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Characterization of a Human Neuroblastoma Cell Line and Its Differentiation into Dopamine Neurons

by

Erika M. Norabuena

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Abbreviations

AADC	Aromatic amino acid decarboxylase
CNS	Central Nervous System
DA	Dopamine
DAT	Dopamine transporter
Gbx2	gastrulation brain homeobox
IsO	Isthmus organizer center
mDA	Midbrain dopaminergic neurons
MHB	Mid-hindbrain boundary
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NURR1	Nuclear receptor 1
Otx2	Orthodenticle homologue 2
PD	Parkinson's disease
PITX3	Pituitary homeobox 3
Shh	Sonic hedgehog
SNc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

ABSTRACT

Dopamine (DA) synthesizing neurons are important because of their role in the control of motor movement, cognition and motivation. The development of DA neurons is marked by the presence of transcription factors and proteins that regulate specific stages during development. Given their specificity, these signals can be used as molecular markers for each developmental stage. The transcription factors NURR1 and PITX3 are two markers for post-mitotic DA neurons (neurons with the potential to become dopaminergic) whereas the presence of the dopamine transporter (DAT), a protein important for the reuptake of DA from the synaptic cleft, is used to identify a mature DA neuron (neurons that can synthesize and reuptake dopamine). The molecular mechanisms that govern DA neurons' development are not fully understood and the lack of ideal cell models that recapitulate the phenotype of DA neurons makes challenging the study of the regulation of DA gene expression. To provide further insights into the role of NURR1 and PITX3 on the transcriptional regulation of DA marker genes such as the DAT, we studied their gene expression in two SK-N-AS cell lines, C line which expresses NURR1 at low basal levels, and E line engineered to over-express NURR1 by two fold. Our results have shown that indeed NURR1 and DAT are expressed in the SK-N-AS cell line, validating previously published research, while PITX3 is not expressed in these cells. Thus we suggest that PITX3

is not required for the expression of *DAT* gene, contrary to some previous data. Additionally, we have characterized the SK-N-AS morphologically by comparing the C-line with the E- line using the transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The most notable difference between the C and the E-line was the cytoplasmic content detected by TEM. Our results point toward the presence of more lysosomes autophagic vacuoles, residual bodies, and multivesicular bodies in the E-line. All these structures represent various stages in a dynamic process of intracellular digestion in which primary lysosomes fuse with their heterophagic vacuoles resulting in the degradation of their contents. As a result of degradation, residual bodies and multivesicular bodies are formed and they are recognized by the presence of a pigment called lipofucsin.

Our findings suggests that SK-N-AS cells are not expressing several of the key signals involved in the DA pathway, so probably it is not an adequate model system to study DA molecular mechanisms. Morphologically, the over-expression of NURR1 in the E line changes the cells phenotypically by incrementing the amount of cytoplasmic content.

INTRODUCTION

Dopamine (DA) is a neurotransmitter found in the brain of most vertebrate species. In humans, approximately eighty percent of the total number of DA neurons present in the brain is found in two nuclei that are located in the midbrain. The presence of DA neurons in this area is fundamental for controlling processes involved in voluntary movement control and regulation of emotions. Degeneration of midbrain DA neurons has been associated with Parkinson's disease, schizophrenia and addictive disorders, causing this brain area to be under intense scientific scrutiny.

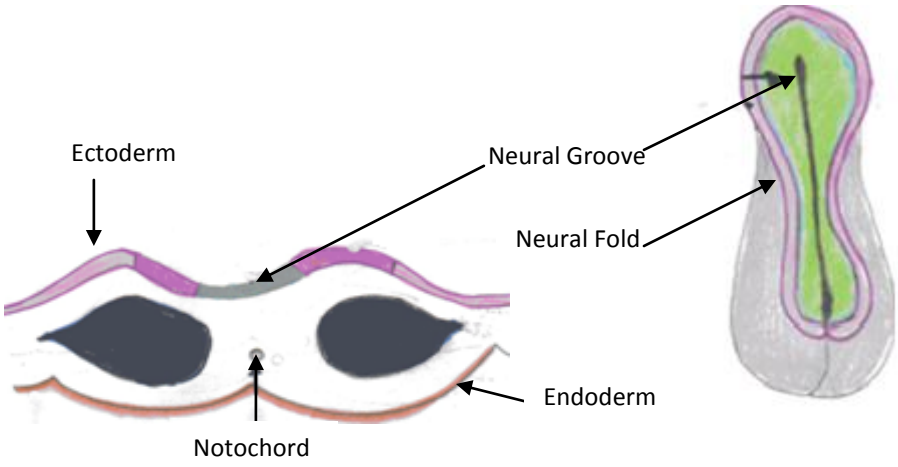
In order to understand the complexity of the DA neuron development I am going to discuss it from the anatomical, developmental and molecular perspective. Lastly, we are going to introduce a neuroblastoma cell line that is been used lately to study the regulation of the expression of DA-related marker genes.

1.1 In vivo development of the nervous system

The development of the central nervous system (CNS) in humans starts with the formation of the neural plate, an ectodermic structure that extends along the surface of the embryo for much of its length (Figure 1). The formation of the

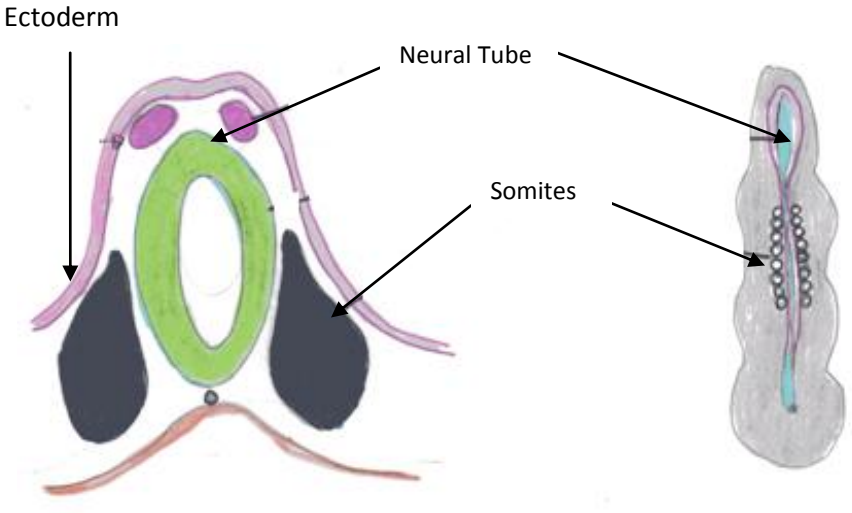
neural plate is begun by induction of the notochord, a structure formed during gastrulation (approximately at day 15 after fertilization) (Kandel, 2000). As the embryo continues to develop, a groove is formed down the midline of the neural plate deepening until it forms the neural tube (Carnegie stage 11- week 4, 23-24 days) (Figure 2).

During this stage, the neuroepithelium of the neural tube contains only undifferentiated stem cells that with time, will give rise to neurons and supporting glial cells (Siegel, 1999).



A. Cross section of the neural tube **B. Dorsal view of the neural tube**

Figure1. Formation of the neural plate. After gastrulation, the ectoderm gives rise to the neural groove. This transformation is possible due to the signals released by the notochord. In the figure we can see a cross section of the neural tube (A) and the dorsal view (B).



A. Cross section of the neural tube **A. Dorsal view of the neural tube**

Figure2. Neural tube formation. The neural plate undergoes morphogenesis and forms the neural tube during neurogenesis. The entire neural region undergoes convergent extension movements, lengthening the embryo along the anterior to posterior axis.

As the stem cells continue the process of cell proliferation (mitotic activity), they start migrating away from the neuroepithelium toward the piamater, one of the three layers of the meninges surrounding the surface of the brain (Figure 3).

The proliferation and migration of these cells result a thickened and layered neuroepithelium (Siegel, 1999) that will give rise to the *ventricular zone* (VZ), where the cells are adjacent to the ventricle and retain their proliferative properties; the *intermediate zone* (IZ), in which the cells stop dividing and

become post-mitotic; and the most external zone, the *marginal zone* (MZ) in which we find mature cells (fig. 3).

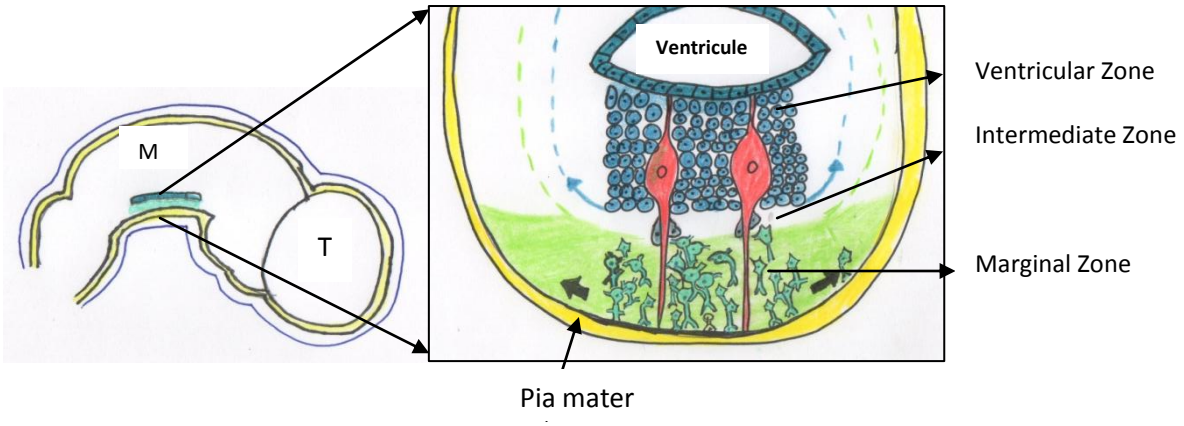


Figure3. Cross section through the wall of the neural tube. The progenitor cells are projection neurons born from cells in the ventricular zone (VZ) and migrate radially toward the pia mater. The cells in this layer are in the S phase of the cell cycle. By the time they reach the apical surface they enter mitosis (G0). As cells move down, they cross the Intermediate Zone (IZ) and finally reach maturity when they arrive at the Marginal Zone (MZ) where they start to migrate laterally. The correct positioning of neurons during development is achieved during migration. For reference purposes T is telencephalon and M is mesencephalon.

The thickening of the neural tube causes its dilatation and the formation of the three vesicles that are considered the primary brain vesicles: proencephalon (forebrain), metencephalon (midbrain) and rhomboencephalon (hindbrain) (Bears, 2007). Soon after, these 3 vesicles will split into five and each one will form the different areas of the CNS (figure 4).

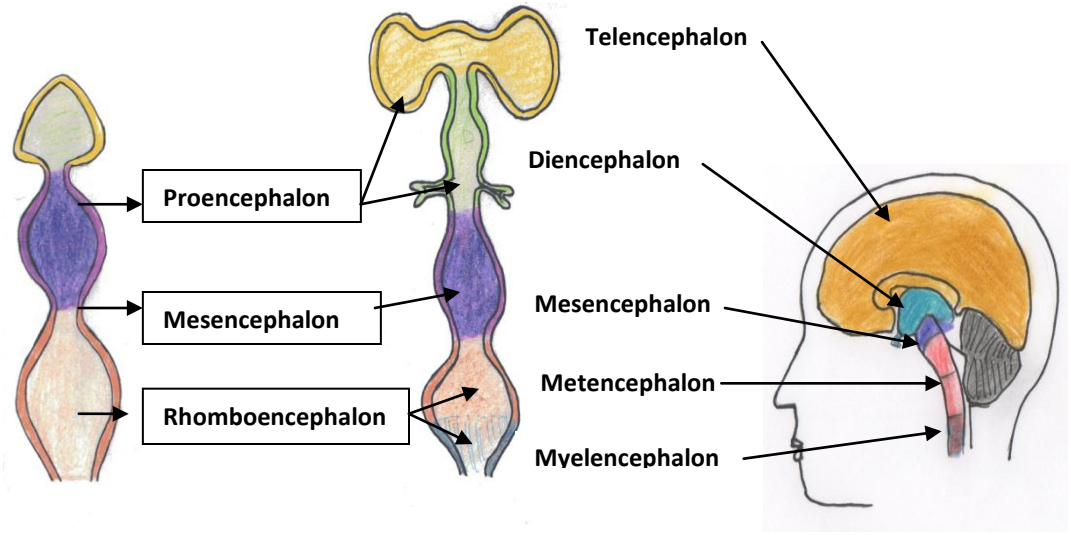


Figure 4. Primary brain vesicles. The closure and thickening of the neural tube gives rise to the 3 primary vesicles. These vesicles will give rise to the five main areas of the brain: Telencephalon (cerebral hemispheres), diencephalon (thalamus and hypothalamus), mesencephalon (midbrain: tectum and tegmentum), metencephalon (cerebellum and pons) and myelencephalon (medulla oblongata).

As development continues each of these structures forms clear boundaries (e. g the midbrain/hindbrain boundary) and will start to generate its own neural lineage. The presence of these boundaries is important because these areas can give rise to organizing centers (e.g. the isthmus), which secrete signals that will influence the development of adjacent regions in the neuroepithelium.

Neural cells progress through 3 stages: progenitor cells, post-mitotic precursors, and mature neurons. Each stage is dependent on the interaction of extrinsic and intrinsic signals on the local cells that are activated according to their spatial location, timing, and concentration. Intrinsic signals can be defined as signals

expressed endogenously by a cell that is preprogrammed to do so, while extrinsic signals are signals synthesized by distant cells that can affect the local cells and activate the preprogrammed cells expressions they affect (Edlund, 1999). Some extrinsic signals are based on a gradient signaling mechanism, in which the cell fate is directed by the concentration of the signal present, or based on an antagonist signaling mechanism, in which the secreted factors, the ones in charge of the cell fate, are targeted by inhibitory factors.

Studies performed in different organisms have shown that formation and patterning of the vertebrate neural plate is dependent on signals produced mostly by organizing centers. For example, in the midbrain there are two organizing centers that are very important for the development of the midbrain: these are the midbrain/hindbrain boundary (MHB) and the ventral midline of the neural tube or floor plate (FP) (Prakash, 2006). Studies have shown that interference with the signaling molecules secreted by these organizing centers can result in malformations or even absence of the midbrain (Gale, 2008). The normal development of these centers results in the acquisition and maintenance of their respective neuronal phenotype. For example, for the neurons located in the midbrain, the neuronal phenotype will be the acquisition of the dopaminergic phenotype.

1.2 Development of the midbrain

Most what it is known about brain development has been discovered by studying mice brain embryos. The embryo can be classified according to its age, size, or its morphological characteristics. These criteria are then used to identify the embryo in a standardized system of 23 stages known as *Carnegie stages* (Table 1). This classification is useful for staging and comparing the embryological development of most vertebrate. For humans, this classification only takes in consideration the first 58 days after fertilization (first 8 weeks), period after which the embryonic stage is over and the fetal stage begins. Similarly, the mice embryological stage consists of the first 16 days. After that period, the mouse fetal stage begins.

Table1. A summarized example of equivalence using Carnegie stage classification

Specie	**Stage**	**1**	**2**	**..**	**7**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Mouse*	Days	1	2	..	7.5	9.5	10	10.5	11	11.5	12	12.5
Human*	Days	1	2-3	..	15-17	17-19	20	21	22	23	24	25

Studies using mice as a model system to study brain development have shown that the formation of the midbrain occurs at embryological stage 7.5 (E7.5) which in comparison to humans would be approximately after day 15-17. Its development is due to the presence of the *isthmus*, an organizing center located at

the junction of the metencephalon and mesencephalon (midbrain/hindbrain boundary or MHB) (Kandel, 2000) (figure 5) and the organizing center located in the ventral midline of the neural tube or floor plate (FP) (Prakash, 2006).

