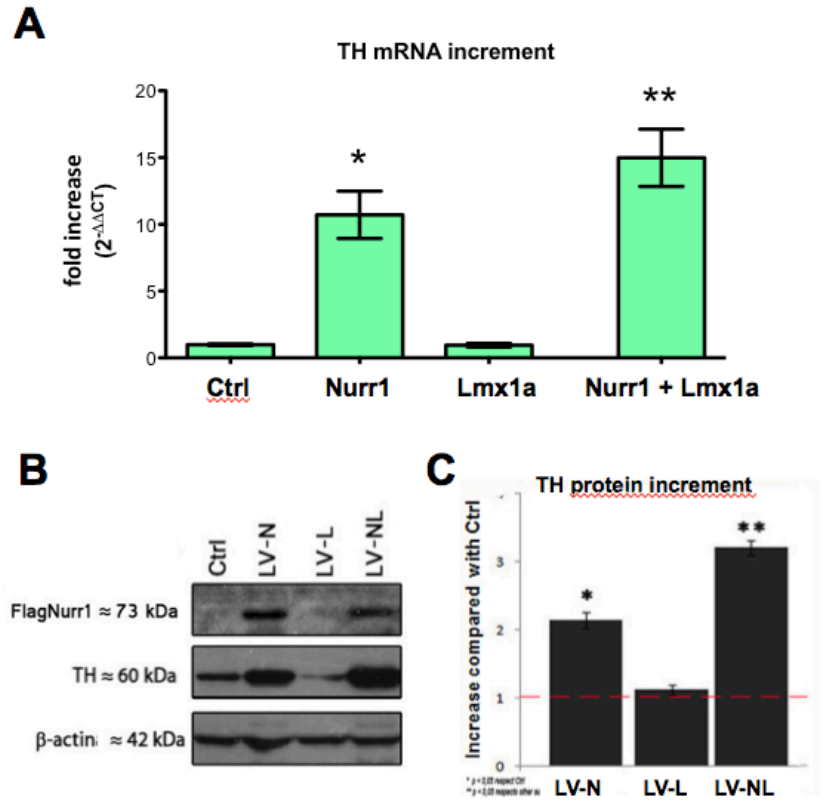

Figure 3.3: TH up-regulation in mE12.5-PCs over-expressing Nurr1 and Lmx1a.
(A) The diagram show the TH mRNA level in differentiated mE12.5-PCs over-expressing Nurr1, Lmx1a or both. TH expression were normalized on Hprt mRNA and compared to control (Ctrl=1; $2^{-\Delta\Delta CT}$ method). Bars represent mean ± SEM of quadruplicated samples. * $p \leq 0.05$ relative Ctrl and Lmx1a; ** $p \leq 0.01$ relative to Ctrl and Lmx1a and $p \leq 0.05$ relative to Nurr1. p values were calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.
(B) Western blotting images of differentiated mE12.5-PCs over-expressing 3xFlag-Nurr1 (LV-N), Lmx1a-V5HIS (LV-L) or both (LV-NL). Nurr1 protein over-expression was evaluated by rabbit anti-Flag antibody, TH protein by mouse anti-TH and β-actin by anti-mouse β-actin.
(C) Quantitation of TH proteins. Bars represent the densitometric values of TH western blot signals normalized with those of β-actin in the same samples and compared to the control indicated as dotted line (Ctrl = 1; mean ± SEM; n = 3).

I also analyzed TH expression by immunofluorescence. At DIV12 differentiated mE12.5-PCs express Tuj1; however they form clusters of cells (Fig. 3.4A) that makes it more difficult to quantify the percentage of mesDA neurons. To bypass this problem, I combined immunofluorescence analysis with high-contents automated confocal microscopy to acquire high-resolution images from an extended area (270 images for each condition) that allow counting of TH^+ cells. This analysis revealed that the combined over-expression of Nurr1+Lmx1a increased the number of TH+ cells doubling the effect of the single over-expressing Nurr1 (Fig. 3.4A-B).

These data suggest a potential cooperation between the transcription factors Lmx1a and Nurr1, at least as regulator of TH expression.

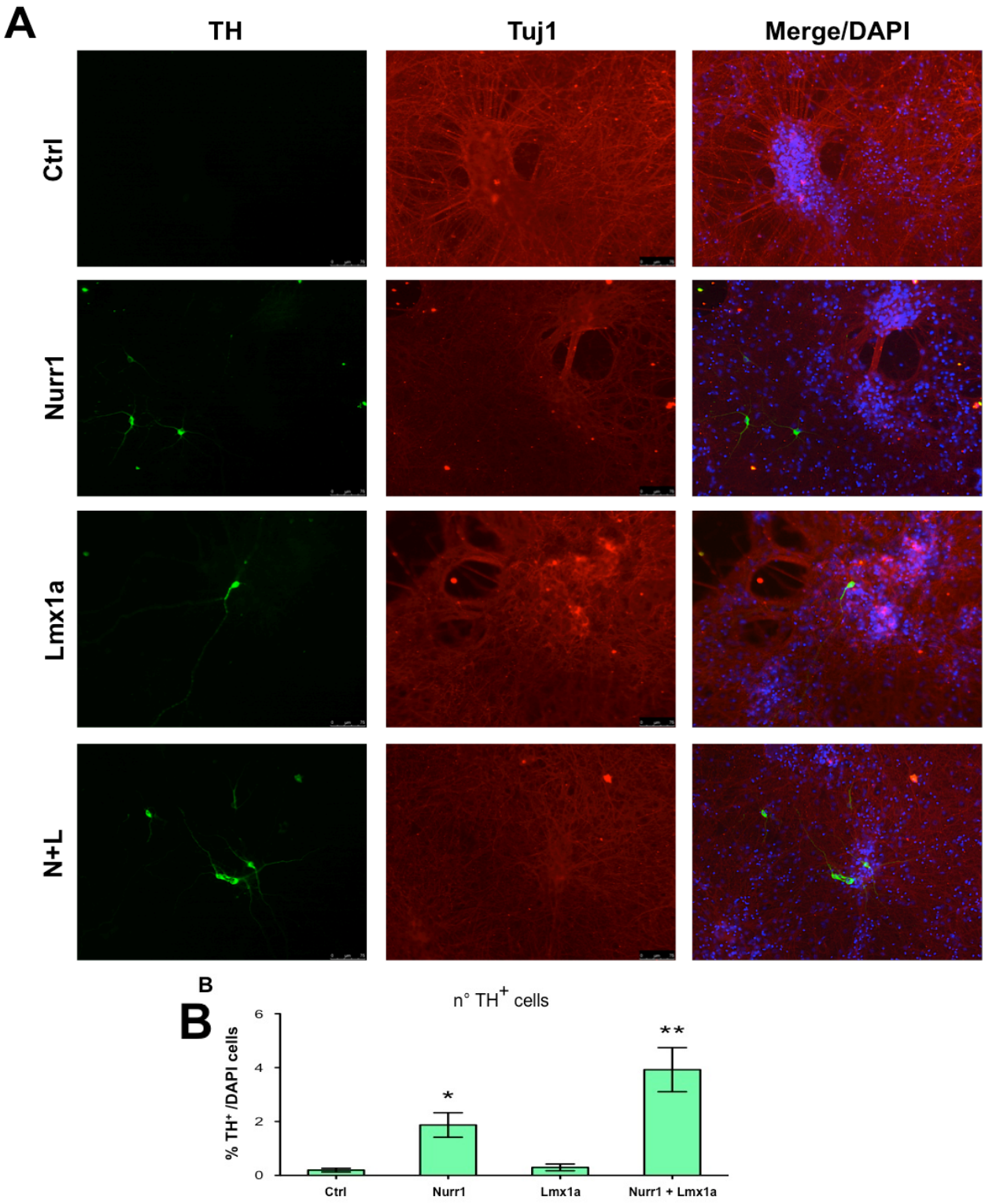

Figure 3.4: Nurr1 and Lmx1a increase the number of TH⁺ cells.
(A) Differentiated mE12.5-PCs over-expressing Nurr1 and Lmx1a were immunostained with specific antibodies against the neuronal marker βIII-tubulin (anti-Tuj1 in red) and TH (green). Images were acquired with DMI6000 Leica microsystems at 20x magnification.
(B) The bar plot represents the percentage of cells immunopositive for the nuclear marker DAPI and for the antibody anti-TH. Means ± SEM; n = 270 ± 12; * $p \leq 0.05$ relative to Ctrl and Lmx1a; ** $p \leq 0.01$ relative to Ctrl and Lmx1a and $p \leq 0.05$ respect Nurr1. p values were calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.

3.1.3 - Over-expression of Lmx1a and Nurr1 promotes maturation of midbrain neurons

To verify whether the over-expression of Nurr1 with Lmx1a effectively promotes mesDA differentiation, I analyzed the levels of expression for other DA markers in differentiating mE12.5-PCs. Over-expression of Nurr1 (LV-N or LV-NL) up-regulated the mRNA levels for *Pitx3*, *Vmat2*, *Dat*, GDNF receptor (*c-Ret*) and *Bdnf* (Fig. 3.5A). Lmx1a potentiates the effect of Nurr1 on *Vmat2*, other than *Th*. Lmx1a alone had no effects on any of these genes but controls the expression of *Msx1* and *Ngn2*, already described as direct targets (Fig. 3.5A; Yan et al. 2011, Hong et al. 2014).

