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**Essay: Induced pluripotent stem cells as a source for dopaminergic neurons to treat and understand Parkinson’s disease.**

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**Summary**

Parkinson’s disease (PD) is the most common neurodegenerative disease in elderly. It is characterized by the degeneration of the midbrain dopaminergic neurons in the substantia nigra leading to a decrease in dopamine production. For that reason, several treatments have been tested to improve dopamine production without offering a final solution in long-term for PD patients. Moreover, other approaches using cell implantation of fetal midbrain tissue into the brain of PD patients left unanswered questions considering the variability of conditions within each study. Since using fetal and embryonic cells involve issues as tissue availability and ethical bases, other alternatives sources as induced pluripotent stem cells (iPSC) have been proposed. Further, iPSC from mice and human sources have been tested in parkisonian animal models showing fair integration into the brain and improvement of motor behavior by using different protocols. In order to improve the efficiency of mDA neurons yield is important to underlie its differentiation process and mature phenotype to generate a proper protocol and to extend it to the clinic in a risk-safe way. In this review the main protocols used to generate iPS-mDA neurons to treat PD will be addressed.

**Introduction**

Parkinson’s disease (PD) is a neurodegenerative disorder that is characterized by symptoms of motoric impairment as bradykinesia, resting tremor and rigidity, as well as cognitive deficits, in particular in the later stages of the disease (Chaudhuri 2011; Playfer 2008; Ebadi 2005). Importantly, PD is one of the most common neurodegenerative diseases with a prevalence of 200 per 100 000 individuals worldwide; it forms a significant, economic burden on the society (Chaudhuri2011). The cause of PD has not yet been found, however, it is thought that a combination of genetic factors, environmental factors and pathogenic mechanisms (e.g. abnormal protein processing, oxidative stress, mitochondrial dysfunction and apoptosis, among others) contribute to the development of PD (Playfer 2008; Ebadi 2005).

In this complex disease, the main pathological hallmark is the degeneration of midbrain dopaminergic (DA) neurons in the substantia nigra causing a decrease of dopamine levels in particular in the striatum since the nigrostriatal connections are the most prominent dopaminergic connections of the substantia nigra. (Playfer 2008; Ebadi 2005). Of note, dopamine is not the only neurotransmitter affected in PD (Ebadi 2005).

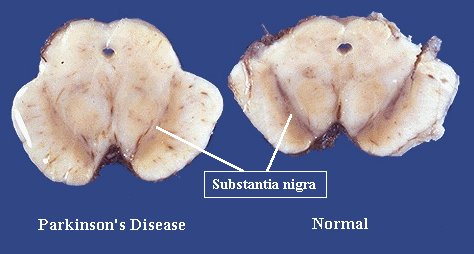


Figure 1: Postmortem sections of a normal and a parkinsonian midbrain showing the characteristic pallor due to the loss of dopaminergic (DA) neurons in the substantia nigra.

In the midbrain (or mesencephalon), dopaminergic neurons can be divided into the ventral tegmental area (VTA) neurons and the substantia nigra pars compacta (SNc) neurons; the SNc neurons are the ones predominantly affected in PD (Ebadi 2005). In post mortem brains of PD patients, loss of the DA neurons with their typical neuromelanin pigment can be observed macroscopically; the neuronal loss in combination with Lewy body formation can be studied in detail microscopically (Figure 1) (Chaudhuri 2011; Playfer 2008; Ebadi 2005). Administration of a precursor of dopamine (L-DOPA) has been found to be beneficial in the early years of the disease and has become the current treatment of PD. L-DOPA is the immediate precursor to dopamine which can enter the brain by crossing the blood brain barrier and can be decarboxylated by the aromatic L-amino acid decarboxylase (AADC) enzyme producing dopamine helping to restore the low levels of dopamine (Chaudhuri 2011; Playfer 2008; Ebadi 2005). The advantage of using L-DOPA to convert it into dopamine relies on the independent activity of AADC, since this enzyme is produced by non-dopaminergic cells as well, therefore it is not completely affected in PD. Long term use of L-DOPA may result in lower efficacy and may cause dyskinesia as a side effect (Chaudhuri 2011; Playfer 2008; Ebadi 2005). As an alternative approach to restore dopamine levels in the striatum of PD patients PD patients received intrastriatal cell implantations of dopamine-producing neurons from different sources that also produce other sustaining factors that promote survival of native DA neurons (Ebadi 2005).