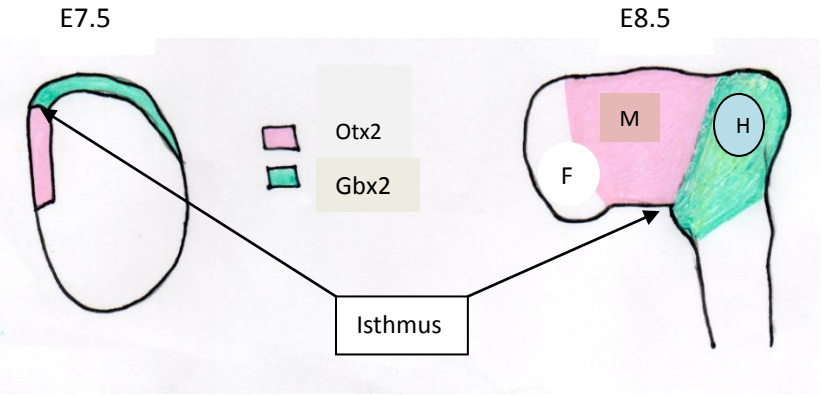


Figure5. Lateral view of an embryonic mouse brain. The isthmus appears at E7.5 and is characterized by the presence of two transcription factors Otx2 and Gtx2. The brain continues its development and the isthmus changes and starts adopting its characteristic morphology. By embryological stage 8.5 Otx2 is expressed in the forebrain (F) and midbrain (M) whereas Gtx is expressed in the anterior Hindbrain (H).

The position of the isthmus is due to the presence of the transcription factors Orthodenticle homologue 2 (Otx2), which is required for the development of the forebrain and midbrain, and the gastrulation brain homeobox (Gbx2), which is required for the development of the anterior hindbrain (Maxwell, 2005). It is the Otx2 and Gbx2 antagonistic signaling interaction that causes the positioning of the midbrain/hindbrain boundary (Gale, 2008) (figure 6). The isthmus determines the position of the midbrain and the pattern of cells inherent to it (Kandel 2000).

At E 8.0 in mice, the transcription factor Paired box gene 2 (Pax2) is expressed in the isthmus across the OTX2/GBX2 border (Wurst, 2001). The protein Wnt1 and Fgf8 are also being expressed but they show regionalization. Wnt1 is expressed only in the Otx2 domain while Fgf8 is expressed in the Gbx2 domain (Maxwell, 2005) (Figure6).

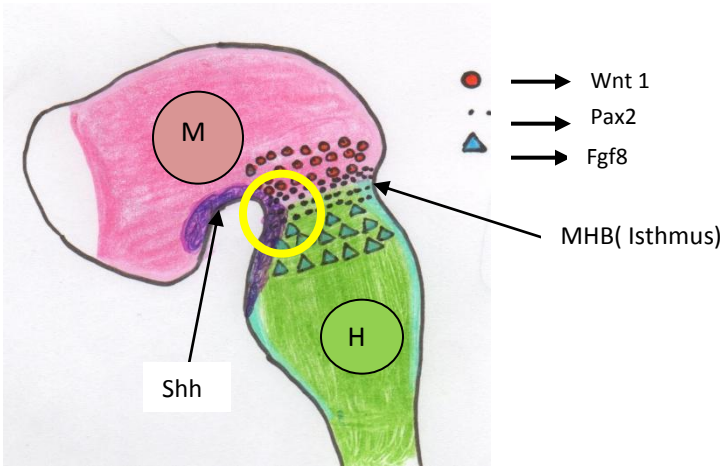


Figure6. Interplay between signals that specify neural identity in the mid-hind border (MHB). Sagittal view of the mouse neural tube shows distribution of the signals around the MHB. The MHB is the boundary between the midbrain (M) and Hindbrain (H). Pax2 is secreted across the Isthmus. The signal protein Wnt1 is found in the Otx2 region whereas Fgf8 is secreted in the Gbx2 area. The neurons from the midbrain will originate at the intersection of the signal Shh and Fgf8 (yellow circle)

Following this event, a cascade of signals that involves different transcription factors starts to be expressed across the organizing center (Maxwell, 2005).

Patterning of the dorsal-ventral axes starts right after the formation of the neural tube, and it is influenced by two signaling centers that will specify the neural tube into floor plate, alar plate, and roof plate (Smidt, 2011) (Figure 7).

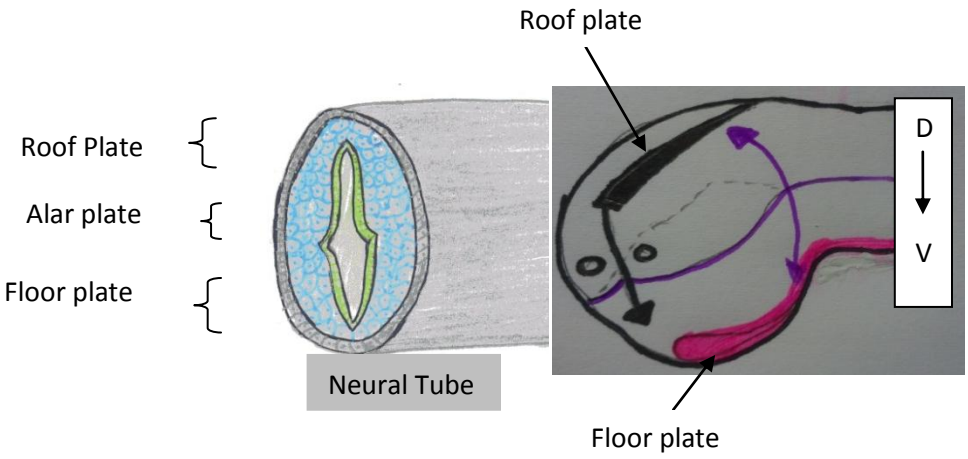


Figure7. Dorsal- ventral specification of the neural tube. The roof of the neural tube is exposed to the protein BMP 4 and 7 while the floor plate is exposed to Shh protein from the notochord. This process happens at E8.5 in mouse.

These regions are important because the different classes of neurons are based on their location along these areas and on the differential exposure to inductive signals.

The cells from the organizing center located in the floor plate (FP) secrete the morphogen Sonic hedgehog (Shh). The midbrain dopaminergic neurons are originated, just rostral to the isthmus, at the location where FgF8 and Shh signaling intersect (Maxwell, 2005). Fgf8 regulates the anterior-posterior polarity

in the developing midbrain (Edman, 2009) and it induces a cascade of different gene expression profiles as the distance from the isthmus increases (Prakash, 2006).

By the end of E9.0 in mice, the regionalization of the midbrain has been completed through the converging signals of Otx2, Gbx2, Wnt1, Fgf8 and Shh that originate from both organizing centers of the midbrain, the MHB and FP.

During the formation of the midbrain at E11.5 mouse (Carnegie stage 14 in humans) two regions of the midbrain start to be delimited: the dorsal region and ventral region. Within them, specific cells groups arise; the dorsal region will give rise to the *tectum* and the ventral region will form the *tegmentum* (Bears, 2007). The *tegmentum* will give rise to two of the areas with high concentration of neurons synthesizing the neurotransmitter dopamine (DA): the substantia nigra *pars compacta* (SN_C) and the ventral tegmental area (VTA).

1.3 Development of post mitotic midbrain DA (mDA) neurons:

One of the best animal models to study mammalian brain development is the mouse because mice can be genetically modified, they reproduce fast, and embryologically they are similar to humans. Most of what is known today about midbrain DA (mDA) neuronal development is because of the data obtained from studying the mouse brain at the different embryological stages.

Studies have shown that after regionalization has occurred and the progenitor cells have developed and positioned slightly anterior to the MHB and FP, the cells become post-mitotic and initiate the process of specification. Post mitotic neurons are still immature neurons between the embryological stages E10.5 to E12.5 (Prakash, 2006). The expression of specific signals and/or factors at specific developmental stages allows the distinction of progenitor, post mitotic and mature DA neurons. Some of these molecular markers used to identify the different neuronal stages are transcription factors (e.g. NURR1, PITX3), enzymes (e. g TH) while others are signaling proteins (e.g. Fgf8).

1.4 Factors involved in the development of post-mitotic neurons

Transcription factors are proteins containing DNA binding domains (DBD) that play important roles during the process of gene expression. The transcription factors recognize and bind specific sequences of DNA regulating the transcription of these genes. At a post mitotic level the main players are the transcription factors NURR1 and PITX3.

1.4.1 Nurr1: Nr4a2

Nr4a2 is an “orphan” (no cognate ligand binds to it) nuclear receptor that belongs to the steroid/thyroid hormone receptor family (Law, 1992). Nuclear receptors are transcription factors that contain a conserved DNA binding domain (DBD) and

two transactivation domains (AF1,AF2) that allow them to affect gene transcription (Martinez-Gonzales, 2005) . Nurr1 has been show to act on DNA as monomer, homodimer or heterodimer, interacting with the retinoid receptor x (RXR), to increase gene expression (Maruyama et al, 1998; Sacchetti, 2002). Recent structural studies have shown a large presence of hydrophobic amino acid chains close to the NURR1 binding domain making it very difficult for a ligand to bind (Wang, 2003). These results suggest that NURR1 acts as ligand-independent receptor and that its activation is controlled by other mechanisms.

Developmentally, NURR1 is expressed in different areas of the central nervous system, but it shows a strong expression in the ventral midbrain starting at approximately E10.5 in mice (Zetterström, 1997, Wallen, 1999; Prakash, 2006). Nurr1 peak of expression varies according to the position in the ventral midbrain; for SNc the peak is between E13-14 whereas for the VTA, the peak of expression is between E14-15 (Maxwell, 2005).

The role of NURR1 has been shown mainly by gene knock-out experiments (Zetterström, 1997; Saucedo-Cardenas, 1998, Semina, 1997, Smidt, 2003). All of these findings confirmed that in the absence of NURR1, the development and maturation of mDA neurons were affected. One of the first studies showed that the absence of NURR1 resulted in a failure to generate mDA neurons (Zetterström, 1997). Further studies found that Nurr1-/- mice do not express the early DA marker tyrosine hydroxylase (TH) (Zetterström, 1997; Castillo, 1998;

Siegel, 1999), which is necessary for the synthesis of DA. In addition, the expression of genes such as the aromatic amino acid decarboxylase (AADC/DCC), the vesicular monoamine transporter (VMAT2) and the dopamine transporter (DAT) was never initiated or the expression was delayed after birth (Zetterström, 1997; Castillo, 1998; Saucedo-Cardenas, 1998). More recent studies have shown that the transcriptional activity of the late dopaminergic marker dopamine transporter (DAT) is also increased in a NURR1 dose-dependent manner *in vitro* (Semina, 1997) and *in vivo* (Smidt, 2003) in mice. These results have validated previous studies performed *in vitro* on the human DAT gene (Sacchetti et al, 1999, 2001). Complementary studies using NURR1-/- mice have shown that other dopaminergic markers such as the aldehyde dehydrogenase 2 (AHD2), the tyrosine kinase receptor (c-RET), VMAT are affected by the absence of NURR1 (Martinat, 2006; Smidt, 2003).

When Nurr1 is expressed, the transcriptional activity of the DAT increases (Sacchetti, 1999). Subsequent studies have suggested that the mechanism involved in this upregulation is not due to the presence of the RXR receptor but to a different and indirect mechanism that might involve the interaction with other proteins (Sacchetti, 2001).

A recent study suggests that NURR1 regulates directly the transcription factor PITX3 in a dose dependent manner (Volpicelli, 2012), meaning that NURR1 might be acting as a master regulator in the maturation of DA neurons. Overall

these studies suggest that NURR1 not only plays an important role during development but also is involved in the maintenance of DAergic neurons and thus is used as an important marker for post-mitotic DA neurons.

1.4.2 Pitx3: (pituitary homeobox gene3)

Pitx3 is a transcription factor that belongs to the bicoid-related Pitx gene family (Semina, 1997; Gage, 1999). It is classified as a bicoid-related homeobox protein because its homeodomain contains a lysine at the residue fifty, which is typical of the small subfamily of homeoproteins related to the Drosophila *bicoid* protein (Gage, 1999; Drouin, 1998).

In humans and mice, the Pitx3 gene has a very restricted expression in comparison to the other two members of its family, Pitx1 and Pitx2. Pitx3 is transiently expressed outside the CNS in the eye lenses, tongue, and head muscles (Gage, 1997). In the CNS Pitx3 has a restricted pattern in the mDA neurons (Smidt, 1997).

During mDA development, Pitx3 is expressed at approximately at E.11.5 in mice (Smidt, 1997). This expression is maintained throughout development of the SNc and VTA and continues in the adult rodent and human brain (Smidt 1997, Smidt, 2004). Because of this restriction and permanent expression in the DA neurons

located in the ventral midbrain, PITX3 is used as a neuronal marker for post-mitotic DA neurons.

Given the fact that homeobox genes are important for development of an organism because they behave as transcription factors, the presence of PITX3 might suggest that it is involved in the control of certain mDA developmental processes. Studies using aphakia mice that lack eye lenses due to the absence of Pitx3 expression have shown that Pitx3 is required for TH expression in a subset of mature mDA neurons and for the survival of primary SNc and VTA neurons (Hwang et al., 2003, Nunes et al, 2003, van den Munckhof et al., 2003; Smidt et al., 2004, Maxwell et al., 2005). Further experiments showed that Pitx3 can bind to response elements on the TH promoter, suggesting that Pitx3 could be an activator of TH (Cazzorla, 2000). In fact, Pitx3 and TH are expressed almost simultaneously at approximately E11.5 (Smidt, 1997) thus suggesting a regulatory or modulator role for Pitx3in the initiation of TH expression. Experiments using null Nurr1 (Nurr1-/-) mice showed that DA neurons adopt a normal ventral position and express the marker Pitx3 at E11.5 but they lack the expression of TH, and failed to induce a dopaminergic phenotype in the cells (Saucedo-Cardenas, 1998). As development continues, these Pitx3 expressing neurons degenerate inducing apoptosis in the ventral midbrain of newborn null mutant mice (Saucedo-Cardenas, 1998). Experiments using the human neuroblastoma cell line Neuro2A showed that the effect of Nurr1 alone on the transcription of the TH was very weak, despite the location of a binding site for Nurr1 in the TH promoter

sequence studied (Cazzorla, 2000; Sakurada, 1999). This same study showed that in the presence of Pitx3 and Nurr1, TH expression was enhanced (Cazzorla, 2000). A difference is that over-expression of Nurr1 affects the expression of TH and DAT; however, Pitx3 over-expression by itself does not affect the expression of DA markers such as TH, AADC, cRET and D2 receptors (Sakurada, 1999).

One of the most recent studies suggests that NURR1 regulates directly the transcription factor PITX3 in a dose-dependent manner (Volpicelli, 2012) in DA neurons. Although the mechanism is not clear yet, it seems that Nurr1 and Pitx3 interact to produce a mature DA neuron. These data suggest that Pitx3 and Nurr1 cooperate to induce tyrosine hydroxylase expression while Pitx3 might also be involved in the expression and maintenance of this gene (Cazzorla, 2000). Further experiments have suggested that NURR1 and PITX3 might also cooperate to promote the acquisition of the DA phenotype by upregulating the expression of late markers, like the *DAT* gene (Martinat, 2006).

It still remains unclear what is the exact role of Pitx3 in the two nuclei of the midbrain, known as SNc and VTA. Some studies in which TH positive cells were co-localized with Pitx3 positive neurons showed that Pitx3 is expressed all over the midbrain in the adult (Smidt, 2004), while other data (Van den Munckhof, 2003) suggest that there are subpopulations of cell expressing Pitx3. In fact, their data showed that almost all TH/Pitx3 double labeled neurons were located in the

SNc whereas only 50% of these double labeled neurons were present in the VTA. (Van den Munckhof, 2003).

1.5 Dopaminergic Neurons: late markers

A successfully mature neuron has the ability to communicate with another neuron through action potentials, in a process that involves depolarization and re-polarization of the cell membrane. Action potentials are a highly specialized way of cellular signal transmission.

The process of transmission involves the secretion of neurotransmitters, and cells can be classified according to the type of neurotransmitter they release; in the case of dopaminergic neurons, they synthesize the catecholamine dopamine (DA).