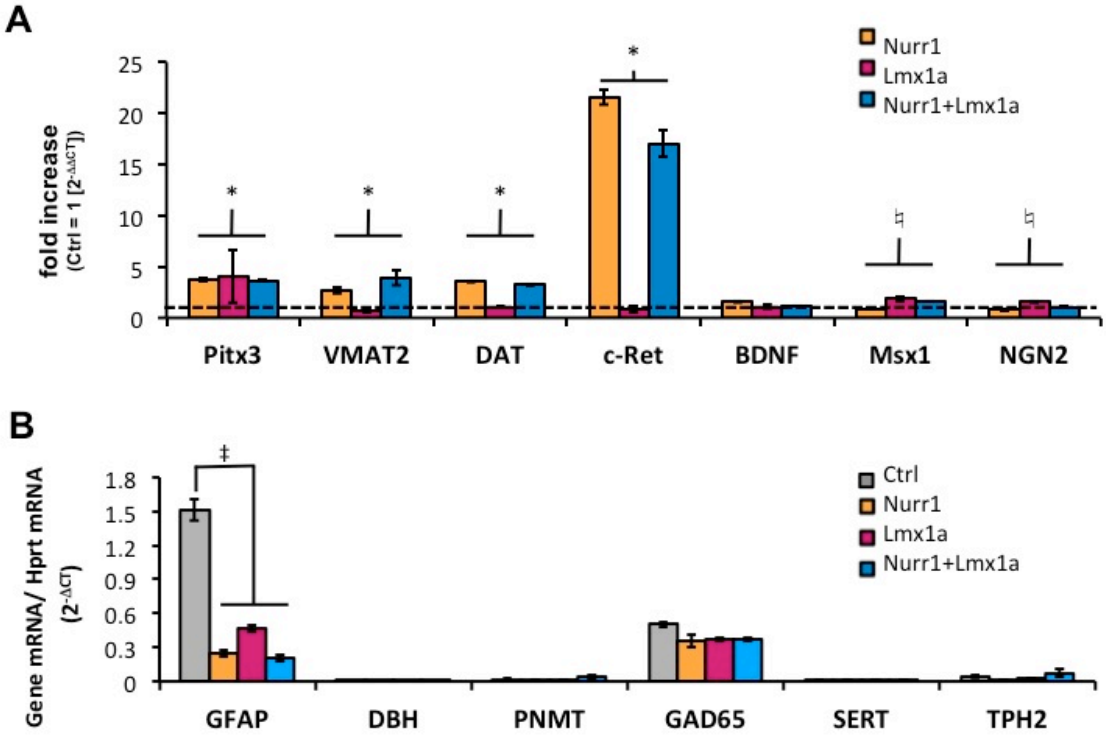

Figure 3.5 (*previous page*): Characterization of mE12.5-PCs over-expressing Nurr1 and Lmx1a.
Real time PCR analysis of dopaminergic (A) and non-dopaminergic (B) genes in mE12.5-PCs after infection with lentiviral particles carrying Nurr1 (orange), Lmx1a (pink) and both (blue). In (B) values of the mRNA genes were normalized on that of *Hprt* ($2^{-\Delta CT}$ method) and in (A) compared to average of control points ($2^{-\Delta\Delta CT}$ method). Means ± SEM, n = 6.
* $p \leq 0.05$ between Nurr1 or Nurr1+ Lmx1a respect Ctrl; ǂ $p \leq 0.05$ between Lmx1a respect Ctrl; ‡ $p \leq 0.01$ between Ctrl and all other samples. *p* values were calculated after one-way analysis of variance and Dunnett's post hoc test to compare all columns to control.

At the same time, Nurr1 and Lmx1a reduced *Gfap* expression (marker of glial and precursor cells; Fig. 3.5B), suggesting that these two TFs promote pro-neuronal differentiation. Remarkably, the neuronal phenotype obtained is dopaminergic-specific, as revealed by almost undetectable levels of others catecholaminergic markers such as *DBH* and *PNMT* and serotonergic markers (*SERT* and *TPH2*; Fig. 3.5B). In culture few GABA-like cells were present and Nurr1 and Lmx1a reduced the mRNA levels of GABAergic marker *GAD65*.

Therefore mE12.5-PCs differentiate in mesDA neurons through the over-expression of Nurr1 and Lmx1a, resulting a good *in vitro* model to study the mesDA phenotype.

3.1.4 - Pro-mesDA effects of Lmx1a and Nurr1 co-expression in mes-*c-myc*-A1 cell line.

Starting from the protocol described on mE12.5-PCs I also used the murine mesencephalic cell line mes-*c-myc*-A1 to generate DA cells. These cells have been already characterized for their commitment to the dopaminergic phenotype (A1; Colucci-D'Amato et al. 1999) and have been used as an alternative model to recapitulate dopaminergic differentiation. In details, A1 cells were plated at low density in presence of SHH (50 ng/mL) and FGF8 (10 ng/mL). At DIV2 cells were infected with the inducible lentiviral vectors carrying Nurr1 and/or Lmx1a (LV-N and LV-L) and at DIV4 differentiation was induced by activation of exogenous genes with doxycycline. After 10 days (DIV14), cells were harvested to perform mRNA and IF analysis. First of all the efficiency of over-expression was verified by *real time* PCR (data not shown); as expected differentiated A1 express *βIII-tubulin* also in control (Fig. 3.6A). Over-expression of Nurr1 alone or in combination with Lmx1a decreased *Gfap*, *Nestin* and *Sox2* expression therefore boosting neuronal differentiation (Fig. 3.6A). Similarly to mE12.5-PCs, Lmx1a alone did not influence *Gfap* level and increased *Sox2* mRNA. The evaluation of mesDA markers shows that similarly to primary cultures, Nurr1 alone or in combination with Lmx1a increased both TH mRNA levels (Fig. 3.6B) and the number of TH^+ cells (Fig. 3.6C) if compared to control samples. Moreover the TH mRNA enrichment in A1 cells was higher of that observed in primary cultures, most probably due to the very low levels of TH in undifferentiated A1 cells respect to primary cultures. Similarly to primary cultures, the over-expression of Lmx1a enhances the action of Nurr1 on TH induction, supporting the hypothesis of a cooperative action between the two factors. The up-regulation was observed also for *Vmat2* and *Dat* genes, even if in A1 cells Lmx1a alone was able

to increase the mRNA levels for *Dat*. These data confirm that A1 cells behave in part differently from primary cultures. Most probably they are more responsive to transcription factors since they have been engineered to constitutively express *c-myc*.

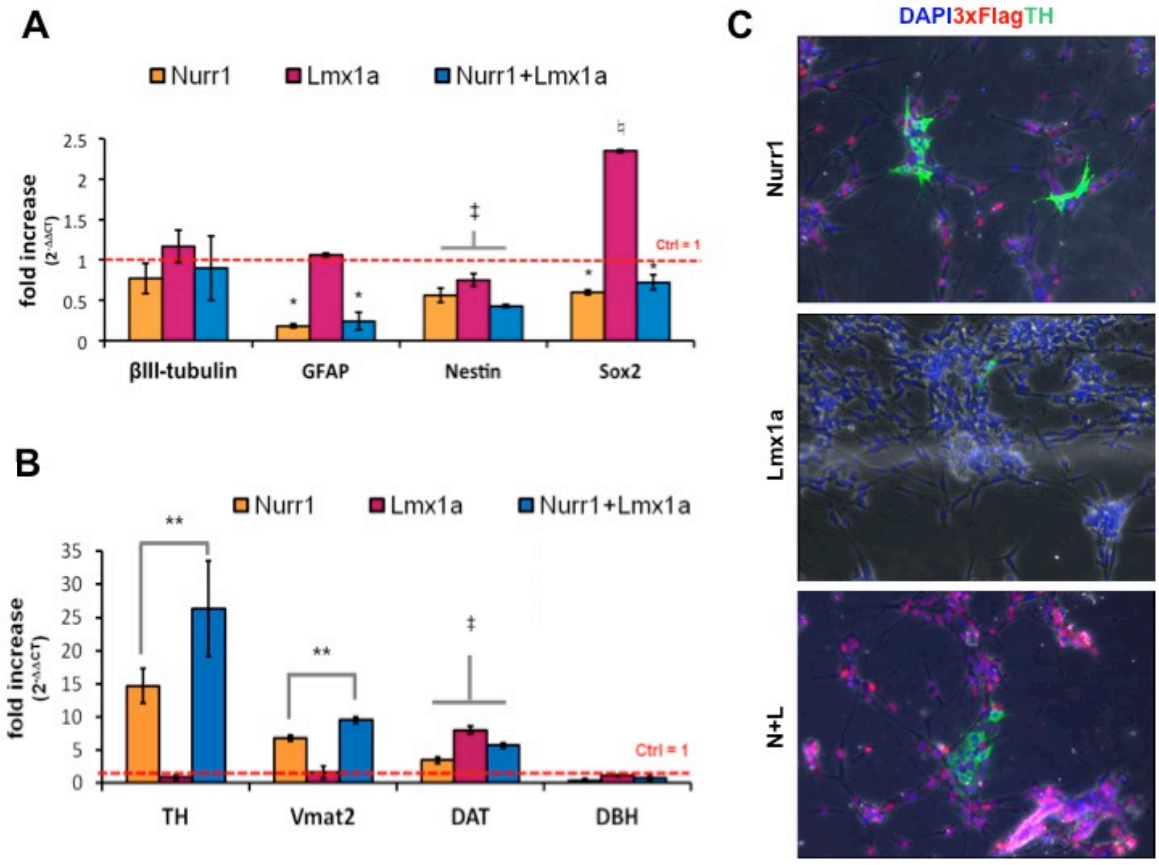

Figure 3.6: Characterization of mes-*c-myc*-A1 over-expressing Nurr1 and Lmx1a.
Real time PCR analysis of neuronal and glial marker (A) and cathecolaminergic genes (B) in mE12.5-PCs after infection with lentiviral particles carrying Nurr1 (orange), Lmx1a (pink) and both (blue). mRNA genes levels are normalized on that of *Hprt* and compared to average of control points represented as dotted line (Ctrl=1; $2^{-\Delta\Delta CT}$ method). Bars represent means ± SEM, n = 5. * $p \leq 0.05$ of samples compared to control. ** $p \leq 0.05$ between linked samples and of both with control. ‡ $p \leq 0.05$ of all samples compared to control. $^{\natural}$ $p \leq 0.05$ with others samples. *p* values were calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.
(C) Immunofluorescence analysis of A1 over-expressing Nurr1, Lmx1a or both (N+L). Exogenous Nurr1 was stained in red (anti-3xFlag). TH was marked in green. Pictures displays also phase contrast images and DAPI staining. 20x magnification.