After DA has been released into the synaptic cleft, the specialized transmembrane proteins, DAT, are responsible for the reuptake of the DA from the synaptic cleft. The presence of these transmembrane proteins can be used as a marker of "mature" cells since it indicates that the neurons should be functional. At this late stage in the DA development the presence of enzymes (TH, AADC) and transporters in charge of storage (vesicular monoamine transporter 2 or VMAT2), release and reuptake of their respective neurotransmitters (dopamine transporter2 or DAT) marks the beginning of mature DA neurons (Smidt, 2003; Prakash, 2006).

Transcription factors are very important in the development of DA neurons. However, they are not the only players in the functioning of these neurons, as we have seen previously; other proteins also participate in the functioning of the cells due to their enzymatic capacity which make them necessary for biosynthesis of neurotransmitters.

1.5.1 Dopamine transporter: (SLC6A3)

The dopamine transporter gene (*DAT*) codes for a transmembrane protein located in the DA neuron's terminals of the SNc and VTA (Bannon et al 1997, 2000). It is in charge of reuptake dopamine after its release into the synaptic cleft.

DAT is a member of a large family of Na^+/Cl^- dependent transporters. Structural analysis has revealed that it includes twelve transmembrane domains with both ends (N and C termini) in the cytoplasm (Bannon et al, 2000).

Even though dopamine neurons are located in several areas of the CNS, the *DAT* gene is highly expressed only in the subset of DA neurons located in the midbrain; for this reason DAT can be used as a marker of late mDA neurons.

The expression of DAT varies within the midbrain too. Studies in rodents and humans have showed that DAT mRNA is found highly concentrated in neurons of the SNc and in lesser degree in the VTA (Bannon, 2000). Other areas where DAT can be found in lesser degree are the diencephalon (arcuate nucleus, ventral

thalamus and the para and peri ventricular hypothalamus nuclei). Additionally DAT can be found co-localized in the TH positive cells of the olfactory bulb (Bannon, 2000). This distribution matches the distribution of dopamine neurons throughout the CNS.

As mentioned earlier, it has been suggested that upregulation of *DAT* is partially due to a cooperative interaction between NURR1 and PITX3. The 8.3 kb of the 5' flanking regulatory region of the human *DAT* gene has been isolated and potential transcription binding sites have been identified on this sequence (Sacchetti, 1999). Further analysis showed that NURR1 increases the transcriptional activity of several human DAT (hDAT) constructs containing different fragments of the 5' flanking region of this gene (Sacchetti, 2001). Later studies using luciferase assay plasmids that contain DAT regulatory regions transfected into murine and human embryonic stem cell cultures showed that over-expression of NURR1 or PITX3 or both factors at the same time enhanced DAT gene expression (Martinat, 2006). These experiments suggest that NURR1 and PITX3 are both necessary to induce the expression of *DAT*; however, further studies are necessary to determine the precise location of binding sites that could explain the cooperation between these two transcription factors on the promoter region of the *DAT* gene.

1.5.2 Tyrosine Hydroxylase (TH)

TH is best known for its important role in the biosynthesis of catecholamines, which are molecules that contain a catechol group consisting of benzene and two hydroxyl side groups (Siegel, 1999) in their structure. Dopamine, epinephrine and norepinephrine are catecholamines.

The role of TH is to convert the amino acid L-tyrosine to L-DOPA, which is a precursor of dopamine (Siegel, 1999). Besides mDA neurons, TH is expressed in the hypothalamus, olfactory bulb, noradrenergic neurons of the locus coeruleus (brainstem) and the adrenergic neurons of the brainstem (Cazzorla, 2000) but TH transcriptional activity varies among them (Wong, 1994). TH mRNA midbrain expression occurs at E11.5 (Cazzorla, 2000) and its expression becomes permanent throughout the adult brain. Given that TH is the first enzyme that will catalyze the DA precursor, and this assures its dopaminergic background, some of the studies use TH for co-localization studies.

1.6 Dopaminergic Neurons: Biosynthesis

The biosynthesis of DA starts with the presence of the amino acid tyrosine. Tyrosine is a conditionally essential amino acid because its supply depends on the catalytic reaction of the essential amino acid phenylalanine (obtained through

ingestion of food), in which a hydroxyl group is added to the end of the phenylalanine ring, transforming it to tyrosine (figure 8).

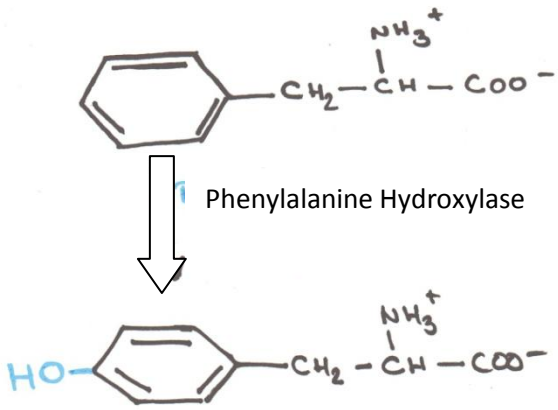


Figure 8. Conversion of phenylalanine to tyrosine via phenylalanine hydroxylase. Phenylalanine is an aminoacid necessary for the formation of tyrosine. Phenylalanine hydroxylase add a hydroxyl group to the end of the phenylalanine ring, transforming it to tyrosine

L-tyrosine is the precursor for all catecholaminergic transmitters, including dopamine, epinephrine and norepinephrine (Kandel, 2000). The biosynthetic pathway starts with the hydroxylation of L-tyrosine by the enzyme tyrosine hydroxylase (TH), which gives L-Dopa as a product. Subsequently, L-Dopa is converted into dopamine by the enzyme dopa- decarboxylase (Siegel, 1999). After synthesis, dopamine is packaged into vesicles via the monoamine transporter VMAT2 for its subsequent release into the synaptic cleft in response to action potentials. Most of the DA that has been released will be taken back up

by the DAT, which is located on the membrane of the pre-synaptic bouton (Figure 9).

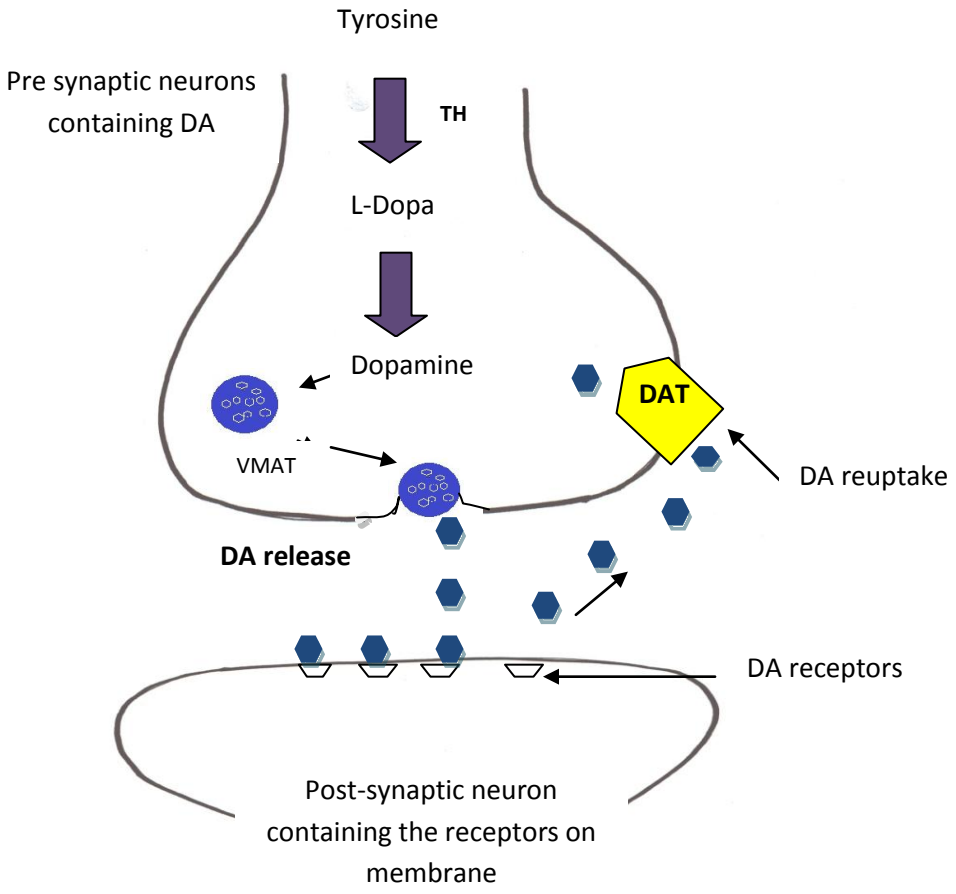


Figure 9: Schematic view of DA synthesis in the midbrain. DA biosynthesis starts in the pre-synaptic terminal. DA is synthesized and packaged into vesicles by the monoamine transporter called VMAT. VMAT is transported to the membrane and fuses with it, allowing the release of DA into the synaptic cleft. After the nerve impulse is finished, DAT will take back up the DA found in the synaptic cleft.

Dopamine neurons in mammals are located in different areas of the brain and they have a specific nomenclature according to their location (Prakash, 2006)

e.g. dopamine neurons in the midbrain are located in the retrorubral area (A8), the SNc (A9) and the VTA (A10) (figure 10).

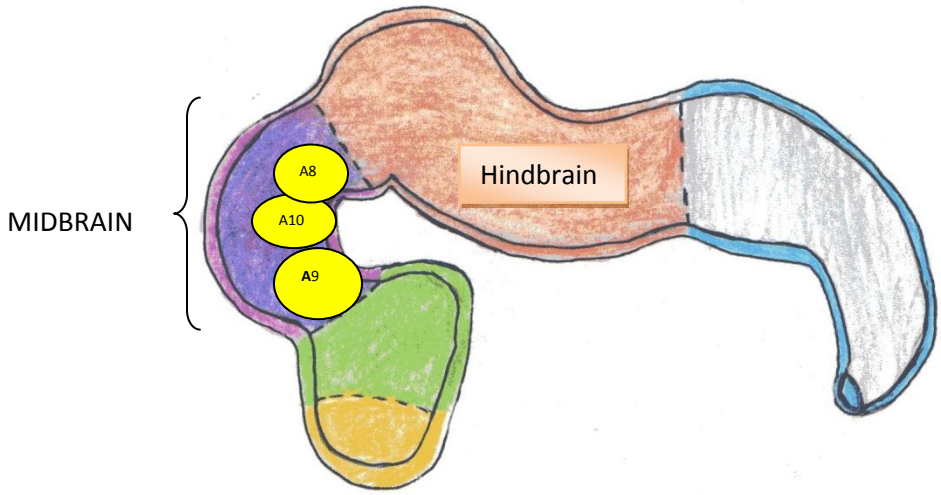


Figure 10. Distribution of mDA neurons during embryogenesis

1.7 Dopamine Pathways

Based on their spatial location, each DA nucleus sends projections to specific areas. For example, neurons from the SNc send projections to the dorso-lateral striatum forming the Nigrostriatal pathway. DA neurons from the VTA send their projections to the ventral striatum forming the Mesolimbic pathway (Figure 11) (Prakash, 2006). These two pathways are of great importance because they play roles in several body functions and behaviors. DA neurons from the SNc are involved in the control of voluntary movement and body posture and their degeneration has been associated with Parkinson's disease (Prakash, 2006).

Mesolimbic pathway neurons are involved in reward and cognitive behavior and alteration of this pathway has been associated with schizophrenia and addictive disorders (Prakash, 2006).

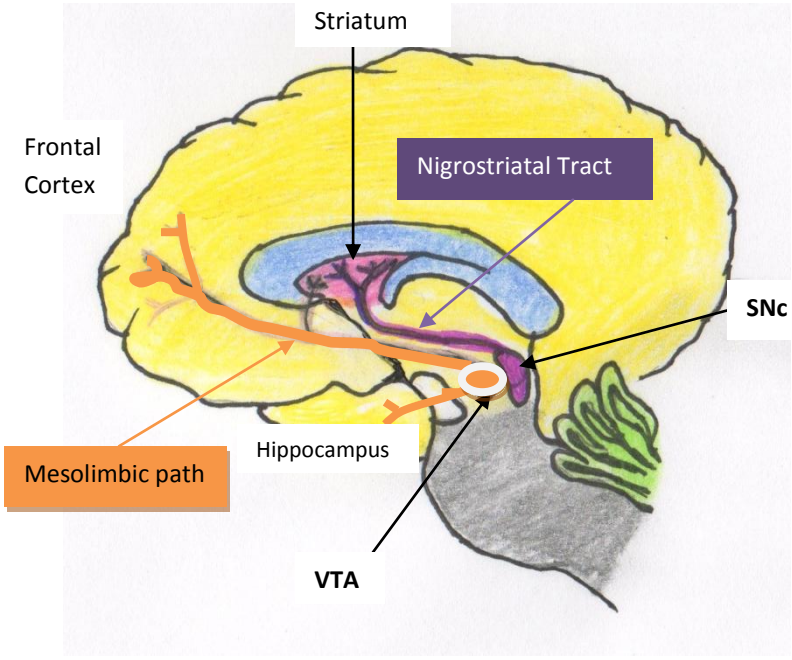


Figure 11. Dopaminergic Pathways in the midbrain. The cell bodies from DA neurons are located in high concentration in the SNc and VTA. Neurons from the SNc send their projections to the striatum (Nigrostriatal pathway). Neurons from the VTA send their projections to the hippocampus, Striatum (Nucleus accumbens) and to the frontal cortex (Mesolimbic pathway).

1.8 Parkinson's Disease (PD)

Parkinson's disease is a progressive neurodegenerative disorder that is usually diagnosed in older people (age 60 or more). Most of the symptoms involve

alteration in movement since PD is due to the death of DA neurons located in the SNc. These DA neurons send projections to the basal ganglia, which is an area of the brain that controls movement and coordination. By the time PD manifests its more classical symptoms, approximately 80% of the DA neurons of the SNc have been lost (Spina, 1989).

The degree of expression of the symptoms depends on the stage of the disease. Nonetheless, one of the first noticeable symptoms includes tremors in just one hand or in the toes and eventually these will develop into shaking, rigidity, bradykinesia (slowness of movement) and difficulties during ambulation. In advanced stages of the disease, cognitive and behavioral problems may arise due to the high loss of DA neurons (Jankovic, 2008).

Although the symptoms of PD and the area of the brain affected are well known, the exact causes of the disease are still unclear. PD is idiopathic (no known cause) although approximately 10% of the cases have a genetic component.

The discovery that certain drugs contaminated with 1-methyl-4-phenyl-4-propionoxypiperidine (MPTP) selectively damaged DA neurons from the SNc and thereby caused Parkinson's-like symptoms (Langston 1983,1996) was the first evidence that suggested that PD can be caused by external factors such as toxins. Subsequent findings found that environmental factors, such as certain pesticides, also increase the chances of developing PD (Lonneke, 2006).

For years, research on Parkinson's disease has been limited because of the lack of understanding of the molecular mechanisms that control DA neuron development and the lack of animal models to study and test new drugs and treatments.

An alternative way to study these molecular mechanisms is to use cell culture models. No cell culture models exist that completely recapitulate the phenotype of DA neurons and would allow the development of studies aimed at understanding the regulation of gene expression in DA neurons. Recently, the neuroblastoma cell line SK-N-AS has been shown to express the late dopaminergic marker DAT (Johnson, 2011), making this the most appropriate cell culture model for studies on transcription regulation of DA marker genes.

1.9 DA neurons in cell culture

Cell cultures are cells that are grown *in vitro* under controlled conditions. They are used as model systems to perform investigations of molecular mechanisms that involve cellular differentiation, regulation, development and toxic processes because they can be manipulated by addition of external factors (e.g. growth factors) that mimic the *in vivo* environment. Because of these attributes, cell lines can be used to study diseases such as Parkinson's disease and multiple sclerosis, for which it is difficult to perform experiments to reproduce these diseases in live animals, including humans.