3.1.5 – Nurr1 promotes mesDA maturation arresting the cell cycle in mes-*c-myc*-A1

Mature neurons are mitotically quiescent (G0 phase of cell cycle). To understand if Nurr1 could promote neuronal differentiation by arresting cell cycle progression, I performed a cell cycle assay with propidium iodide in A1 cells over-expressing the transcription factors Nurr1, Lmx1a and both or Pitx3. In basal condition only ¼ of cells were in mitotic phase (G0/G1: 57% - G2/M: 24% - S: 19%; Fig. 3.7B). While over-expression of Lmx1a did not affect this distribution (G0/G1: 60% - G2/M: 21% - S: 19%; Fig. 3.7B), Nurr1 over-expression reduced the mitotic population at less than ⅕ (G0/G1: 67% - G2/M: 17% - S: 16%; Fig. 3.7B). Similar effects were recorded with the over-expression of Pitx3, a post mitotic factor (G0/G1: 71% - G2/M: 15% - S: 14%; Fig. 3.7C) or after 48 hours serum withdrawal (G0/G1: 75% - G2/M: 13% - S: 12%; Fig. 3.7C). Interestingly, over-expression of Nurr1 prevented re-entry into cell cycle (**Nurr1-*R***: G0/G1: 75% - G2/M: 13% - S: 12%; Fig. 3.7C). These results confirm that upon Nurr1 expression A1 cells definitely exit from cell cycle and become quiescent mature neurons. Interestingly cells expressing Pitx3 behave in similar way.

In my opinion, Nurr1 determines neural maturation by promoting cell cycle arrest. Most probably this occurs regulating cyclin-dependent kinases inhibitors like that already reported for $p57^{kip2}$ (Joseph et al. 2003). I am actually planning to analyze different cyclins.

3.1.6 – Functional analysis of different Nurr1 domains

3.1.6a – C-terminus domain of Nurr1 is not necessary to Lmx1a cooperation

To better characterize the nature of the functional interaction between Nurr1 and Lmx1a I evaluated the activity of two truncated form of Nurr1 deleted for the N-terminal and C-terminal domain named respectively ***ΔNter-Nurr1*** and ***ΔCter-Nurr1*** (Fig. 3.8A). To this purpose we generated lentiviral particles carrying the truncated forms as well as a mutant transcriptionally inactive named $Nurr1^{C280A/E281/A}$. As shown in figure 3.8B, both ***ΔNter-Nurr1*** and $Nurr1^{C280A/E281/A}$ were transcriptionally inactive in mE12.5-PCs. Differently, the over-expression of ***ΔCter-Nurr1*** increased significantly TH mRNA (Fig. 3.8B) and retained the ability to boost TH expression when over-expressed with Lmx1a, as observed for the combination Lmx1a-Nurr1 (Fig. 3.8B-C).

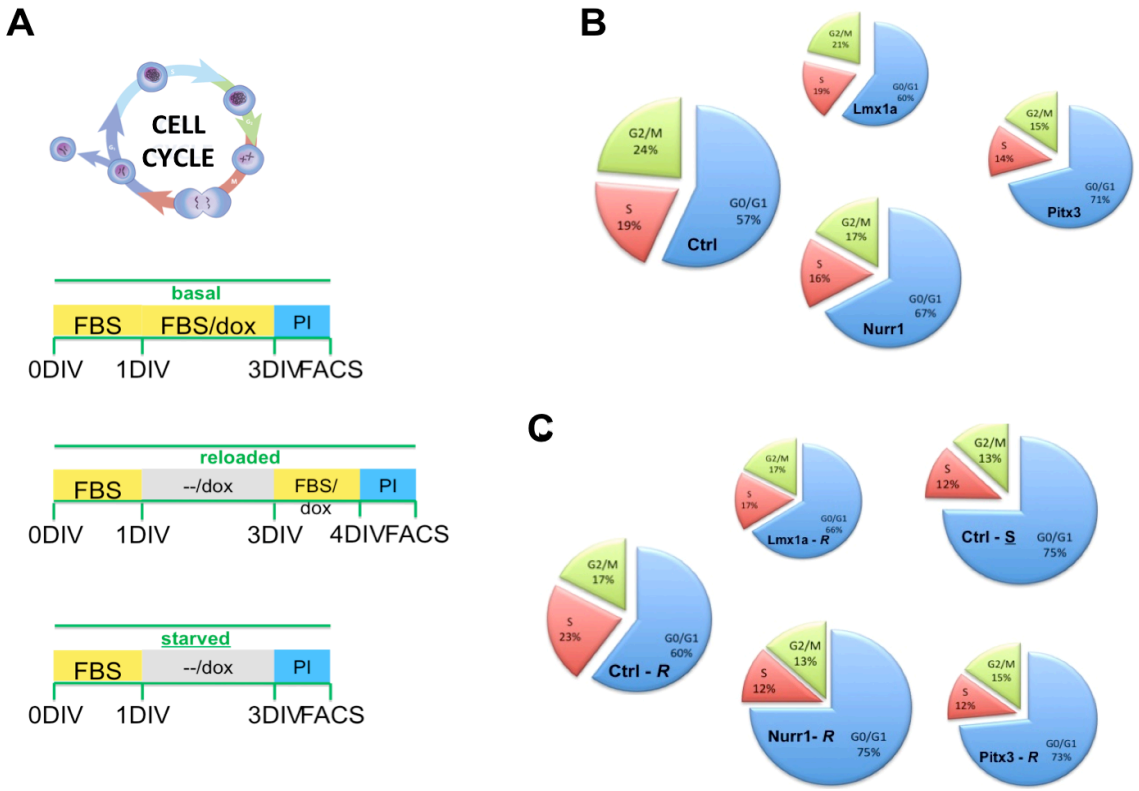

Figure 3.7 (*next page*): Nurr1 promotes the cell cycle arrest.
(A) Experimental design for cell cycle analysis with propidium iodide assays in mes-*c-myc*-A1 cells. In basal condition (***B***) cells were cultured for 3 DIV in presence of serum and doxycycline to express exogenous genes. The reloaded sample (***R***) were starved from serum for 48 h to syncronize cells at G_1 phase and then cell cycle was reactivated with serum addiction for 24 h. The control point starved (***S***) was harvested like ***R*** samples without serum addiction.
(B) Cell cycle analysis of cells over-expressing Nurr1, Lmx1a and Pitx3 in ***B***.
(C) Cell cycle analysis of cells over-expressing Nurr1, Lmx1a and Pitx3 in ***R*** and ***S*** control point.

These data demonstrate that: 1) the N-terminus domain of Nurr1 is necessary to its transcriptional activity (as also described by Nordzell et al. 2004); 2) the interaction with the DNA is highly specific and just two aminoacidic substitutions in the DNA binding domain are crucial to lose its function; 3) the interaction of Lmx1a with Nurr1 does not require physical binding to the C-terminus domain of Nurr1, reported as potential ligand binding domain; 4) the cooperation between Nurr1 and Lmx1a could be directed or mediated by a third factors.

To better describe how Lmx1a synergizes with Nurr1, we performed Co-IP and ChIP analysis. Unfortunately, co-immunoprecipitation was unsuccessful most probably due to poorly efficient antibodies. Instead the results obtained by performing chromatin immunoprecipitation were promising (data not shown), however there was limited enrichment when we immune-precipitated the chromatin-antigen complex, probably due to the relative low amount of mesDA

differentiated cells. To bypass this problem, we generated stem cells (ESCs) stably expressing fluorescent Nurr1 and Lmx1a alone or in combination (see below).

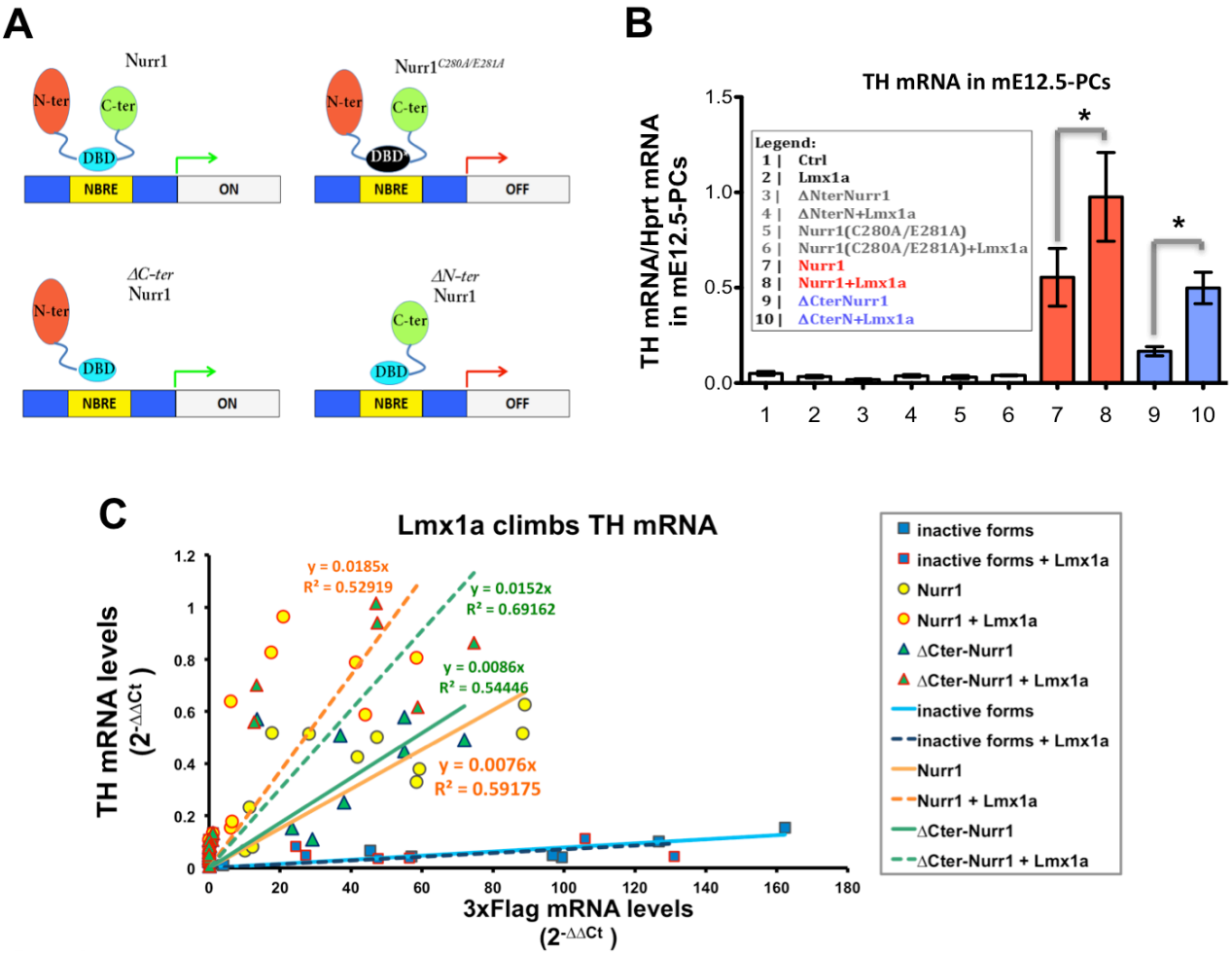

Figure 3.8 (*previous page*): Activity of truncated forms of Nurr1 on TH regulation.

(A) Schematic representations of Nurr1 truncated forms.

(B) *Real time* PCR evaluation of TH expression in mE12.5-PCs after infection with lentiviral particles carrying truncated forms of Nurr1 ± Lmx1a as reported in the legend. Values of TH mRNA were normalized on *Hprt*. Means ± SEM, n = 12. $^{*}p \leq 0.05$ between the samples and $p \leq 0.01$ compared to Ctrl. *p* values were calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.

(C) Linear correlation between TH mRNA and exogenous Nurr1 mRNA. Values of $2^{-\Delta CT}$ for the two genes in the same samples were blotted to generate linear regression. Values of $R^2 \geq 0.5$ indicate that the regression lines fit the data with meaningfulness.

The slopes of inactive forms of Nurr1 are almost near the zero ($m_{\text{inactives+Lmx1a}} = 0.0008$, $m_{\text{inactives}} = 0.0007$), while the addition of Lmx1a to Nurr1 or ΔCter-Nurr1 increases the slope of those points ($m_{\text{Nurr1}} = 0.007$, $m_{\text{Nurr1+Lmx1a}} = 0.016$, $m_{\Delta\text{Cter}} = 0.005$, $m_{\Delta\text{Cter +Lmx1a}} = 0.015$; Fig. 3.8E).

3.1.6b – Subcellular localization of *ΔCter-Nurr1* and *ΔNter-Nurr1*

To verify whether *ΔCter-Nurr1* and *ΔNter-Nurr1* proteins have different sub-cellular localization, I expressed the truncated forms and performed immunofluorescence in HEK293T,

mE12.5-PCs and A1. Surprisingly, while the transcriptionally inactive *ΔNter-Nurr1* was mainly located into the nucleus similarly to the full-length Nurr1, the transcriptionally active *ΔCter-Nurr1* had an unexpected cytosolic distribution (Fig. 3.9). This data contrast what alreasy described previously (García-Yagüe et al. 2013) where two motifs in the C-terminus domain named nuclear exporting signals (NES1 and NES2) absent in our *ΔCter-Nurr1,* have been shown essential for cytoplasmic translocation. This interesting result will be further investigated in future additional experiments, in order to exclude potential artifacts and to find a biological explanation. It will be useful however to understand how the cytoplasmic *ΔCter-Nurr1* promotes DA differentiation. At present the easier explanation is that a small amount of the protein localizes in the nucleus, being enough to promote TH transcription.

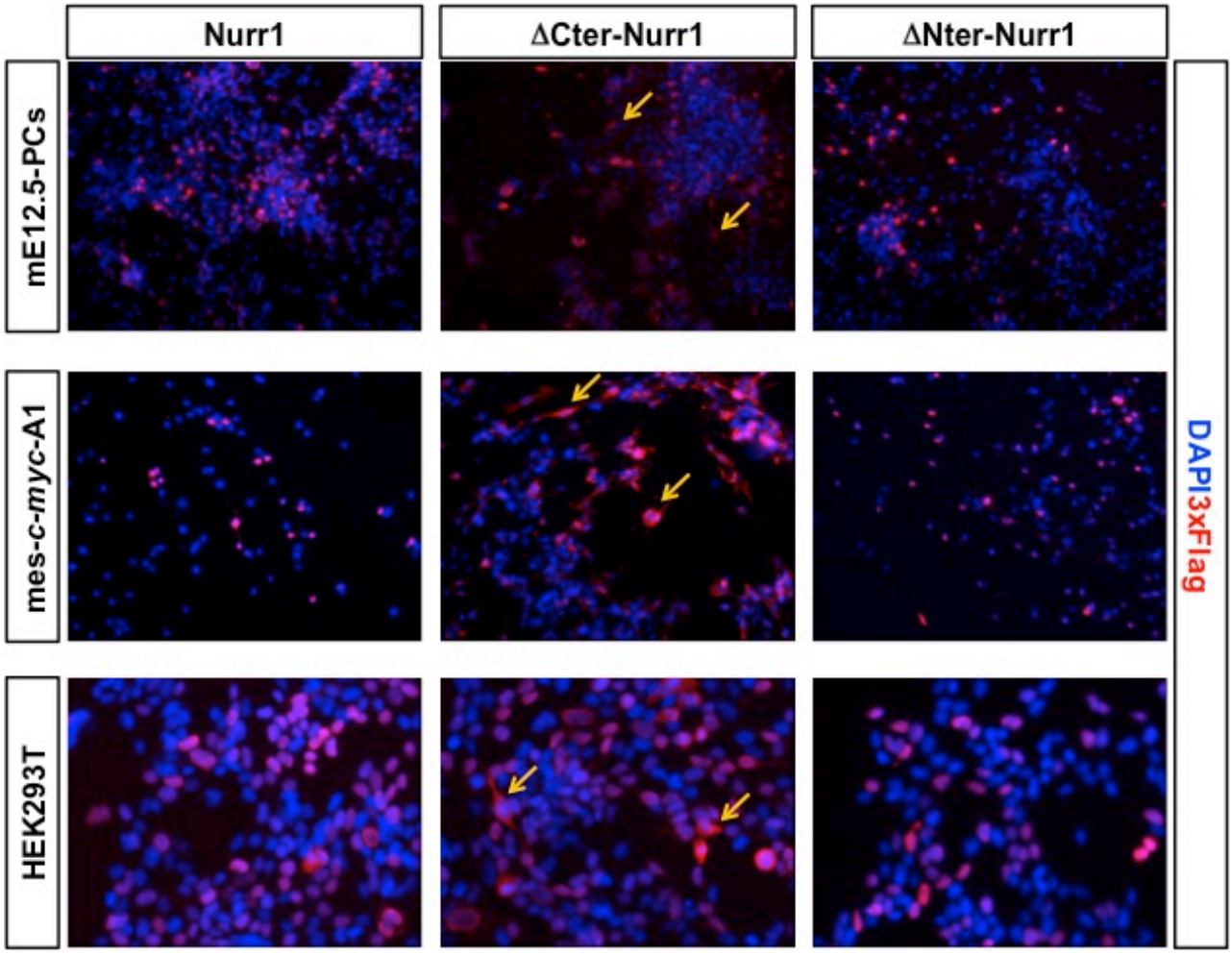

Figure 3.9: Sub-localization of truncated form of Nurr1.
Panel shows over-expressed truncated forms of Nurr1 in mE12.5-PCs, A1 and HEK293T cells, revealed with anti-3xFlag antibody in red. Live cells were stained with the DAPI. Yellow arrows evidence the cytosolic localization of ΔCter-Nurr1 compared to ΔNter-Nurr1 or full length Nurr1. The different morphology of HEK293T cells shows a perinuclear localization of ΔCter-Nurr1. DMI600 20x magnification.

3.2 – Identification of microRNAs involved in dopaminergic development

The cellular model here defined has been extremely useful to identify novel players in dopaminergic transmission.

In the last years a great attention has been dedicated to the role that *non-coding* RNAs play in neuronal differentiation. Among these, particular attention is paid on microRNAs, is mainly due to their potential use as therapeutic molecules (Junn et al. 2012). In addition, miRNAs could be really useful to easily modulate developmental processes and physiological conditions, or to ameliorate DA modeling. Actually, knowledge of their involvement during mesDA development is very limited (Baek et al. 2014). I focused my work on a specific group of microRNAs that have been identified in my host laboratory, because involved in the differentiation of epiSCs into mesDA neurons (De Gregorio et al. *unpublished*), I thus evaluated if over-expression of these miRNAs influences the differentiation of mesDA neurons and if they are modulated by Nurr1 and Lmx1a.