Even though cell culture is a good choice to study diseases that are difficult to study in live animals, the study of cells of the central nervous system is

challenging because there are few cell lines that have an adequate neural profile to mimic molecular mechanisms. Cells useful for research are tumor cells derived from neuroblastoma cells (e.g. SY-SY5Y, PC12, SK-N-AS) (Thiele, 1998). Neuroblastoma (NB) is a type of cancer that originates in tissues of the nervous system. They are usually obtained by surgical resection (Thiele, 1998) after which they are placed in a growing media. With time, some of the cells will attach to the culture dish and some will remain suspended. After removal of the suspended cells by changing the media, only the ones that are attached to the culture dish will be used to give rise to the next group of cells, the ones that will form the primary cell line.

Given the neuronal origin of NB, several cell lines can have neuronal and or neuro-endocrine properties (Thiele, 1998). Therefore, they could be able to synthesize enzymes required for biosynthesis of neurotransmitters (e.g. TH) and express acetylcholine receptors (Thiele, 1998). Another feature characteristic of NB is that they tend to have an adrenergic phenotype by expressing TH and dopamine B hydroxylase (an enzyme necessary to convert DA to norepinephrine) (Ciccarone, 1989).

Choosing a NB cell line to study neuronal cells such as DA neurons is an advantage because they have the proper neuronal background but further validation of their phenotype is needed to confirm that they are expressing our genes of interest. In order to validate their DA lineage characterists, analysis of

the expression of midbrain DA markers such as NURR1, TH, PITX3 and DAT are necessary.

Recently the neuroblastoma cell line SK-N-AS has been shown to express NURR1 and DAT (Johnson, 2011), making it a good cell culture model to study transcription regulation of DA marker genes.

1.9.1 SK-N-AS cell line

The SK-N-AS cell line originates from a neuroblastoma derived from the neural crest. This cell line expresses proteins that are characteristic of midbrain dopamine neurons such as NURR1 and DAT (Johnson, 2011). Therefore it can be used as a model system in which to study the transcriptional regulation of the dopamine transporter gene.

In order to obtain an accurate model with comparable levels of the expression of NURR1 to the DA neurons of the SNc in humans, basal SK-N-AS cells (SK-N-AS C line) expressing low levels of the transcription factor NURR1 were cloned to generate different levels of expression. Two clones were obtained, one that over-expresses NURR1 by two fold, which is referred to as SK-N-AS (E), and a second clone that over-expresses NURR1 by eight fold referred as SK-N-AS (G) (Johnson, 2011).

PROPOSED STUDIES

This thesis focuses on the study of the interaction between NURR1 and PITX3, two transcription factors whose simultaneous expression is limited to a subpopulation of dopamine (DA) neurons residing in the two major nuclei of the ventral midbrain: the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). The objective is to unveil how these two transcription factors interact upstream of the dopamine transporter (*DAT)* gene to upregulate its expression.

Different studies have shown that NURR1 and PITX3 presence are crucial for the normal dopaminergic (DAergic) phenotype. One of these key roles during DA development is the expression of a transmembrane protein (gene product of the *DAT* gene) that allows the reuptake of the neurotransmitter dopamine after it has been released into the synaptic cleft in response to action potentials. When NURR1 is over-expressed, there is an increase in the transcriptional activity of *hDAT* promoter constructs (Sacchetti, 1999). Subsequent studies have suggested that NURR1 enhances *DAT* gene transcription through an indirect mechanism that might involve its interaction with a protein "x" on the 5' flanking region of the human *DAT* (*hDAT*) gene (Sacchetti, 2001). However, protein "x" still remains unknown.

Studies involving PITX3 have suggested that it has a key role in the maturation of DA neurons from the ventral midbrain (Smidt 1997, 2004). Later studies

performed in murine and human embryonic stem cell cultures have suggested that NURR1 and PITX3 interact cooperatively to induce the terminal maturation of DA neuron phenotype (Martinat, 2006). Moreover, the same study suggested that NURR1 and PITX3 interact cooperatively to upregulate the transcription of the *DAT* gene. Subsequent studies have suggested that *DAT* is a direct target gene of PITX3 (Hwang, 2009).

Results of all these studies indicate that in the absence of either Nurr1 by itself or Nurr1 in addition to Pitx3 DAT expression is suppressed. When Nurr1 is present and Pitx3 is absent then, the expression of DAT is significantly reduced, but when both Nurr1 and Pitx3 are present together, then the expression of DAT is increased. These results can be considered as strong evidence that Nurr1 is coupled to Pitx3 and this interaction, either direct or indirect, is upregulating the expression of the *DAT* gene. However, the precise mechanisms through which this occurs and the location of the binding sites where NURR1 and PITX3 act on the *DAT* gene to explain their cooperative action remain unclear.

It is in my interest to study the SK-N-AS neuroblastoma cells to determine if they are a good cellular model to study DA molecular mechanisms that involve NURR1, PITX3 and DAT. My goal is to study NURR1 and PITX3 interaction on the 5' flanking region of the hDAT gene to determine if PITX3 is the protein "x" that might interact with NURR1 to upregulate the expression of the *DAT* gene. It would be also important to determine if there is a direct physical interaction

between NURR1 and PITX3 in order to better understand the molecular mechanism regulating the acquisition of the DA phenotype.

Additionally we would like to characterize the neuroblastoma cell line SK-N-AS using two transmission electron microcopy (TEM) and scanning electron microscopy (SEM). Our goal using these techniques is to determine if there are any morphological differences between the basal C line and the overexpressing NURR1 E line at the nuclear level and if possible also get a general profile of the phenotype of this cell line since little is known about them.

MATERIALS AND METHODS

2. Materials and methods

2.1 Cell lines:

The neuroblastoma cells SK-N-AS (a gift from Dr. Michael Bannon; Wayne State University) basal line C and E line, over expressing NURR1 by two fold, were grown in tissue culture plastic flasks. They were supplied with DMEM media (GIBCO) containing 10% heat inactivated Fetal Bovine Serum, 100 units of penicillin/ mL, 100 ug of streptomycin/ ml and incubated at 37°C in 5% CO_2. To avoid contamination, cell culture manipulations were undertaken inside a laminar flow sterile hood and surfaces and objects were sprayed with 70% ethanol before use.

2.2 Routine culture of SK-N-AS cells:

SK-N-AS cells were cultured in media containing geneticin (1mg/ml). This media was changed every two to three days until the cells reached approximately 80% confluency, approximately 3-4 days for the C line and 6-7 days for the E-line. After the adequate confluency was reached, the cells were ready for passaging and further plating or freezing. When passaging and thawing were required, all media and solutions were warmed to 37°C prior to use.

2.3 Passaging of cells:

To passage the cells, the media was removed using an aspirator then cells were washed with phosphate buffered saline 1x (PBS, pH 7.4; GIBCO). After the wash, trypsin 0.25% (GIBCO) was added in sufficient volume to cover the cell monolayer and the flask was incubated at 37°C for three minutes to facilitate detachment of the cells from the bottom of the flask. The activity of the trypsin was neutralized by the addition of complete media, in a 1: 1 ratio. In order to dilute the cells to the desired concentration (1:5, 1:7.5 or 1:10, depending on needs), tryptan blue was added to an aliquot of cells and cells were counted using a hemocytometer with Neubauer ruling. Then the cell suspension was transferred to a centrifuge tube and centrifuged for five minutes at 1000 rpm for further manipulation. Normally, C-line cells were passaged every 2-3 days and E-line every 6-7 days; no cells were used after passage ten.

2.4 Freezing SK-N-AS cells

After trypsinization and centrifugation, cells were resuspended in 1 mL of freezing mix (normal culture media plus 10% dimethylsulphoxide) at a density of 10^6cells/ml and transferred to a cryotube. Cryotubes were placed at -80°C overnight and transferred to a liquid nitrogen tank for long term storage.

2.5 Thawing SK-N-AS cells

Frozen cryotubes were retrieved from liquid nitrogen storage and placed immediately in a 37°C water bath to allow cells to thaw. Cells were diluted in fresh media and plated in a T75 or T25 flasks (BD Falcon®) with 11 mL or 3 mL of warm media respectively and placed in the incubator. Twenty four hours after, the media containing DMSO was completely removed and new media supplemented with 1mg/ml of geneticin was added and placed on the incubator at 37°C.

2.6 Isolation and purification of RNA:

RNA was extracted from SK-N-AS cells using the RNeasy mini kit (Qiagen) following manufacturer's directions. Briefly, cells were plated in 6-well plates at a density of 750,000 cells/ well 24 hours prior to harvesting. Culture media was aspirated from the wells and they were washed with PBS. A mix containing lysis buffer (350 µL per 9.4/cm^2 well; Qiaqen) and β-mercaptoethanol (10 µL/ml of buffer) was prepared and added directly to the well to disrupt cells. The lysate was collected and transferred to an eppendorf tubes for a homogenization using a syringe and needle (20G). An equal volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The supernatant was added to a Qiagen column and spun. A series of washes using RW1 and RPE buffer were performed directly on the column. RNAse free water was then added and centrifuged for 1 minute at 10,000 rpm to elute the RNA. The RNA concentration

was determined using spectrophotometer that measures nucleic acid concentration at 260 nm and purity using the 260/280 nm ratio.

2.7 cDNA Synthesis:

Purified total RNA was converted to cDNA using the Qiagen® One step RT-PCR Kit. A master mix with a final volume of 20 µL was done using of 2 µL of 10x RT buffer, 2 µL dNTP mix (5mM each), 1 µL random primers (10µM), 0.2 µL RNAse inhibitor (10 units/ µL), 1 µL O-RTase (4 units) and variable amount of RNAse free water. This mix was duplicated per each sample in order to use them for non template control (NTC or RT-) and for our samples (RT+). RT- was used as a negative control. It contained all the components of the reaction except for the template RNA, which was replaced with water and therefore was used as an indicator of possible sample contaminations with exogenous DNA.

After addition of the master mix and purified RNA to our RT+, samples were incubated at 37°C for 60 minutes to allow for cDNA synthesis. If not used immediately, the cDNA samples were stored at -20 ºC until use.

2.7.1 PCR primer design:

Primers specific to human pituitary homeobox 3 (hPITX3), human dopamine transporter (hDAT), and human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) genes were designed using *Primer3 Simgene* and *NCBI/* ***Primer-BLAST*** and synthesized by Integrated DNA technologies (IDT).

The following primers were used to amplify the cDNA's of interest.

For the human PITX3 (hPITX3) gene,

the forward primer,5'- CAACCTTAGTCCGTGCCAGT-3' and

the reverse primer, 5'- AAACACCCCTTTCAGACCCT -3'

gave an amplicon of 155bp.

For the human DAT (hDAT) gene,

the forward: 5'-CCATAGACGGCATCAGAGCATA-3' and

the reverse: 5'-CATGTACCCCAGGAAGGAGA-3'

gave an amplicon of 246bp.

For the human GAPDH (hGAPDH) gene,

Forward: 5'- GGCGATGCTGGCGCTGAGTA-3'

Reverse: 5'- ACAGTTTCCCGGAGGGGCCA-3'.

gave an amplicon of 322bp.

GAPDH is a housekeeping gene and was used as a control for comparison in gene expression data of cDNA samples.

For the human nuclear receptor related protein1 (hNURR1) gene, the following primer sequences

forward, 5'-ATGGACAACTACAGCACAGG-3' and

reverse: 5'-GGGTTCATGGGGACGTGCAG-3')

giving an amplicon of 422bp, were obtained from previously published literature (Bannon, 2005).These specific primers amplify a sequence that is common to all splice variants of hNURR1.

All primers were first resuspended in milliQ water to prepare a stock solution of 100µM. Also, 10 µM working solutions were prepared prior to use in reverse transcription polymerase chain reaction (RT-PCR).

2.7.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR):

Prior to running the PCR, a master mix containing the specific primers detailed above and the desired cDNA samples were prepared. The reaction mixture set up for each sample is detailed in table 2.

Table 2. Reaction mixture for RT-PCR

Initial concentrations	Final concentrations	1X
MgCl (50mM)	1.5 mM	1.5 µL
Buffer (10x)	1x	5.0 µL
TAQ pol (5u/µL)	2.5u/µL	0.5 µL
Primer R (10 µM)	0.5µM	2.50 µL
Primer F (10 µM)	0.5 µM	2.50 µL
dNTP(10mM)	0.2 µM	1.0 µL
H20	----	35.0 µL
Cdna	2µL	2.0 µL
TOTAL	50µL	50 µL

The PCR reaction for GAPDH was ran using 3.0 mM of $MgCl_2$ (50mM).This master mix solution was prepared for each primer set. A set of two PCR tubes per primer pair was labeled as RT+ and RT-. 2 µL of the cDNA was added to the RT+. To the RT – we added 2 µL of our RT negative control which contained water instead of cDNA. All the reactions were run on the Techne Techgene Thermocycler. The temperature profile was constant for all reactions with the exception of the annealing temperature which was changed according to the primers' predicted annealing temperatures. The temperature profile used is outlined in Table 3.

Table 3. Temperatures used for PCR reactions

Stage	**Temperature**	**Time** (per cycle)
Separation	94°C	3 minutes and 45 seconds
Annealing	hNURR1, hPITX3 and hDAT: 54.5 °C hGAPDH: 62.5 °C	30 seconds
Extension	72°C	1minute and 30 seconds
	Number of cycles: 30	
Final	72 °C	10 minutes
Final Hold	4°C	∞

2.7.3 Gel Electrophoresis:

After PCR reactions were performed, the products were analyzed by gel electrophoresis to sort the product based on size and charge.

A 2% agarose gel dissolved in 1x Tris Acetic acid EDTA buffer and supplemented with 5 μL of 10 mg/mL of Ethidium Bromide (EtBr) was prepared to run the PCR products.

Before loading the samples to the well, they were mixed with 6x loading blue dye. Each well in the gel was filled with a total volume of 20 μL (17 μL of PCR sample and 3 μL of 6x loading dye). For reference to estimate the size of unknown DNA molecules, 10 μL of 100bp DNA ladder, a solution of DNA molecules of different lengths, was added to the agarose gel. A 100 bp ladder contains DNA fragments ranging from 100 bp to just above 1000 bp (Company). The gel was run using 100V (voltage) for approximately 45 minutes. The gels were analyzed under the Luminescent Image Analyzer LAS 3000 (Fuji film).

2.8 Microscopy

To characterize and to get a more complete phenotypic profile of this cell line given that little is known about them, scanning electron microscopy transmission (SEM) and electron transmission microscopy (TEM) were used.

For both types of microscopy, the cell lines C and E were plated 24 hours prior to fixation in a sterile six wells plate at two different densities (350,000 cells/ well and 750,000 cells/well) and they were maintained with growth media containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 100 units of penicillin/mL, 100 μg of streptomycin/mL and geneticin at a

concentration of 1µg/mL. Cells were incubated and maintained at 37°C, 5% CO_2 24 hours prior to fixation.

2.8.1 Scanning Electron Microscopy (SEM):

Fixation, Post- fixation and dehydration

SK-N-AS C and E-line were grown on thermanox coverslip overnight and seeded at 350,000 and 750,000 cells/ well. They were maintained with growth media containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 100 units of penicillin/mL, 100 µg of streptomycin/ml and geneticin at a concentration of 1 µg/ml.

All the steps were carried out in the culture wells (2-3 ml solution/ well). Prior to fixation, the media was removed and the wells were rinsed with PBS twice. For fixation, 2% glutaraldehyde in 0.1 sodium cacodylate buffer (pH7.2) was used. The samples stayed immersed for 45 minutes at room temperature. The samples were then rinsed three times, 5 minutes each, with 0.1 M of sodium cacodylate buffer.

For Post fixation, 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer pH 7.2 was used. Samples were submerged for one hour after which they were rinsed 3 times, for five minutes each with distilled water. A stain *en bloc* was done using uranyl acetate for thirty minutes.