3.2.1 – miR-218-1 increases the number of TH^+ cells in mE12.5-PCs

To evaluate if any of the candidate miRNAs could have a role in the development of DA phenotype, I over-expressed each candidate by lentiviral particles in mE12.5-PCs in presence or in absence of Nurr1 and analyzed the number of TH^+ cells by High-Content confocal microscopy. As shown in Fig. 3.10, only the over-expression of miR-218-1 in combination with Nurr1 significantly enhanced the activity of Nurr1, increasing the number of TH^+ cells. This effect is similar to that observed over-expressing Nurr1 with Lmx1a. miR-218-1 alone had no effect on TH^+ cells compared to control (data not shown).

miR-34b/c, miR-148a and miR-210 showed a trend similar to that of miR-218-1 (Fig. 3.10). Although the increment in term of TH^+ cells observed upon their over-expression was not significant (Tab. 3.1), we cannot completely exclude their involvement in dopaminergic differentiation. They may represent a research objective in future projects.

Others analyzed miRNAs (miR27, miR29, miR-132, miR204, miR219, miR-370, miR-375 and miR-494) did not affect the number of TH^+ cells (Fig. 3.10) in the same experimental conditions.

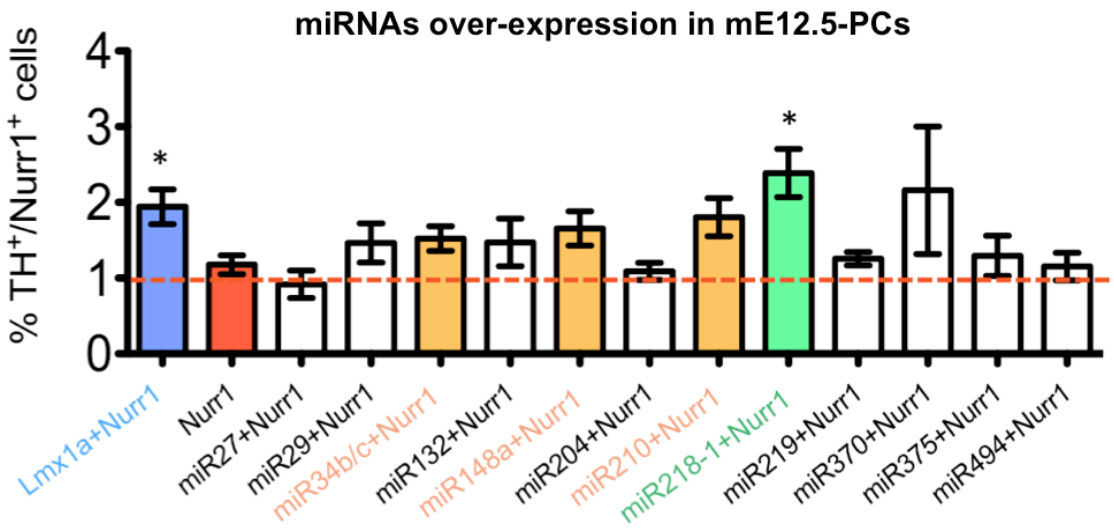

Figure 3.10: miRNA-218-1 enhances the activity of Nurr1 in the induction of TH expression.
High-content screening of miRNA's activity on the number of TH⁺ cells after over-expression of Nurr1 in mE12.5-PCs derived from TH-GFP mice. GFP⁺ cells were counted at automated confocal microscope Opera and normalized on 3xFlag⁺ cells. Means ± SEM, n = 270 ± 60. * $p \leq 0.05$ of the samples respect to the control. p values are reported in Tab. 3.2.

Comparison of x by group (Benjamini-Hochberg)

	Lmx1a+Nurr1	miR132+Nurr1	miR148+Nurr1	miR204+Nurr1	miR210+Nurr1	miR218+Nurr1	miR219+Nurr1	miR27+Nurr1	miR29+Nurr1	miR34+Nurr1	miR370+Nurr1	miR375+Nurr1	miR494+NNurr1
miR132+Nurr1	0.2372												
miR148+Nurr1	0.2641	0.4209											
miR204+Nurr1	0.0003*	0.2307	0.0579										
miR210+Nurr1	0.3516	0.3624	0.4186	0.0133*									
miR218+Nurr1	0.3560	0.1338	0.1350	0.0001*	0.1938								
miR219+Nurr1	0.2324	0.4723	0.4189	0.1520	0.3621	0.1284							
miR27+Nurr1	0.1743	0.3534	0.2917	0.4628	0.2353	0.1200	0.3345						
miR29+Nurr1	0.1293	0.4624	0.3568	0.2362	0.2977	0.0689	0.4194	0.3472					
miR34+Nurr1	0.1550	0.4682	0.4170	0.0739	0.3501	0.0660	0.4840	0.3110	0.4159				
miR370+Nurr1	0.4828	0.3455	0.3818	0.1137	0.4257	0.3779	0.3479	0.2257	0.3056	0.3489			
miR375+Nurr1	0.1672	0.4203	0.3556	0.3575	0.3006	0.1055	0.3826	0.3897	0.4484	0.3878	0.2983		
miR494+Nurr1	0.2877	0.4473	0.3805	0.3783	0.3508	0.1897	0.4194	0.3872	0.4708	0.4164	0.3627	0.4812	
Nurr1	0.0124*	0.3653	0.1959	0.2974	0.1353	0.0031*	0.3383	0.3844	0.3829	0.2592	0.2178	0.4694	0.4597

Kruskal-Wallis chi-squared = 38.4385, df = 13, p-value = 0

Table 3.1: miRNA-218-1 enhances the activity of Nurr1 in the induction of TH expression.
p values derived from statistical analysis on high-content screening of miRNA's activity on the number of TH⁺ cells. p values reported in the table below were calculated after one-way analysis of variance and Benjamini-Hocheberg's post hoc test to compare all samples.

3.2.2 – Lmx1a over-expression up-regulates miR-204 in epiSC-derived mesDA neurons

In parallel I tried to identify if any correlation exists between the TFs Nurr1 and Lmx1a and the class of miRNAs we identified. To this purpose we over-expressed Nurr1 and/or Lmx1a in differentiating epiSCs (Fig. 3.11; see Materials and Methods section) and we analyzed their effect on the expression level of few potentially interesting miRNAs (Fig. 3.12). By performing this

experiment, we observed that miR-204 was significantly up-regulated after over-expression of Lmx1a (alone or in combination with Nurr1; Fig. 3.12C)

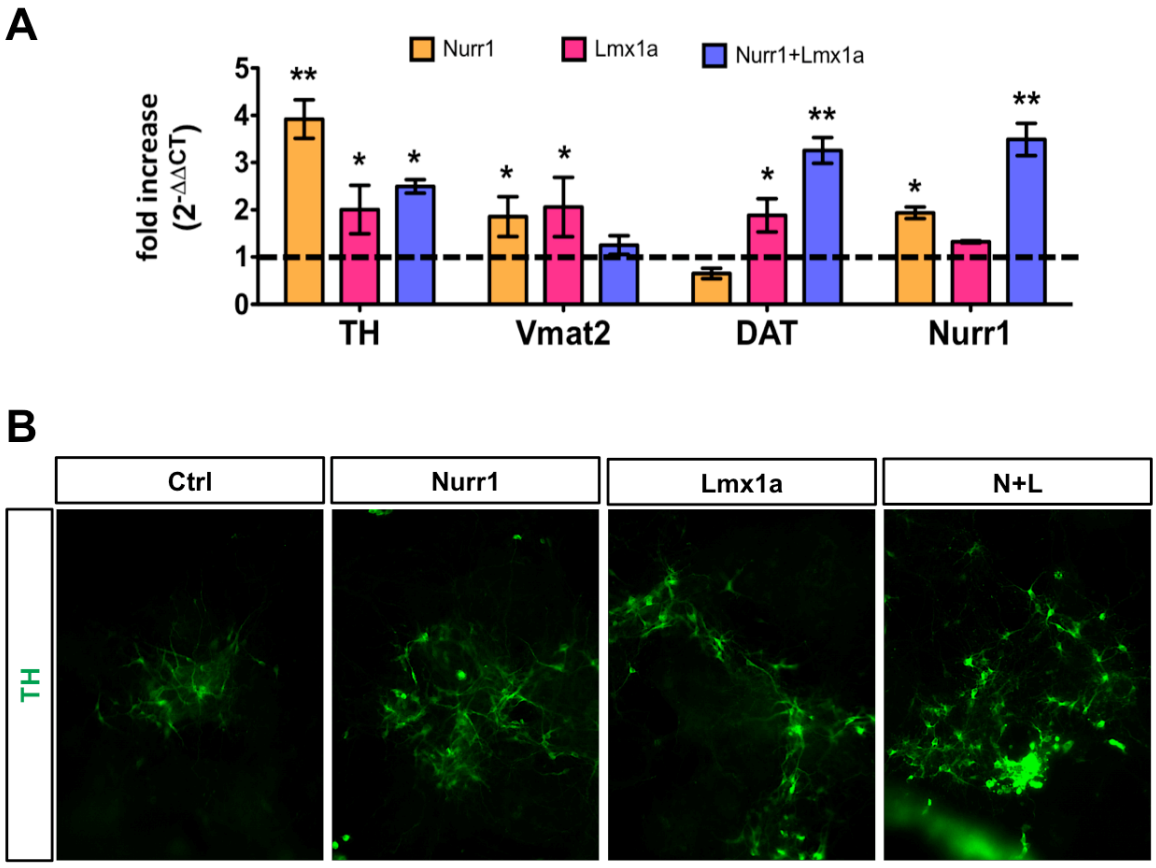

Figure 3.11: Characterization of epiSCs-derived DA cells.

(A) *Real time* PCR evaluation of the expression of dopaminergic markers in epiSCs over-expressing Nurr1 (orange), Lmx1a (pink) and both (blue). Values of the mRNA genes are normalized on the housekeeping gene levels *Hprt*, and compared to average of control points represented by dotted line (Ctrl=1; $2^{-\Delta\Delta CT}$ method). Bars represent means ± SEM, n = 5. * $p \leq 0.05$ of samples compared to control. ** $p \leq 0.05$ compared to all samples. *p* values were calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.
(B) Representative images of TH^{+} cells derived from epiSCs over-expressing Nurr1 and/or Lmx1a and differentiated for 16 days. DMI6000 20x magnification.

Interestingly this up-regulation was specific for miR-204 and not for its homologous miR-211, as revealed by semi-quantitative PCR that discriminates the two pre-miRNA sequences. As shown in Fig. 3.12E, pre-miR-204 was up-regulated after Lmx1a over-expression, while pre-miR-211 was detected at very low levels. Since miR-204 is located intronically in the gene coding for *Trpm3* (while miR-211 is intronic to *Trpm1*) we also analyzed if the expression of these two genes was affected by the over-expression of Lmx1a. As expected, *Trpm3* was up-regulated by Lmx1a, while the expression of *Trpm1* was detected at constant and very low level, independently

on Lmx1a over-expression (Fig. 3.12E). This result was confirmed also in mE12.5-PCs (Fig. 3.12F) and suggests a potential direct regulation of miR-204 by Lmx1a.

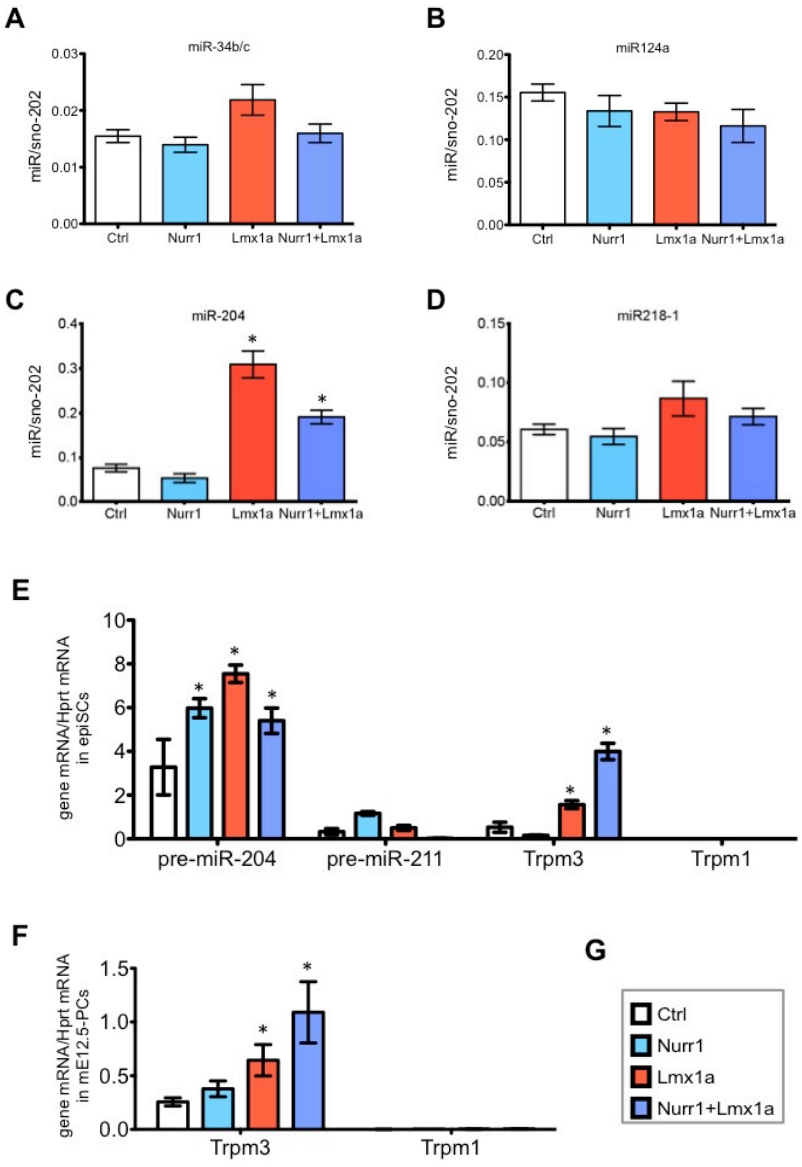

Figure 3.12: Analysis of miRNAs expression in epiSCs-derived DA neurons.
(A-D) qPCR analysis of the expression of several mature miRNAs after over-expression of Nurr1 (cyan), Lmx1a (red) or both (blue) in differentiating epiSCs. RNA samples were harvested at DIV16, 8 days after induction of exogenous genes with doxycycline. miRNA specific Taqman probes were used to perform qPCR. All qPCR data are normalized to the average of the reference small nucleolar RNA 202 (sno-202). Data represent means ± SEM; n = 4. . * $p \leq 0.05$ respect Ctrl; *p* values are calculated after one-way analysis of variance and Dunnett's post hoc test to compare all columns to control. (A) miR-34b/c; (B) miR-124a; (C) miR-204; (D) miR-218-1.
(E-F) *Real time* PCR analysis of pri-miR-204 and pri-miR-211 and their host genes *Trpm3/1* in epiSCs (E) and mE12.5-PCs (F) over-expressing Nurr1 (cyan), Lmx1a (red) or both (blue). mRNA levels are normalized on the mRNA of *Hprt* ($2^{-\Delta CT}$ method). Data represent means ± SEM; n = 6. * $p \leq 0.05$ respect Ctrl; *p* values are calculated after one-way analysis of variance and Dunnett's post hoc test to compare all columns to control. (G) Legend of color scheme.

No other significant variation in the expression of miRNAs analyzed was clearly detected, though miR218-1 and its host gene *Slit2* seemed barely increased (Fig. 3.12D and data not shown), while the small increase in miR-34b/c upon Lmx1a over-expression could be a unspecific effect mainly caused by the low levels of the miRNA itself (Fig. 3.12A).

To better characterize the effect of Lmx1a on miR-204 up-regulation, I used inducible ES cells, over-expressing Lmx1a (see below).

3.2.3 - Generation of ES cells over-expressing Nurr1 and/or Lmx1a

To better appreciate the increase of miR-204 after Lmx1a over-expression I decided to generate mouse embryonic stem cells expressing an inducible lentiviral construct for Lmx1a. To this purpose, ESC cells were plated at low density ($\approx 8 \times 10^3$ cells/ cm^2) and infected with lentiviral vectors carrying for inducible Nurr1 and/or Lmx1a genes tagged with short peptides and conjugated with red and green fluorescent protein (3xFlag-Nurr1_IRES-Cherry and Lmx1a-V5/HIS_IRES-GFP). Following this approach, fluorescent cells ($Cherry^+/GFP^-$, $Cherry^-/GFP^+$ and $Cherry^+/GFP^+$) were sorted and collected in doxycycline-free medium, in order to turn off exogenous expression. After 3 passages, cells were ready for mesDA differentiation in N2B27 medium for 14 days with SHH agonist SAG, FGF8, doxycyxline and ascorbic acid (further details in Materials and Methods section) and finally collected for mRNA analysis. I am currently analyzing miR-204 and mesDA markers expression. The advantage of this approach is that the analyses were performed on a homogeneous population of cells, thus reducing side effects caused by cellular heterogeneity.

The generated cells will be useful to better dissect the previously described synergy between Nurr1 and Lmx1a: comparing ES cells co-expressing Nurr1 and Lmx1a ($ESC_{NL\text{-}RED/GFP}$) with co-cultured single-expressing cells (co-cultured $ESC_{N\text{-}RED}$ with $ESC_{L\text{-}GFP}$), I will be able to discriminate if synergy is *intercellular* or *intracellular*. In both cases, the homogeneous population can be useful to perform ChIP and CoIP analysis to better characterize the mechanisms underlying the observed cooperation between the two TFs.

3.2.4 – Reprogramming into mesDA neurons by miRNAs over-expression

Nurr1 plus Lmx1a can generate mesDA-like cells from neural precursors (mE12.5-PCs and A1) and from epiSC or ES cells. On the other hand, miR-218-1 and miR-204 could be involved in mesDA phenotype. To verify this hypothesis and to further confirm the synergy between Nurr1 and Lmx1a, I *trans*differentiated mouse embryonic fibroblasts (MEFs) and mouse adipose derived adult stem cells (ADAS) into mesDA-like cells using Nurr1/Lmx1a in combination with specific miRNAs (see Materials and Methods section).

The ability to *trans*differentiate into DA neurons any other cell type can be useful for study *in vitro* pathological conditions involved in DA population generating DA-like cells from affected patients. In addition, if a specific miRNA is crucial to *trans*differentiation, it could be a potential therapeutic target.

In agreement with previous observations (Caiazzo et al. 2011) to *trans*differentiate into neurons cells yet committed to other phenotype, I added the pro-neuronal transcription factor Ascl1 (or Mash1) to all experimental points. Indeed, preliminary data on MEFs show that single over-expression of Nurr1 or Ascl1 did not affected TH expression, confirming unpublished data (Piscopo et al. *unpublished*).