The cells were dehydrated using ethanol series of 5- 10 minutes each. The series were as follows: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%. When working with 100% ethanol, the cells were submerged in ethanol 3 times 5-10 minutes each. In every step it was important that the cells were not exposed to air, so they were submerged with a thin layer of ethanol during the entire process.

The coverslips were transferred to a glass petri dish during the 80% step dehydration process. This intermediate step was necessary to get the sample ready for the final step adding Hexamethyldisilazane (HMDS) given that this chemical is corrosive and only can be used in glass.

After the third rinse with 100% ethanol, HMDS was added as follows: 1:1 mixture of 100% ethanol and HMDS for 10 minutes, a 1:2 mixture of 100% ethanol and HMDS for 10 minutes, and 100% HMDS three times for 10 minutes each. After the last change of 100% HMDS a thin layer of solution was left (enough to cover the coverslip) and it was left under to hood overnight with the lid ajar to allow HMDS to evaporate. Samples were then examined under the SEM (QUANTA 200-FEI).

2.8.2 Transmission Electron Microscopy (TEM)

Fixation, Post-fixation and dehydration

Twenty four hours prior to fixation, SK-N-AS cells C and E were seeded at 350,000 and 750,000 cells/well each of them containing a thermanox coverslip. Before starting the fixation, each well was rinsed twice with PBS to eliminate any media residue. Immediately after, samples were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 1 hour at room temperature. Each well was rinsed in the same buffer in 3 cycles of 10 minute rinses each then they were post-fixed for 1 hour in 1% Osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.2). After an hour the wells were rinsed with distilled water (dH_2O) 3 times of 5 minutes each. Then, samples were stained *enbloc* in 1% aqueous uranyl acetate for 30 minutes, dehydrated in a graded series of ethanol (30%, 50%, 70% and 90%). As the last step of dehydration the samples were rinsed 3 times in 100% ethanol 10 minutes each.

Infiltration process

The infiltration process begun after the 3rd rinse with 100% ethanol, for this step we used Spurr's low viscosity resin because it infiltrates the tissue easily and quickly. The volume of components used to prepare this resin is shown in table 4.

Table 4. Spurr's /Quetol extra low viscosity resin (Ellis formulation)

Function	Material	Amount
Epoxy resin	ERL-4221	8.88 g
Epoxy resin	Quetol-651	5.6 g
Hardener	NSA	25.5g
Plasticizer	DER-736	5.7 g
Catalyst	DMAE	0.4 g

The infiltration with resin was done using mixture of ethanol and resin with the amount of acid being decreased gradually until 100 % of resin was added. The series was as follows: 2:1 (ethanol to resin) for 30 minutes, 1:1 for 30 minutes, 1:2 for 30 minutes and 100% resin for 30 minutes. The last step in the infiltration process included leaving the sample in 100% resin overnight.

The next day, the coverslips were transferred to aluminum weighing pans (3 coverslips per pan, avoiding overlapping) with cells facing up. Once they were transferred, a thin layer of resin was poured over them (just enough to cover them completely). The 2 weighing pans were put in the vacuum oven at 50°C for 15 minutes for a posterior polymerization at 70°C for 24 hours.

Samples were mounted for posterior thin-sectioned using diamond knives. Thin sections of 70 µm were trimmed using the Ultracut Microtone and stained with toluidine blue to be observed under the light microscope. Posterior trimmed sections were placed into a grid for post-staining. The post-staining process was performed using 2% aqueous uranyl acetate for 40 minutes and Reynold's lead

citrate for 7 minutes to obtain a better contrast. The resulting grids were observed on a Philip CM100 TEM operating at 80 kV.

Negatives of the pictures taken using the microscope were scanned under the light box to determine the correct focus for a later enhancement using Photoshop CS5 to improve contrast and brightness.

RESULTS

3.1 RT-PCR Analysis of *NURR1, PITX3* and *DAT* expression in SK-N-AS C and E lines

The electrophoretic results of the RT-PCR products were used as a qualitative means to confirm that the SK-N-AS cell line was a suitable model to study the gene expression in dopamine cells. Also, these results were used to validate the endogenous expression of NURR1 and DAT in SK-N-AS cells since only a very few labs are working with this neuroblastoma line as a model to study dopaminergic molecular mechanisms.

After obtaining cDNA and performing PCR, the samples were evaluated by gel electrophoresis to detect gene expression. Gene expression is identified by the presence of proper banding patterns, corresponding to the expected base pair length of each primer set amplicon.

In each of the gel electrophoresis experiments (figure 12-14) I used a 100 bp DNA ladder, positive and negative controls. The negatives controls (RT-) only contain buffers, reagents and water, so it shouldn't amplify any DNA and, no bands should be observed. For every set of primers I used its respective RT- and, as expected, in all my results, the RT- controls showed no band, validating the performance of the PCR.

Figure 12 shows the gene expression of NURR1 between the C and E line. GAPDH, whose amplicon is 322 bp in length, was used as a positive control and, as we can see, both lines express it. When analyzing NURR1 expression, whose amplicon is 422bp in length, the well defined presence of a band in the SK-N-AS E line confirmed that it expresses the transcription factor NURR1 whereas in the C line its presence is very low and does not show a clear band.

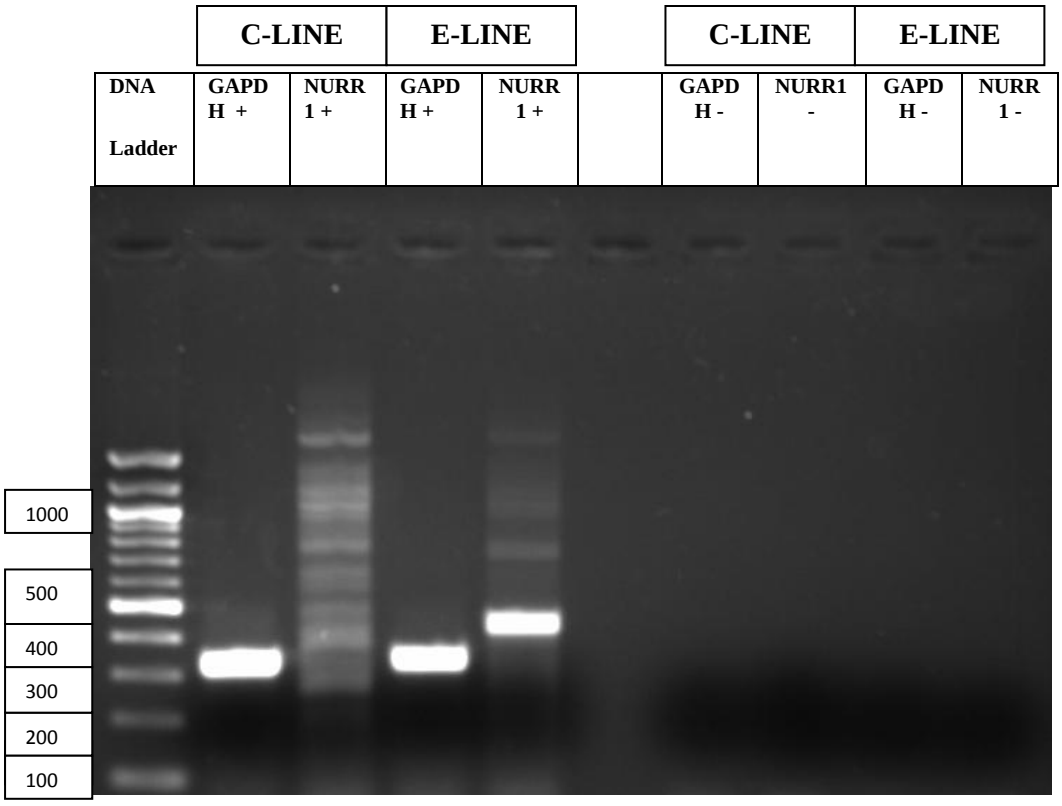


Figure12. Comparison of NURR1 expression between C and E line. A 100bp DNA ladder was used as a reference to estimate the size of unknown cDNA fragments. NURR1 in the C line does not appear as clear band whereas in the E line there is a well defined band around 400bp. GAPDH is used as internal control control(expected band around 300bp).

The gel electrophoresis shown in figure 13 was run to analyze and to compare DAT and PITX3 gene expression in both C and E cell line. As shown in the graph, the only band that was present belonged to the expression of DAT gene from the E line. Our RT- controls show no bands as expected. Our results showed that PITX3 is not expressed in the SK-N-AS cells (neither control nor NURR1 over-expressing E line) but yet DAT is present in the E line.

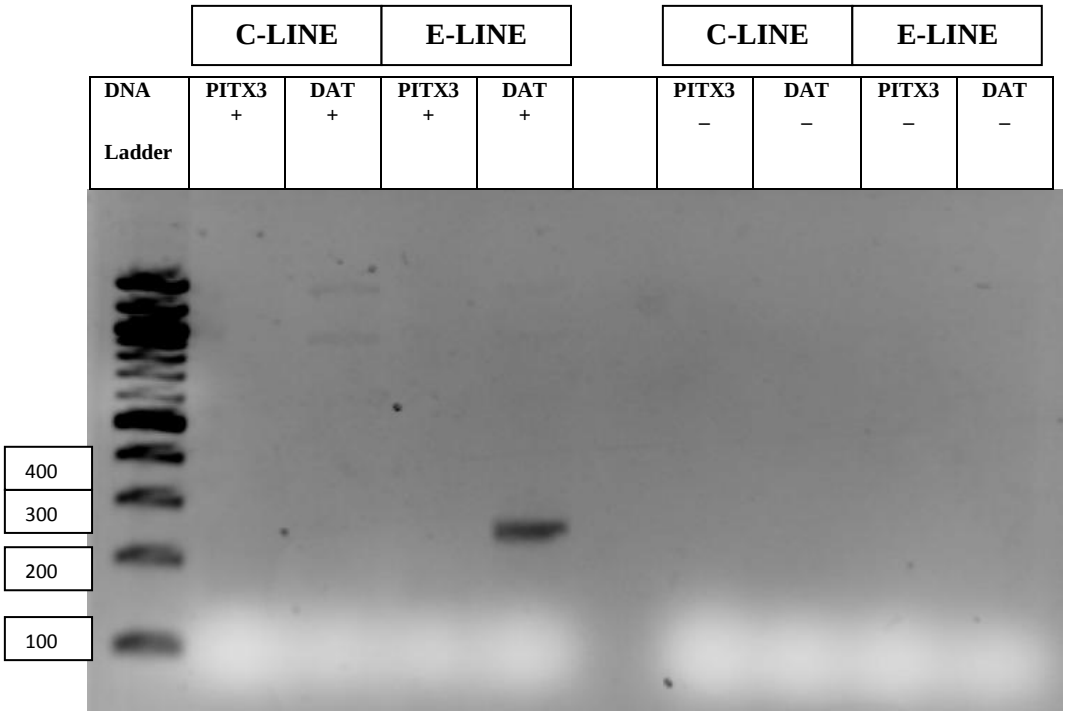


Figure 13: Comparison of PITX3 and DAT gene expression in SK-N-AS cell lines C and E. A 100bp DNA ladder was used as a reference to estimate the size of unknown DNA molecules. The only band present belonged to the DAT E- line, with an amplicon of 246bp. Notice the band is between the 200bp and the 300bp. RT- control showed no bands as expected.

According to our results, SK-N-AS cells C and E are not expressing the transcription factor PITX3 (Figure 14). We tried to optimize the PCR protocol using different concentrations of $MgCl_2$ (1.5 mM and 3.0 mM), cDNA (2 µL vs 3 µL) and Taq polymerase but our results only confirmed that PITX3 was not being expressed in either cell line.

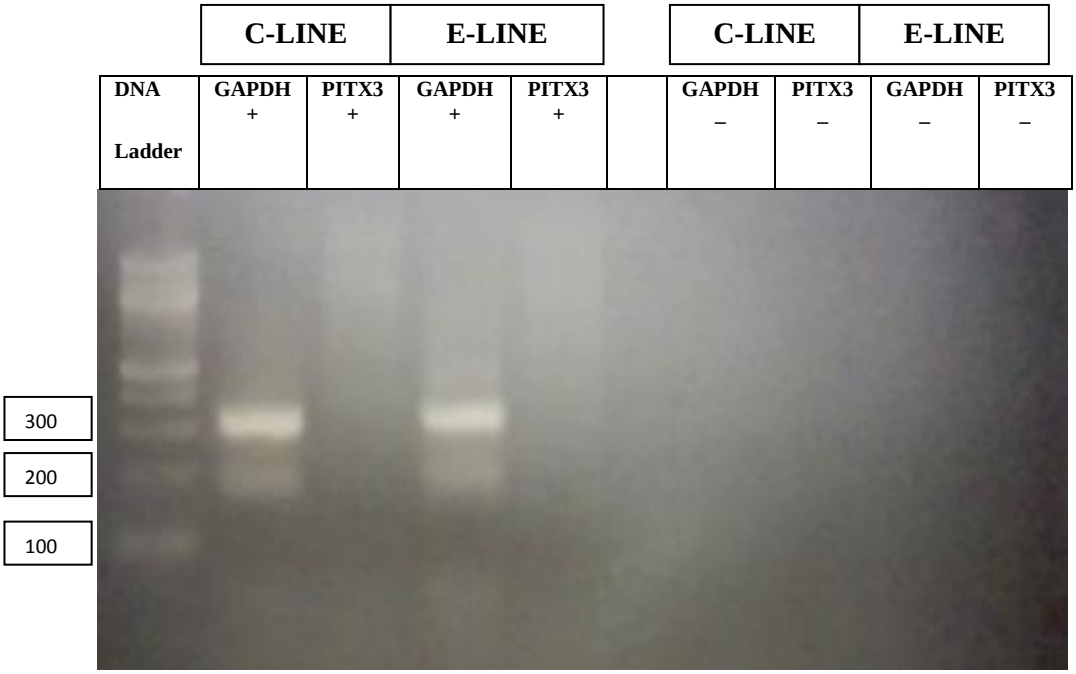


Figure14. PITX3 gene expression analysis in SK-N-AS C and E lines. 100bp DNA ladder was used as a reference to estimate the size of unknown DNA molecules. We used GAPDH as a positive control for our experiments. We expected to find a band in between 100 and 200 bp given that the amplicon for PITX3 was 155 bp in length. PITX3 expression was not detected in either line. RT- control showed no bands as expected.

RESULTS FOR MICROSCOPY

3.2 SK-N-AS C and E cell line under the Scanning Electron Microscopy

SEM is a type of microscope that uses a beam of electrons instead of light to form an image. Because the SEM utilizes vacuum conditions and a beam of electrons, all water must be removed from the sample. The sample is then covered by a conductive element, in this case, gold, by using a device called “sputter coater”. The sputter coater uses an electric field and argon gas. The sample is placed in a small chamber that will bombard the samples with a thin layer of gold. A critical point procedure is required to dry the cell or tissue without collapsing or deforming the structure of the specimen. Pressure is involved in the process and this can damage samples that are soft such as cells, so an alternative way is to use hexamethyldisilizane (HMDS). The sample is then able to be looked at under the SEM microscope.

First of all we wanted to get a characterization of how these cells look like under the SEM because there is very little known about them. I didn’t know what to expect, but given that the E line is overexpressing a transcription factor NURR1, I was curious to see if this affected somehow the structure of the nucleus. Additionally to see if the cells change structurally when they are at low density versus high density we plated the C and E-line at two different densities (350,000 cells/well and 750,000 cells/well).

When the cells were found in low confluency, they tend to be isolated but with their filipodia trying to reach other cells (Figures 15 and 17).

When comparing the nucleus of the C-line cells (figure 15, A and 15 B) with those of the E line cells (figure 17 E and F), it seems that there is a difference in the shape of nucleus. The nucleus of the C line (Figure 15 A and B) seemed to have a more elongated nucleus with less definition in shape than the E-Line (figure 17 E and F). In the E line, the nucleus seems to have a more rounded well defined shaped. This pattern was observed throughout our samples when looked under the SEM but further repetitions with different cell samples taken at different passages need to be done before stating a conclusive result.

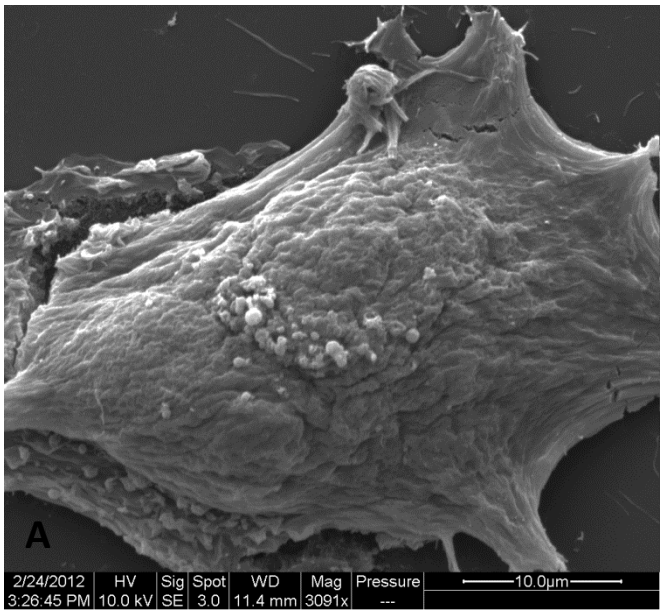
When comparing the C –line that was plated at an initial density of 750,000 cells/ well (figure 16) with the E line (plated at the same density of the C-line) we were able to see a monolayer formation in both cell lines and no difference between them.

One detail impossible to ignore about these cells (both C and E) is that some of they two nuclei. We are not sure if there is a fusion of cells or if one cell has two nuclei. As far as my research allowed me to find, neuronal cells do not have two nuclei but because these cells are mainly cancer cells, I think that might be the reason.

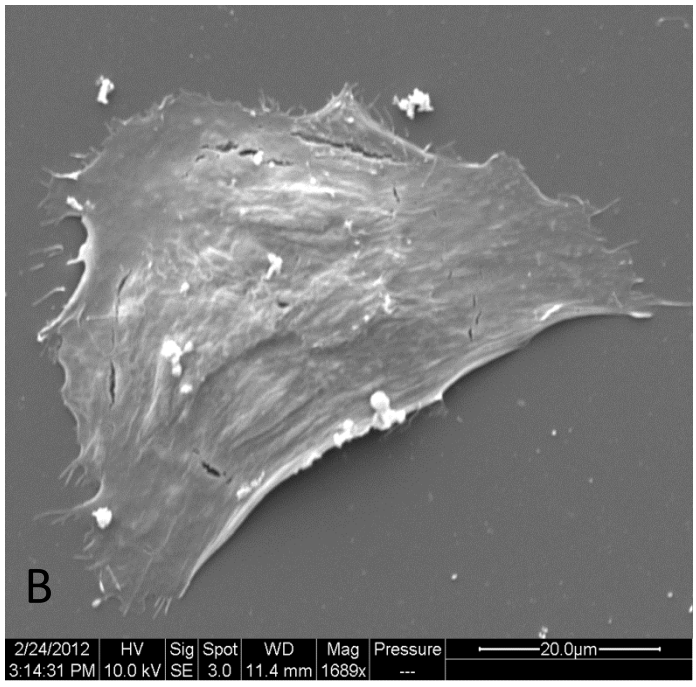
Figure 15. Scanning electron micrograph image of an SK-N-AS C- line. After the cells were coated with gold, they were analyzed under the SEM. Representative SK-N-AS cells plated at 350,000 cell/ well.

In (A) The high magnification (3091x) allows us to have a closer look at an individual cell. The nucleus is centrally located and is bulging with no well - defined shape.

In (B) another view of a cell looked at different magnification (1689x) show the presence of what seem to be 3 centrally located nuclei.

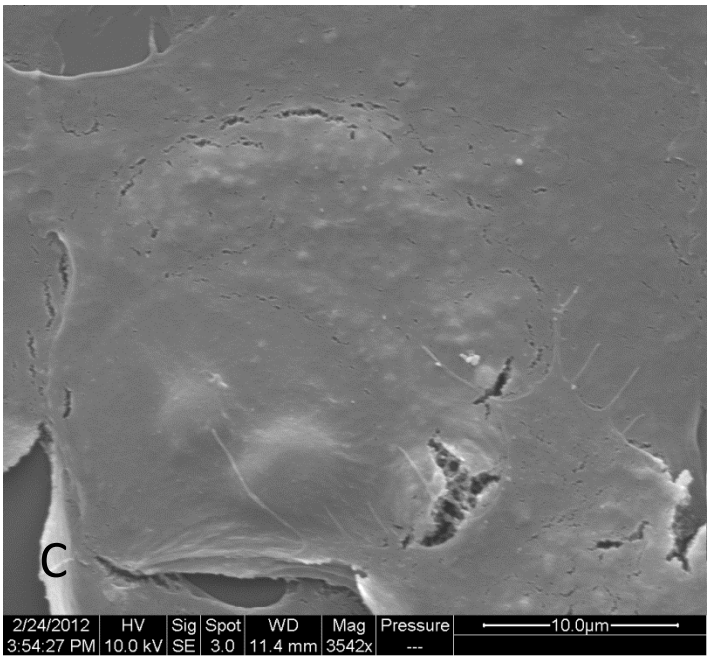

A
2/24/2012
3:26:45 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
11.4 mm
Mag
3091x
Pressure

10.0µm


B
2/24/2012
3:14:31 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
11.4 mm
Mag
1689x
Pressure

20.0µm

Figure 16. Scanning electron micrograph image of an SK-N-AS C-line plated at higher density. Cells were plated at 750,000 cells/well. The analysis of their morphology determined that they have a monolayer structure with filipodia extensions. (C) and (D) shows different angles suing different C-cell samples.


C
2/24/2012 3:54:27 PM
HV 10.0 kV
Sig SE
Spot 3.0
WD 11.4 mm
Mag 3542x
Pressure ---
10.0µm

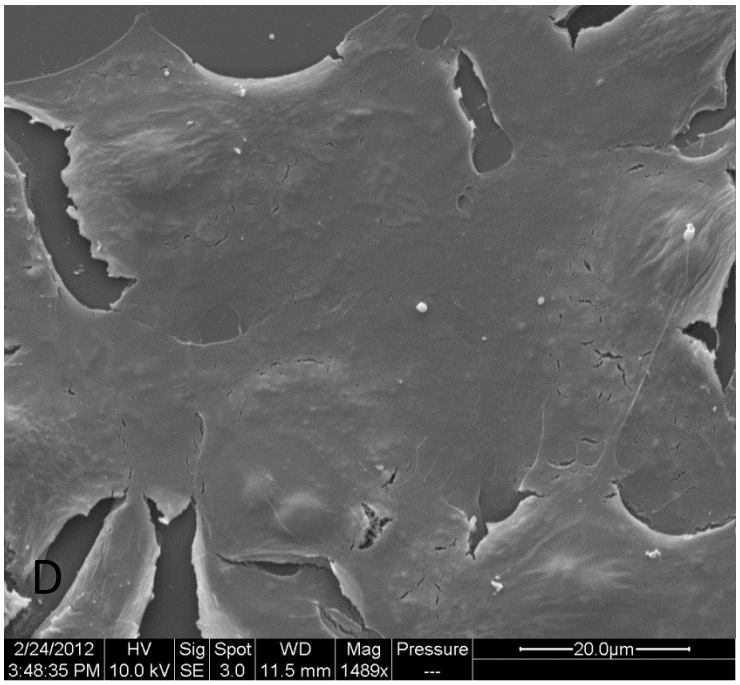
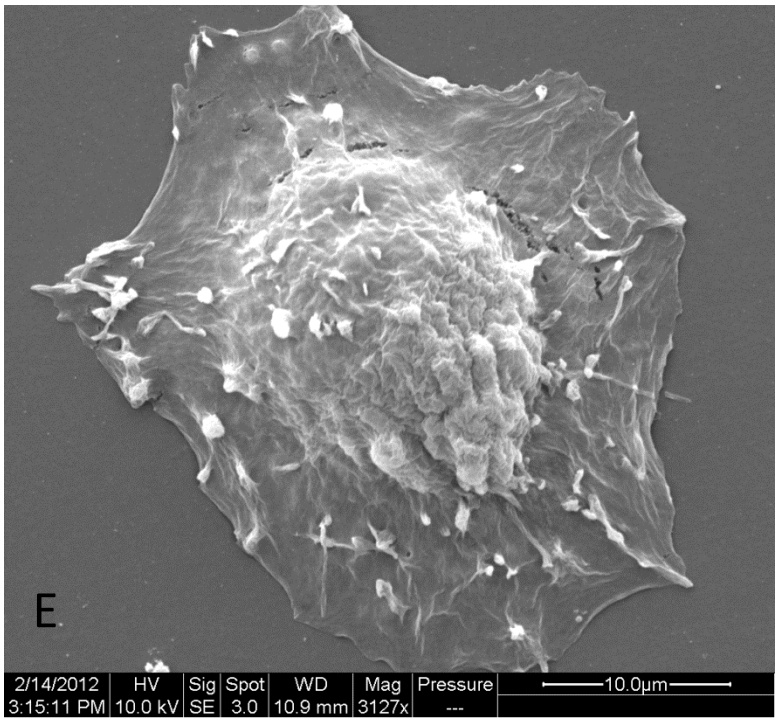

D
2/24/2012 3:48:35 PM
HV 10.0 kV
Sig SE
Spot 3.0
WD 11.5 mm
Mag 1489x
Pressure ---
20.0µm

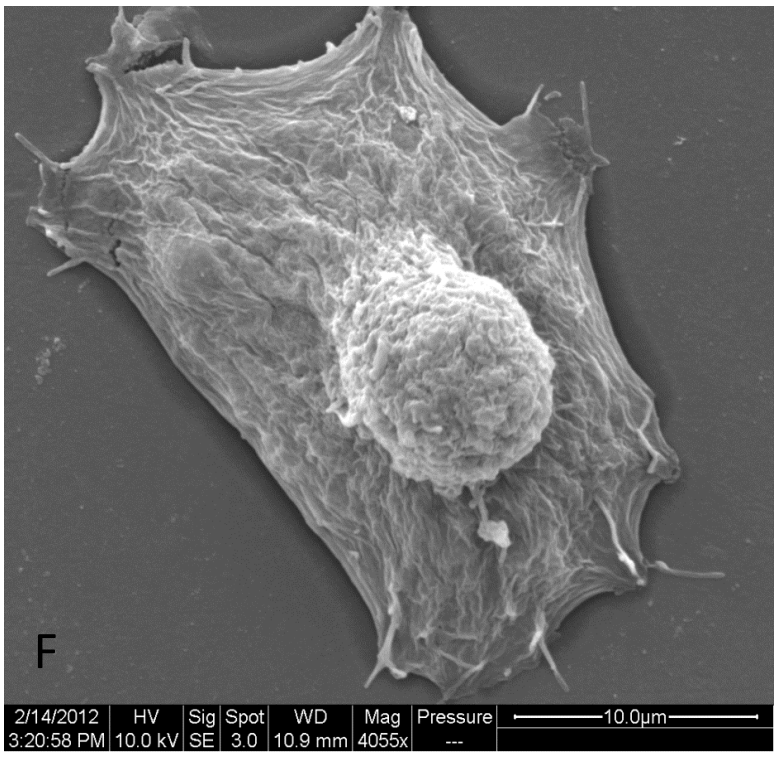
Figure 17. Scanning electron micrograph image of an SK-N-AS E-line at low density. The E-line was plated at 350,000cell/ml which allowed looking at each cell individually.

(E) the nucleus has a well defined shape and a flat cytoplasm. Notice the difference between the SK-N-AS C-line (figure 15A) and SK-N-AS E-line. This pattern was observed throughout the samples.

(F) Another representative of the E line at higher magnification (4055x). The same different nuclear shape is present.

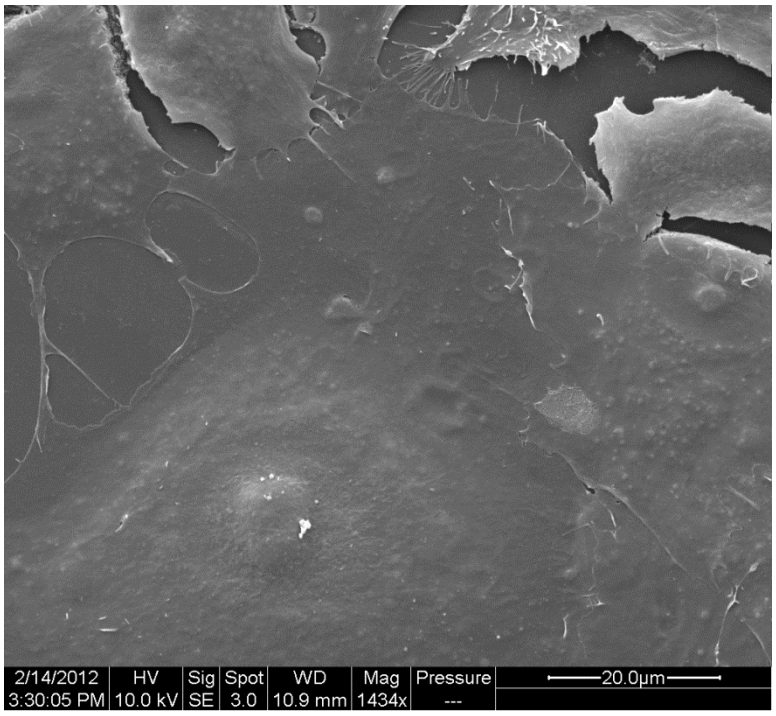

E
2/14/2012
3:15:11 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
10.9 mm
Mag
3127x
Pressure

10.0µm

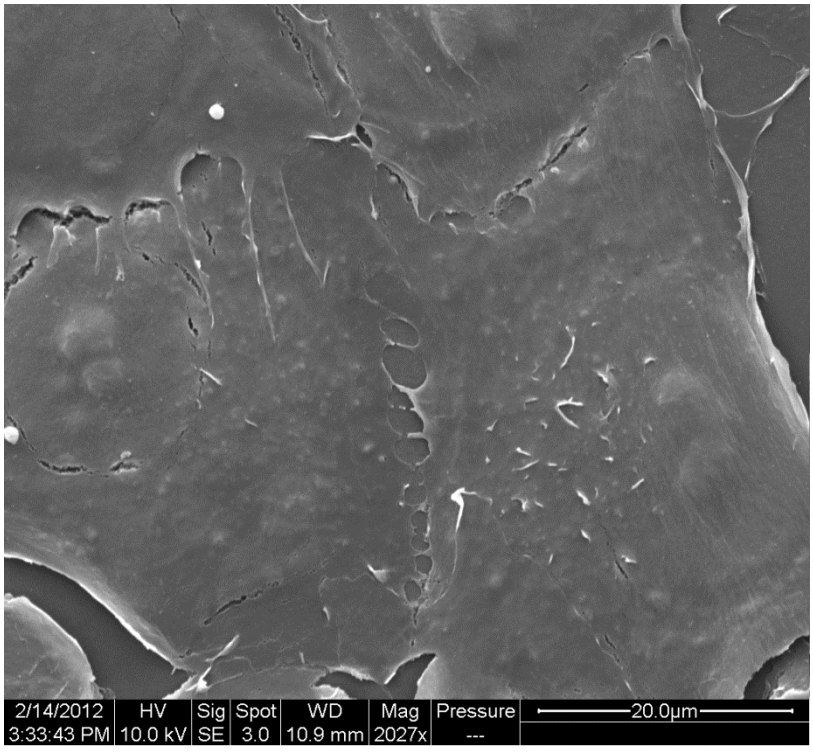

F
2/14/2012
3:20:58 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
10.9 mm
Mag
4055x
Pressure

10.0µm

Figure 18. Scanning electron micrograph image of an SK-N-AS E-line at high density. The E-line was plated at a density of 750,000 cells/well. (A) and (B) are two different angles showing different groups of cells. At high density, the E-line form a monolayer structure with a flattened cytoplasm. Some of the cells seemed to be multinucleated.