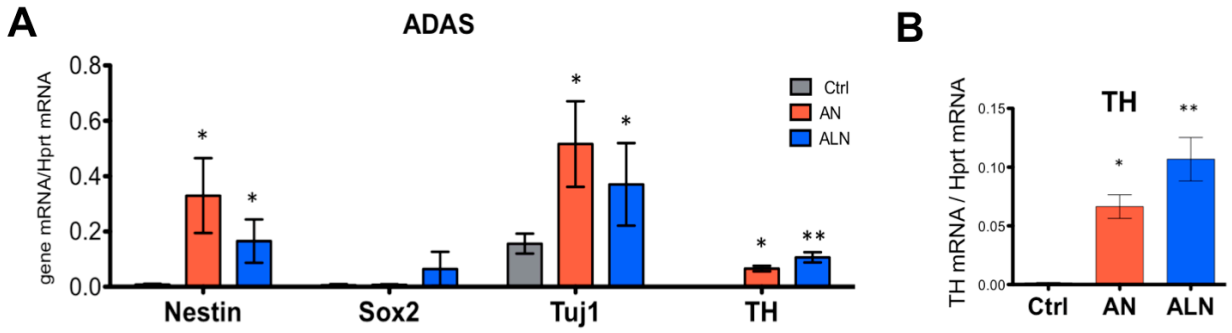

Figure 3.13: DA *trans*differentiation from ADAS.
(A) *Real time* PCR analysis of *trans*differentiating ADAS over-expressing Nurr1 (cyan), Lmx1a (red) or both (blue). (B) Magnification on TH mRNA expression. mRNA levels are normalized on the mRNA of the housekeeping gene *Hprt* ($2^{-\Delta CT}$ method). Data represent means ± SEM; n = 5. * $p \leq 0.05$ respect to Ctrl; ** $p \leq 0.05$ respect to all samples. p values are calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.

Interesting, TH and Tuj1 expression was measured on ADAS and MEFs after over-expression of transcription factors Ascl1, Nurr1 and Lmx1a (ALN). As showed in Fig. 3.14A neuroblast and

neuronal markers were increased (*Nestin, Sox2, Tuj1*) in ADAS over-expressing ALN. Importantly TH mRNA was increased in AN (Ascl1 + Nurr1), and the addition of Lmx1a (ALN) potentiates this effects (Fig. 3.14B) corroborating previous data. Starting from these observations, I will analyzed the effect of over-expressing specific miRNAs together with AN and ALN in ADAS.

In parallel preliminary data obtained on *trans*differentiated MEF confirmed that the miR-218-1 enhances the activity of Nurr1 in term of total amount of TH^+ cells. Indeed after over-expression of Nurr1 with Ascl1 (AN), 50% of $Tuj1^+$ cells are also TH^+. This effect is boosted at 60% when miR-218-1 was added to the TFs (Fig. 3.15).

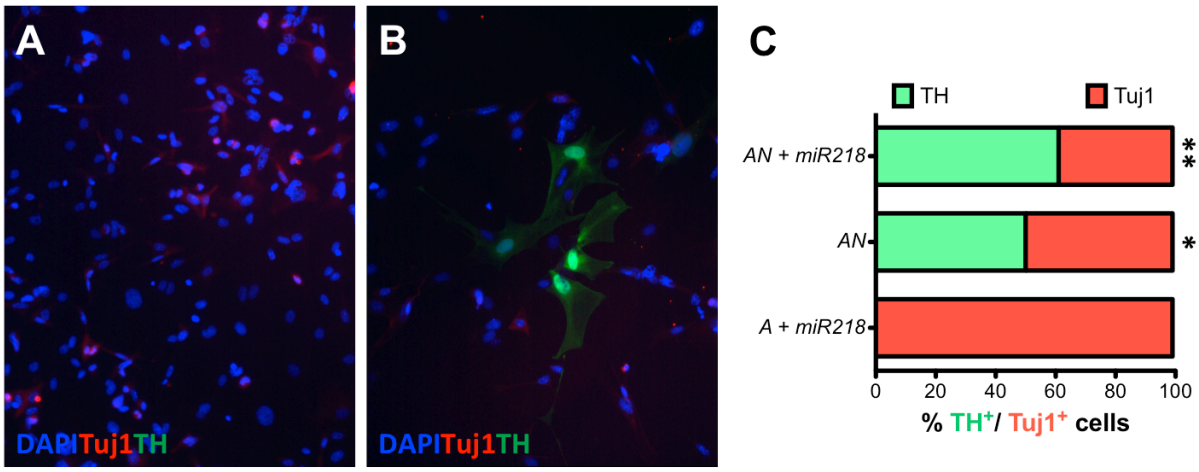

Figure 3.14: miR-218-1 *trans*-differentiate MEF into TH^+ cells.
(A-B) Exemplificative images of MEF derived from TH-GFP mice *trans*differentiated into mesDA neurons after over-expression of Ascl1+miR-218-1 (A) or Ascl1 + Nurr1 + miR-218-1 (B). Green = TH, red = Tuj1, blue = DAPI. 20x magnification.
(C) Percentage of TH^+ cells on $Tuj1^+$ cells. Means ± SEM, n = 13. * $p \leq 0.05$ respect Ctrl; ** $p \leq 0.05$ respect all samples. p values are calculated after one-way analysis of variance and Newmann-Keuls post hoc test to compare all pairs of columns.

4 – DISCUSSION

Brain is one of the most fascinating structures in nature. In human being it is composed of about 10^{11} neurons interspersed with 10 times more glial cells (Azavedo et al. 2009). Neurons communicate between themselves by generating 9 trillions of synapses and represent the most complex network known in nature.

The functions of this complex network allow defining the brain as a "living calculator", able to provide answers to every day life problems in few milliseconds. During the evolution brains evolved by committing different nuclei of the Central Nervous System to specific tasks in order to better answer to individual requirements aimed to species preservation. Of course there is the involvement of several brain areas for complex tasks and cognition.

Among these regions, the midbrain appears as one of the most interesting structure since the dopaminergic neurons, here located, are involved both in primitive brain functions and in emotional and motivational behavior. Thus, the dopaminergic fibers generate a "bridge" that integrates primordial stimuli higher with functions, such as the reward responses of the limbic system. Alterations of midbrain circuits are involved in severe neurological and psychiatric disorders.

How specific classes of neurons acquire their phenotype and how they correctly connect themselves forming the functional network in the developing brain is still an open question. Indeed the specific factors that precisely orchestrate molecular events are not yet completely identified. Likewise, midbrain development has a lot of unknown features since it is involved in many aspects of brain function and therefore many molecules influence its development.

The development of DA neurons is articulated in a continuous multi-step process in which can be recognized mainly four phases: induction, specification, differentiation and maturation. For every step a great number of molecules is involved, and some of them have different roles in different phases, mostly depending from their concentration.

Among these molecules, the transcription factors *Nurr1* and *Pitx3* are considered master genes of DA neuron development and differentiation, as shown by specific alteration in mesDA nuclei observed in knock-out mice (Zetterstrom et al. 1997, Rieger at al. 2001). The hierarchy between

those two factors has been unveiled only in 2012, when experiment performed in my host lab (Volpicelli et al. 2012) described the direct regulation of *Pitx3* by Nurr1.

My PhD work has been aimed to recapitulate *in vitro* the development of mesencephalic dopaminergic neurons (mesDA) and to dissect the molecular events that orchestrate this process. To this purpose I firstly set up an *in vitro* mesDA differentiation protocol by over-expressing the transcription factors **Nurr1** and **Lmx1a**, and validated the flexibility and reproducibility of this protocol into different cell types. Thus, I used the mesDA-like cells 1) to study the relationship between the two factors Nurr1 and Lmx1a, and 2) to identify new partners and target genes regulated by these transcription factors.

As a starting point, midbrain neuronal progenitor cells (NPCs) were isolated and cultured to promote neuronal differentiation and survival of TH^+ cells with SHH and FGF8, as described previously (Volpicelli et al. 2004). The over-expression of Nurr1 enhances TH expression, and the combination with Lmx1a potentiates the pro-dopaminergic enrichment not only in NPC but also in different cellular models I used such as A1, reprogrammed ADAS and MEF. This effect was absent in epiSCS, most probably due to the low infection rate achieved in this cell model. Which are the molecular bases of this interaction is still an open issue. In agreement with known data there are different possibilities: Lmx1a may act as a precursor factor that promotes mesDA phenotype by inhibition of alternative fates in developing midbrain (Andersson et al. 2006); or being part of a autoregulatory loop directly regulating *Nurr1* and *Pitx3* (Chung et al. 2009). Probably, both hypotheses are true. In epiSC, the over-expression of Lmx1a is able to increase TH mRNA most probably through the activation of the Wnt's pathway and in NPCs Lmx1a promotes *TH* expression only in combination with Nurr1.

Thus I hypothesized that Lmx1a cooperates with Nurr1 to enhance *TH* regulation by several possible mechanisms of action::

- Lmx1a binds Nurr1 and modulates its activity directly or indirectly by a third factor;
- Lmx1a influences the binding of Nurr1 on *TH* promoter by epigenetic modifications of the DNA binding motif itself.

Since I observed TH^+ cells that do not express both transcription factors ($TH^+/Nurr1^+/Lmx1a^-$ or $TH^+/Nurr1^-/Lmx1a^+$), I couldn't exclude that this cooperation occurs by intercellular pro-differentiating molecules released by $Lmx1a^+$ on $Nurr1^+$ cells, or vice versa.

Thus, to dissect the mechanisms of the cooperation between these two TFs, I proceeded in different directions.

Firstly, since Nurr1 is an orphan nuclear receptor, I considered that its activation could be modulated by the binding of one or different co-factors to the LBD (Perlmann et al. 2004). To investigate the possibility that Lmx1a could bind the LBD, thus potentiating the activity of Nurr1, I generated lentiviral vectors deleted for the LBD (ΔCter-Nurr1); as control I over-expressed Nurr1 lacking the N-terminal transactivation domain (ΔCter-Nurr1) or mutated in DBD. As expected these last two forms are transcriptionally inactive, while over-expression of ΔCter-Nurr1 in mE12.5-PCs increased TH mRNA levels showing the same trend of the full length Nurr1 also cooperating with Lmx1a. These results indicate that the synergy between Nurr1 and Lmx1a does not require LBD and that this domain is not necessary to Nurr1 activity. Thus, my data corroborate the idea that the activity of Nurr1 is ligand-independent, but can be modulated by other factors like Lmx1a. To verify this idea additional experiments, such as Co-immunoprecipitation of the two proteins, will be done.