2/14/2012
3:30:05 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
10.9 mm
Mag
1434x
Pressure

20.0µm


2/14/2012
3:33:43 PM
HV
10.0 kV
Sig
SE
Spot
3.0
WD
10.9 mm
Mag
2027x
Pressure

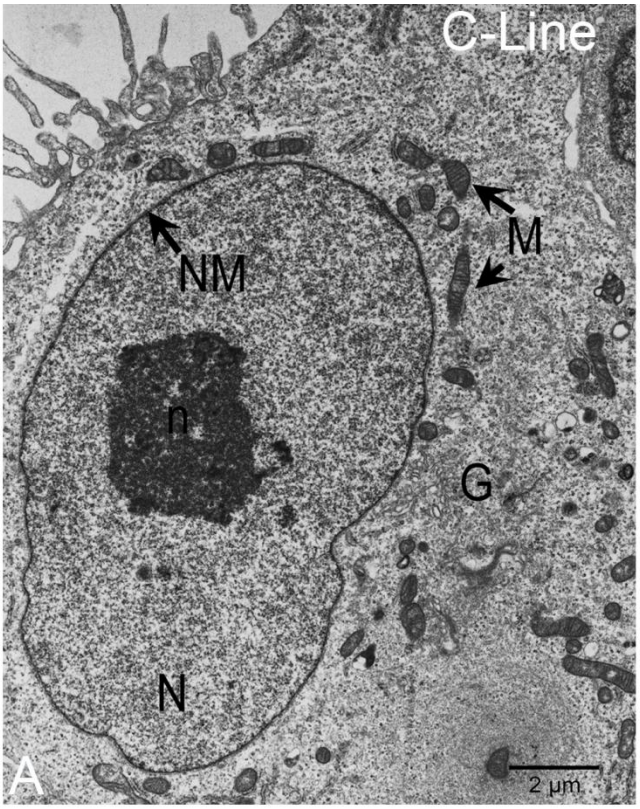
20.0µm

SK-N-AS C and E cell line Transmission Electron Microscopy

This type of microscopy use electrons as “light source” and its capacity to detect much lower wavelengths makes it possible to obtain resolutions thousand times higher than with a light microscope. Usually, transmission electron microscopy will give us information about the morphology and composition on samples.

The analysis of the SK-N-AS using transmission electron microscopy was intended to provide a comparative analysis of the morphological differences between the two cell lines. Based on literature we know that the C line expresses low levels of the transcription factor NURR1, whereas the E line is overexpressing it by two folds and as a consequence, other proteins such as DAT are being expressed. I wanted to look at them to see if there was any remarkable difference between both lines because to date there is very little information about how these cells look like under the TEM and SEM.

Figure 19.Low power TEM of SK-N-AS C and E comparison. Both cell lines, the SK-N-AS –C line (A) and the SK-N-AS E- line (B) share some similar organelles and such as the nucleus (N) which is delimited by the nuclear membrane (NM) and contains the nucleolus (n), a non-membrane bound structure composed of proteins and nucleic acids. The cytoplasm (C) contains the organelles. At this low magnification we can identify two organelles: mitochondria (M) which are considered centers of energy because its main function is to supply energy to the cells and the Golgi apparatus (G) that is involved in sorting and packaging of proteins. All cellular content is the same for both C and E except in that in the E-line (B) the presence of, what it seems to be, secondary lysosomes labeled with the letter (A).


C-Line
NM
M
n
G
N
A
2 µm

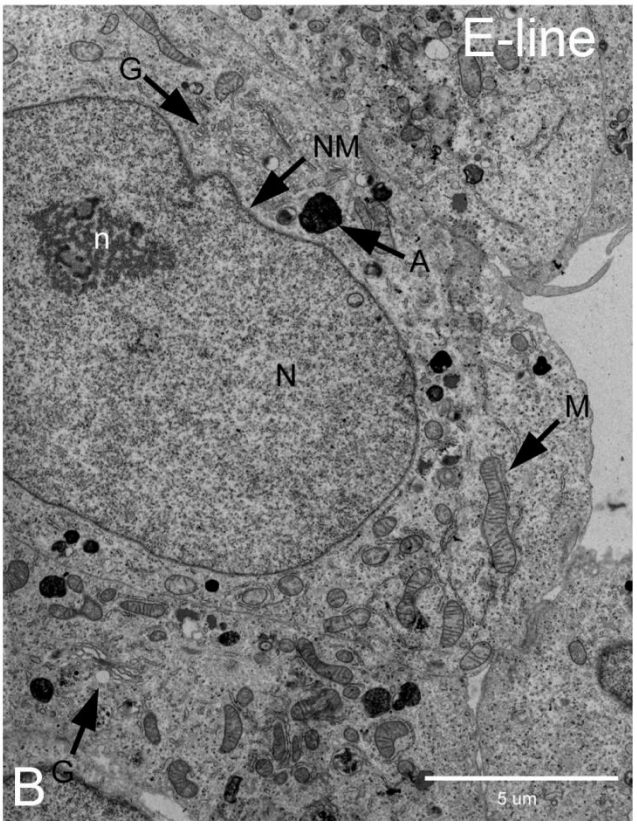
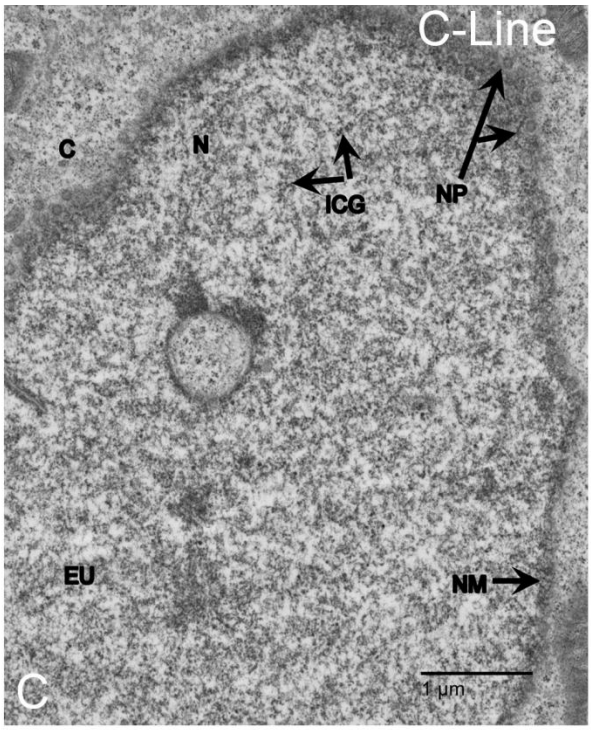

E-line
G
NM
n
A
N
M
B
G
5 um

Figure 20. Nucleus of the neuroblastoma SK-N-AS cell C and E-Line. The nucleus of both cell lines C and E-line (represented by C and D respectively) share the same characteristics and no difference has been found. The nucleus is one of the cell structures that vary in shape and size according to the type of cells. It is enclosed by the nuclear envelops (NM) which is a double membrane that fuses together at intervals to give rise to the nuclear pores (NP).The main function of the nuclear pore is to allow the movement of not only small molecules and ions but also of larger molecules such as proteins. The latter are carefully controlled given that they are required for both gene expression and chromosomal maintenance. The euchromatin (EU) comprises the most active portion of the genome within the cell nucleus. Another nuclear component is the interchromatic granules (ICG) which are a type of nuclear body that measures approximately 20-25 nm in diameter. These ICGs are distributed throughout the interchromatin spaces, linked together by thin fibrils. Notice the presence of dark areas (not labeled) around the nuclear membrane. These dark areas are only being present in the E line.


C-Line
C
N
ICG
NP
EU
NM
C
1 µm

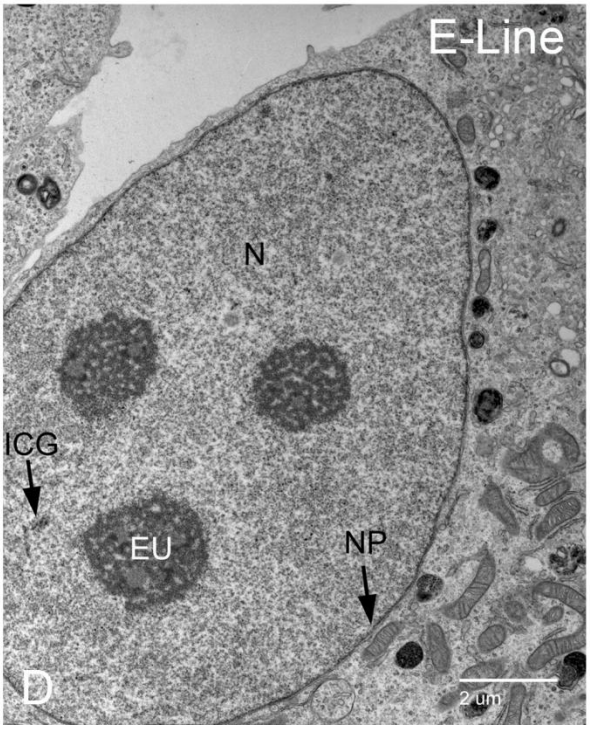
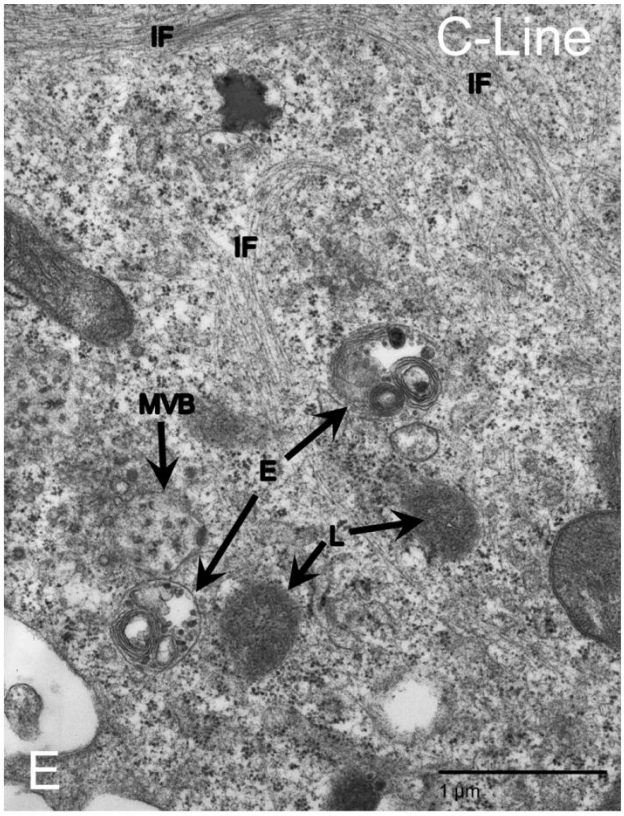

E-Line
N
ICG
EU
NP
D
2 µm

Figure 21. Comparison of cytoplasmic content between SK-N-AS C and E-line. Both cell lines, C and E line (represented by the E and F respectively) reveal the presence of intermediate filaments (IF), which are one of the cytoskeleton components. Other structures that form part of the cytoplasm are the endosomes (E) and multivesicular bodies (MVB). The latter are round to oval cell organelles that show usually multilamellar and multivesicular structures that contain acid phosphatases. Another cellular component that has an important role in the organization of intracellular membrane dynamics are the endosomes (E) which are formed by the invagination and pinching off of the cell membrane during the process of endocytosis. Lysosomes (L) are single layered organelles that are also involved in endocytosis. They vary in shape and size, which makes their identification difficult sometimes. Their main function is to serve as digestion compartments for cellular materials and cell debris in the process known as autophagy.


C-Line
IF
IF
IF
MVB
E
L
E
1 µm

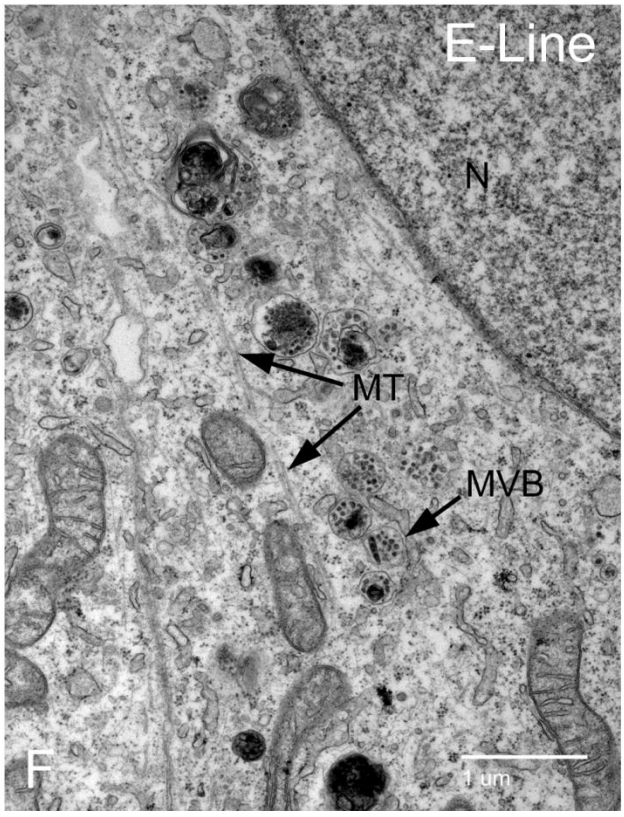
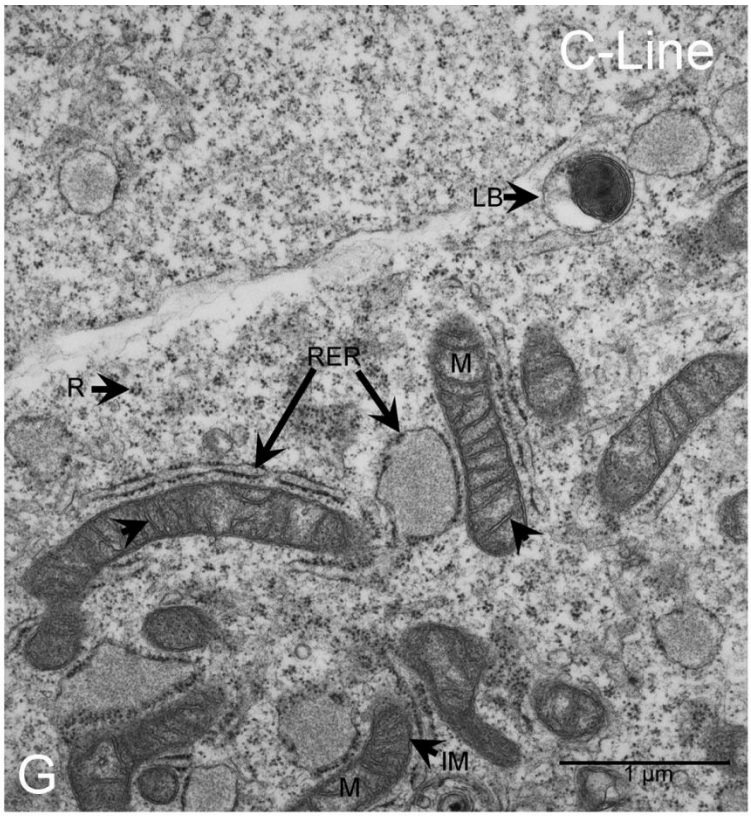

E-Line
N
MT
MVB
F
1 um

Figure 22. High magnification TEM of different organelles in both C and E-line. A comparison between organelles presence in both cell lines C and E line (G) and (H) respectively. One organelle that makes the difference between both lines is the presence of the secondary vesicles (arrows) A mitochondrion (M), The inner membrane (IM) folds inward and forms the cristae (arrow heads), which play important roles in the energy-chemical reactions of cellular respiration. Another component of the cells are the ribosomes (R) that are present in two forms: "membrane bound", in which they are bound to the surface of the rough endoplasmatic reticulum (RER) and "free" ribosomes which are located throughout the cytosol; in either case their function is protein biosynthesis. Another membrane –bounded organelle present is the lamellar body (LB) which is in charge of the storage and secretion of various substances (phospholipids, glycoprotein and acid phosphates). They can be arranged in several forms: tightly packed concentric, membrane sheets or lamellae. Other organelles present (G) Golgi apparatus in the E line (H).