To analyze if Lmx1a binds TH promoter and changes its responsiveness to Nurr1, ChIP experiment was performed on mE12.5-PCs, however the mesDA population is too much heterogeneous to give precise data. To perform reliable ChIP assays these experiments will be made in ESCs expressing permanently Nurr1, Lmx1a or both. Furthermore, the ESCs expressing both Nurr1 and Lmx1a could be compared to co-cultured cells expressing $Lmx1a^+$ and $Nurr1^+$ alone to verify whether the cooperation observed is intracellular or intercellular.

The data here presented support the capability of Nurr1 and Lmx1a over-expression to induce mesDA phenotype. In particular mE12.5-PCs and A1 cells showed an increase of mature markers of mesDA neurons such as VMAT2 and DAT, and of survival markers Pitx3 and GDNF receptor c-Ret, while markers of cathecolaminergic neurons (DBH and PNMT), of other neuronal subtype (SERT, TPH2 and GAD65) or of glial (GFAP, Nestin and Sox2) population were reduced or absent.

From a functional point of view it is important to mention the ability of Nurr1 to induce cell cycle arrest. Indeed the over-expression of Nurr1 in A1 cells promotes cell cycle arrest and put the cells in quiescence (G_0), preventing the serum-induced reactivation of mitosis. This activity of Nurr1 is essential to allow maturation of mesDA neurons *in vitro* and most probably depends on cyclins regulation, as previously proposed for $p57^{Kip2}$ (Joseph et al. 2003). Interestingly, also the over-expression of Pitx3 induces cell cycle exit similar to that induced by Nurr1. Since Nurr1 directly regulates *Pitx3* (Volpicelli et al. 2012), we could argue that Nurr1 activates Pitx3 thus mediating cyclin regulation. We may hypothesize that neurons exit from cell cycle to form stable synapses and complete maturation as last phases of their development (Frade et al. 2015).

My results confirm that it is possible to reproduce *in vitro* mesDA phenotype from different cell types in order to generate model systems useful to better understand the process involved in development and neurodegeneration of mesDA neurons. Indeed, the *in vitro* model generated can be suitable to identify the synergic relationship between Nurr1 and Lmx1a.

Starting from the data obtained during dopaminergic differentiation of epics, it was possible to demonstrate that miR-218-1 and miR-204 play a role in mesDA development. Indeed the over-expression of miR-218-1, in combination with Nurr1, enhances the number of TH^+ cells derived from mE12.5-PCs and MEFs while the over-expression of Lmx1a up-regulates miR-204. Recently it has been described that both miR-218 and miR-204 are involved in important cellular processes associated to motor-neuron development (Amin et al. 2015, Thiebes et al. 2015) and retinal disease (Conte et al. 2014), respectively. Nevertheless the molecular and cellular events controlled by these microRNAs are largely unknown. Thus, since these miRNAs are expressed in dopaminergic neurons, we could expect a role also in mesDA neurons development.

In future our goals will be to identify the Lmx1a activated pathways and to characterize the role of Lmx1a in Nurr1 activity modulation.

These information, combined with the capability to generate mesDA neurons from non-neuronal cells, can lead to the obtainment of a more powerful in vitro model system for the study of PD or other diseases affecting the dopaminergic system. Moreover, the ability to generate patient-specific mesDA from adult differentiated cells could pave the way for cell replacement therapies.

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6 - LIST OF ABBREVIATIONS

A

A1	mes-*c-myc*-A1 cell line
A	Lentiviral particles carryng *Ascl1* cDNA
A*(n)*	Catecholaminergic nuclei
AADC	Aromatic L-amino acid decarboxylase
ADHD	Attention deficit hyperactivity disorder
ADAS	Adipose derived adult stem cells
AF1	*Trans*activation domain of Nurr1
Ahd2	Aldehydedehydrogenase2
AK	Aphakia mice
AN	Lentiviral particles carryng *Ascl1* and *Nurr1* cDNAs
ANL	Lentiviral particles carryng *Ascl1, Nurr1* and *Lmx1a* cDNAs
Ascl1	Achaete-scute homolog 1

B

BDNF	Brain deriven neurotrophic factor
bFGF	Basic fibroblast growth factor (FGF2)

C

cAMP	Cyclic Adenosine Mono-phosphate
CDNF	Cerebral dopamine neurotrophic factor
ChIP	Chromatin immunoprecipitation
CNS	Central nervoussystem
CoIP	Co-immunoprecipitation
COMT	Catechol-O-methyltransferase
c-Ret	GDNF receptor

D

ΔCter-Nurr1	Nurr1 deleted of C-terminus portion
ΔNter-Nurr1	Nurr1 deleted of N-terminus portion
DA	Dopamine / Dopaminergic
DBD	DNA binding domain
DBH	Dopamine ß-hydroxylase
DRD	DA receptor
DAT	Dopamine active transporter (Slc6a3)
DIV	Days *in vitro*
Dmrt5	Doublesex and mab-3related transcription factor 5
DOPAC	3,4-dihydroxyphenylacetic acid
Dox	doxycyxline
Dsh	Dishevelled

E

E	Embryonic day

En1/2	Engrailed 1 and 2
epiSC	Epiblast stem cells
Eph	Ephrines
ESC	Embryonic stem cells

F

FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF8	Fibroblast growth factor 8
FP	Floor plate
FoxA1/2	Forkhead box protein A1/A2
Fz	Frizzled
Fz3	Frizzled 3
Fz6	Frizzled 6

G

GABA	γ-Aminobutyric acid
GAD65	Glutamate decarboxylase 2
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPe	external Globus pallidus
GPi	internal Globus pallidus

H

HEK293T	Human embryonic kidney cell line 293T
hESC	Human embryonic stem cells
hiPSC	Human inducedpluripotentstemcells
HPRT	Hypoxanthine Phosphoribosyltransferase
HVA	Homovanillic acid

I

IAC	Istituto per le Applicazioni del Calcolo
iDA	Induceddopaminergic cells
iN	Induced neuronal cells
IF	Immunofluorescence analysis
IGB-ABT	Institute of genetics and biophysics Adriano Buzzati Traverso
iPSC	Induced pluripotent stem cells
IsO	Isthmic organizer

K

KO	Knock-out

L

LBD	Ligand binding domain
L-DOPA	L–3,4–dihydroxyphenylalanine (levodopa)
LIF	Leukemia inhibitory factor
Lmx1a	LIM-homeodomain factor1a
Lmx1b	LIM-homeodomain factor1b
LV	Lentiviral particles

M

MAO	Monoamine oxidase
mDA	Midbrain dopaminergic neurons
MHB	Mid-hindbrain boundary
Mb	Midbrain
MCLp	Mesocorticolimbic pathway
mE12.5-PCs	Mouse embryonic primary cultures from E12.5 midbrains
MEF	Mouse embryonic fibroblasts
miRNA	microRNA
mRNA	messanger RNA
Msx1	Msh homeobox 1

N

NBRE	NGF rensponsive elements
NC	Notocord
NEAA	Non-essential aminoacids
NES	Nuclear exporting sequences
Ngn2	Neurogenin 2
Nkx2.2	NK2 Homeobox 2
Nkx6.1	NK6 Homeobox 1
NLS	Nuclear localization sequences
Nr4a1	Alias for Nurr77
Nr4a2	Alias for Nurr1
Nr4a3	Alias for Nor-1
NSC	Neuralstemcells
NSp	Nigrostriatalpathway
Nurr1	Nuclear Receptor Related 1 protein
$Nurr1^{C280A/E281/A}$	Nurr1 mutated in two aminoacidic residues

O

OB	Olfactory bulbs
Otx2	Orthodenticle Homeobox 2
OV	Otic vescicle

P

$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 1C
PABP	poly(A)-binding protein
PD	Parkinson's disease
Pen/Strep	Streptomycin and Penicillin
PhD	Doctor of Philosophy
Pitx3	Pituitary homeobox 3
PNMT	Phenylethanolamine N-methyltransferase
pre-miRNA	Precursor-miRNA
pri-miRNA	Primary-miRNA
PSF	Splicing factor PTB-associated
PTB	Polypirimidine–tract-binding-protein
Ptc1	Patched 1
PKA	Protein Kinase A

R

RA	Retinoic acid
RP	Roof plate

RRF	Retrorubral field
RT-PCR	*real time* PCR
RXR	Retinoic acid receptor

S

SAG	Smoothened Agonist
SC	Spinal cord
SEM	Standard error of the mean
SERT	serotonin transporter
SHH	Sonic Hedgehog
Slit2	Slit Guidance Ligand 2
Smo	Smoothened
SN	Substantia Nigra
SNc	Substantia Nigra *pars compacta*
SNr	Substantia Nigra *pars reticulata*
Sox2	SRY (sex determining region Y)-box 2
STN	Subthalamic nucleus

T

TF	Transcription factors
TGFβ	Transforming growth factor β
TH	Tyrosine Hydroxylase
TIGEM	Telethon Institute of Genetics and Medicine
TPH2	Tryptophan hydroxylase 2

U

UTR	Untranslated regions

V

Vmat2	Vesicular monoamine transporter
VTA	Ventral Tegmental Area

W

Wnt	Wingless-Type MMTV Integration Site Family
wt	Wilde type

7 - SUMMARY OF FIGURES AND TABLES

8 - ACKNOWLEDGEMENTS

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