C-Line
LB
R
RER
M
G
M
IM
1 µm

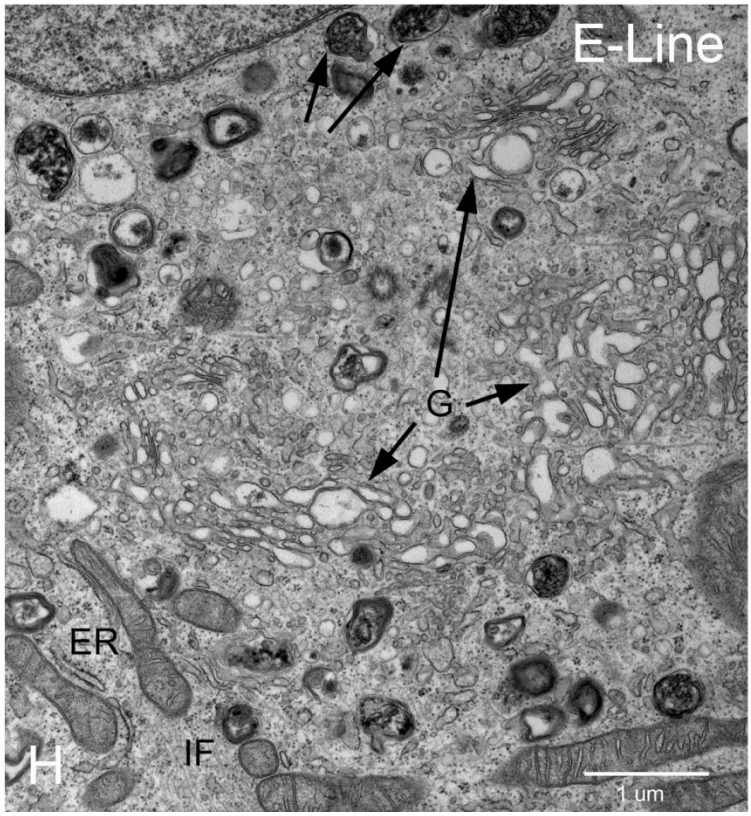
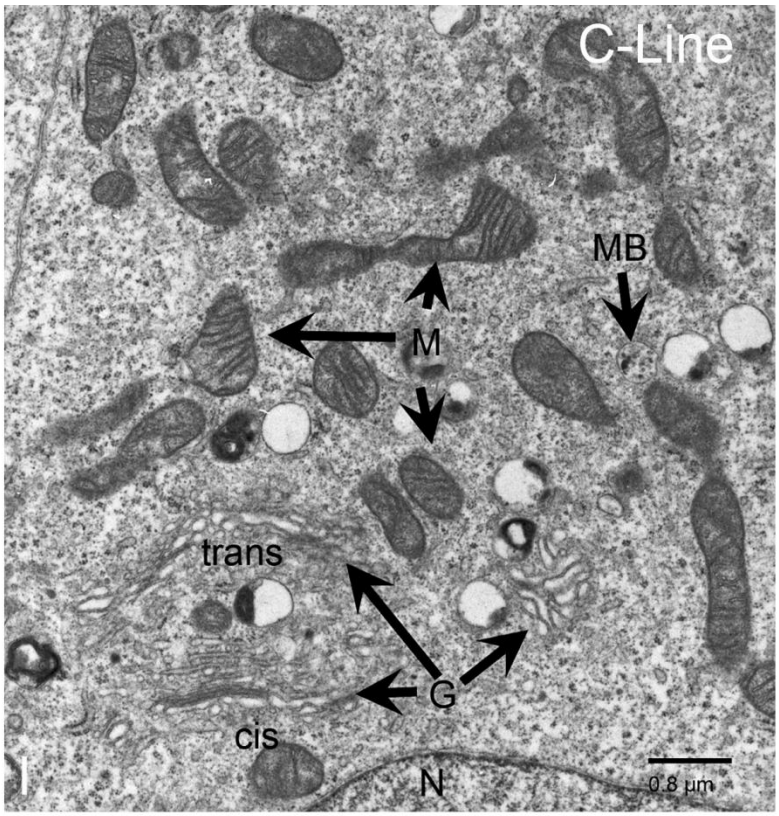

E-Line
G
ER
H
IF
1 um

Figure 23. Comparison in cytoplasmic content between C and E line. The Golgi apparatus (G) is a membranous system of cisternae and vesicles distributed throughout the cytoplasm or confined to a zone near the nucleus (N). The Golgi apparatus is a major metabolic center for the cell because it modifies proteins and lipids that have been built in the endoplasmic reticulum and prepares them for transport either to other locations in the cell or to the outside of the cell. One remarkable characteristic of this organelle is that it has two ends or faces: a forming "Cis" (cis) and a maturing "trans" (trans). The "Cis" face is at the end of the organelle where substances from the endoplasmatic reticulum enter for processing while the "Trans" face is where they exit in the form of detached vesicles. The latter is closer to the plasma membrane. Another membrane-bound body found in the cytoplasm are the Multivesicular bodies (MB). These organelles belong to the secondary lysosomes which contains a number of small vesicles that enclose acid phosphatase. Other organelles present are (M) mitochondria. Notice the amount of possible secondary lysosomes (arrow) in the E line but none in the C –line.


C-Line
MB
M
trans
G
cis
N
I
0.8 µm

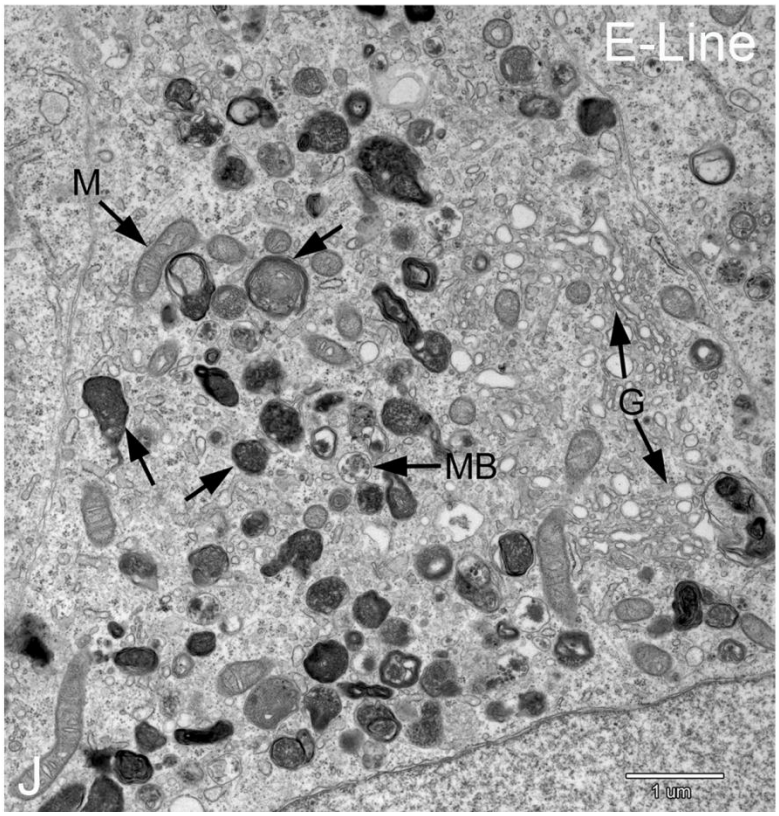
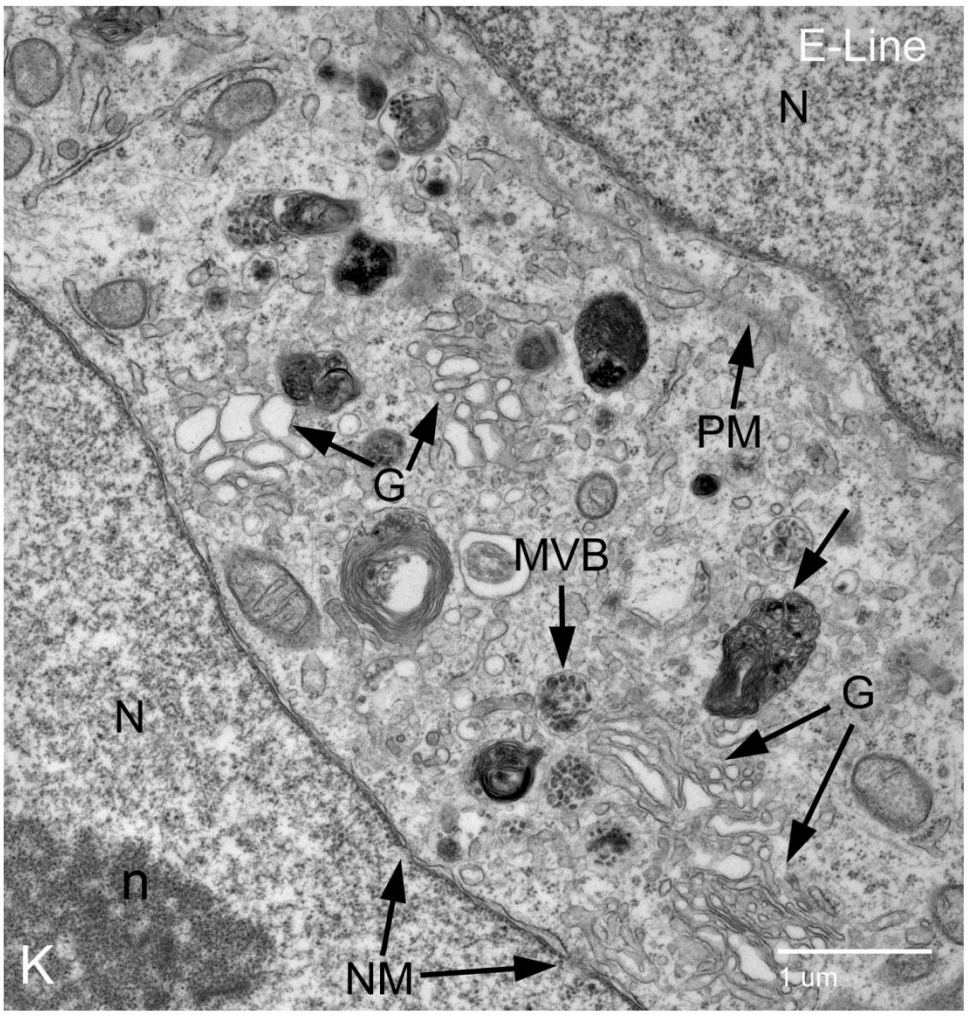

E-Line
M
G
MB
J
1 um

Figure 24. A high magnification of the cytoplasm in between two SK-N-AS E-cells. This figure show more clear components present in the E-line, such as Golgi Apparatus (G), multivesicular bodies (MVB). The dark organelles seems to be secondary lysosomes. Other organelles and structures presents are the nucleus (N), nucleoli (n), and nuclear membrane (NM).


E-Line
N
PM
G
MVB
G
N
n
K
NM
1 um

DISCUSSION

4.1 RT-PCR

The aim of this project was to study the interactions between NURR1 and PITX3 in the neuroblastoma cell line SK-N-AS. My objective was to unveil how these two transcription factors are interacting on the dopamine transporter (*DAT)* gene to upregulate its expression. To approach the problem, the cell line SK-N-AS was used given the recent studies showing its dopaminergic background. The first question to be determined was to see if the SK-N-AS cells were expressing both transcription factors. To approach this question an RNA extraction was required from the C and E line. Following RNA extraction the cells were treated to obtain a purified RNA. Reverse transcriptase protocol allowed the conversion from RNA into cDNA following an amplification of it by performing PCR. The amplified cDNA was used as template to run the gel electrophoresis.

The results of the gel confirmed that NURR1 is being expressed in both SK-N-AS C and E-line, confirming what other studies have suggested (Johnson, 2011). When comparing the difference in the C and E NURR1 band patterns, we noticed clearly that the E line has a very intense, well defined band suggesting that NURR1 expression is higher in the E line than in the C line. This result confirmed

prior studies that suggested that SK-N-AS C has low level of NURR1 whereas the E-line is overexpressing NURR1 by two fold (Johnson, 2011).

Similarly, we analyzed DAT gene expression and compared it in the C and E line. In this case, DAT expression was not present in the C line whereas in the E-line a well defined band was present. This result was very important because used in conjunction with the first data suggested that DAT expression is not only NURR1 concentration dependent but also that NURR1 presence is required for DAT expression. This confirmed previous studies that suggest that NURR1 is essential for the acquisition of a complete DA phenotype (Saucedo-Cardenas, 1998; Sacchetti, 2001). It contradicts suggestions that NURR1 alone is insufficient to drive the DA phenotype but requires PITX3 for full activation of target gene expression (Jacobs, 2009).

In order to analyze the expression of PITX3, we ran the gel expecting to see a band in the 155 bp. Against our expectation, PITX3 was not expressed in either the SK-N-AS C or E-line. Several attempts to improve the PCR protocol were ineffective. PITX3 was not expressed by SK-N-AS cell lines. Different studies have suggested that NURR1 and PITX3 interaction is fundamental for the expression of DAT (Martinat, 2006; Hwang, 2009; Smidt, 2009) but based on our data, DAT expression does not require the presence of PITX3 in the SK-N-AS cell line.

Up to date the mechanism that involves the acquisition of the DA phenotype is not known although main players have been discovered. There is a good amount of evidence that PITX3 and NURR1 are interacting to allow the DA neuron to convert into a mature DA neuron, but the question of how this interaction occur still remains.

4.2 Scanning electron microscopy and Transmission electron microscopy

Under the TEM microscope we were able to see differences between the C and the E line. Even though this difference wasn't localized at the nucleus level, there was a noticeable difference in the cytoplasmic content. More, of what it seems to be, secondary lysosomes presence was found. Secondary lysosomes are generally 0.2-3 micrometer in diameter and present a heterogeneous appearance under the electron microscope because of the wide variety of materials they may be digesting. Some of the material they digest are indigestible compounds and these are retained within the vacuoles, which change the name to residual bodies. In some neurons, large quantities of residual bodies accumulate and are called lipofucsin and the presence of these pigmented granules are considered one of the aging pigments found among others in nerve cells. Given the neuronal background of this cell it would be possible that the organelles present in the E line are actually secondary lysosomes and residual bodies.

Even though it is very premature to give conclusion about the presence of Golgi apparatus, at a naked eye, while observing all the samples under the TEM it seemed to me that the amount of Golgi found per view segment was higher in the E line than the C line. Cells have different amount of Golgi apparatus but I think it would be interesting to continue with a deeper analysis of cell content.

CONCLUSION

Different studies have shown that NURR1 and PITX3 interaction is fundamental for the development and maintenance of mDA neurons. The next question is how are these two transcription factors interacting? Up to date we only have models that explain possible interactions but no study has the final word yet. It is crucial to understand how these two transcription factors are interacting to drive the terminal differentiation of a DA neuron.

Recent studies have suggested that NURR1 over-expression up-regulates PITX3 in a dose dependent manner (Volpicelli, 2012) and that NURR1 has concentration- dependent effects on different target genes (Johnson, 2011). If time would have allowed me to continue working on this project, I would have liked to make a comparison of gene expression of the second clonal SK-N-AS cell which is overexpressing NURR1 by eight fold to see if PITX3 is being expressed due to an over-expression of NURR1 or if it is not. Based on the data collected during this project it seems that the SK-N-AS cells are not an ideal model system to study the molecular mechanism underlying the dopaminergic phenotype because they are not expressing critical signals such as PITX3 and TH (data not shown).

Nonetheless, this cell line seems to be a good model in which to study molecular mechanism that involved NURR1 target genes that are dependent on concentration.

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