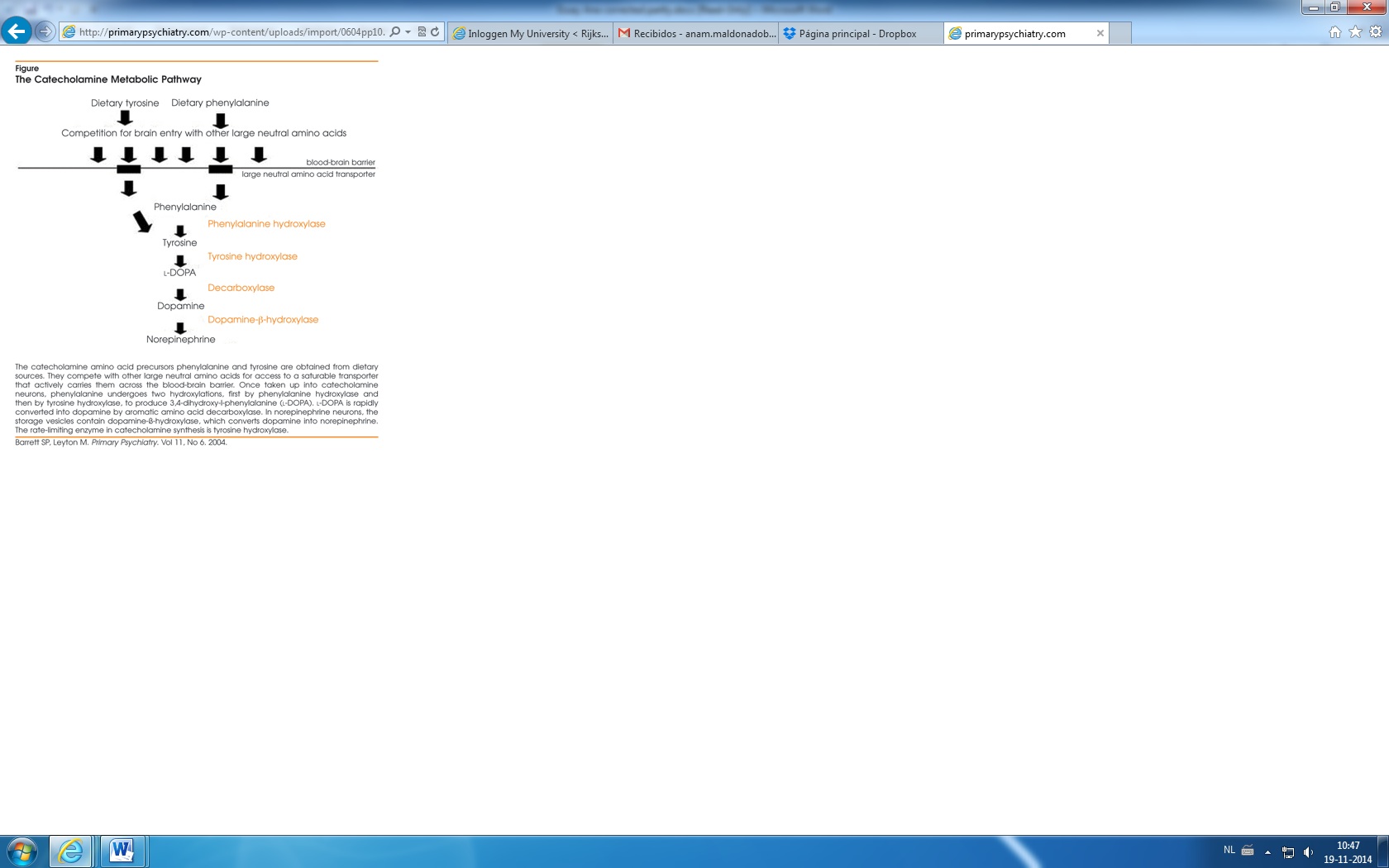


Figure 2: The amino acids are obtained from diet and are used as precursors for neurotransmitters. Phenylananine and tyrosine compete to cross the blood-brain barrier. Once in the neurons, phenylalanine is twice hydroxylated, first by the Phenylalanine hydroxylase and then by Tyrosine hydroxylase (TH). Later it is converted into dopamine by aromatic amino acid decarboxylase (AADC). The drug L-DOPA can enter through the blood-brain barrier in order to be converted in dopamine.

There have been several experiments with cell implantation into the brain of PD patients; the first one was in 1987 by implanting fetal mesencephalic tissue into two patients resulting in modest improvement which disappeared after a year in one of them (Lindvall *et al* 1989). Later on, more than 300 patients received similar transplantations of human fetal abortion-derived DA neurons under different conditions and trial designs, having considerable variations in the outcome (Clarkson *et al* 2001). Clarkson generated a database with some of these experiments, leaving aside the ones that did not stipulate L-DOPA doses. This author mentions that the clinical improvement in various implantation studies was measured with different parameters, making it difficult to compare their results (Clarkson *et al* 2001). In general, the intrastriatal implantations resulted in a temporary improvement from 6 to 24 months after administration in most of the patients leading to a reduction of the L-DOPA dose (Clarkson *et al* 2001).

Freed et al. compared the outcome of fetal DA neuronal implantation in a young group of Parkinson patients (60 years old or younger) with that in an older group of Parkinson patients (older than 60 years old) (Freed *et al* 2001). They found a significant improvement only in the younger group still with dystonia and dyskinesias appearing after a year in 15 percent of the patients. (Freed *et al* 2001). Aside from this, the implanted neurons survived from a period of 3 to 4 years having beneficial outcomes in the rest of the younger patients (Freed *et al* 2001). More recently, the research group of Mendez et al. observed the successful survival of human fetal cell suspensions containing DA neurons when implanted in two PD patients. These patients had a favorable clinical evolution without developing motor complications which was confirmed by postmortem brain analysis; yet the low number of patients makes it a difficult to draw general conclusions from this study (Mendez *et al* 2005).

Due to practical and ethical concerns the clinical use of fetal, abortion-derived, DA neurons was no longer feasible (Clarkson *et al* 2001). New alternative sources have emerged: embryonic stem cells (ESC) and induced pluripotent cells (iPSC).

ESCs can be obtained from human blastocysts and offer the possibility of combining cell and gene therapy (Ebadi 2005). There have been various studies in which human ESCs (hESCs) were differentiated into midbrain DA neurons and transplanted in 6-hydroxydopamine (OHDA)-lesioned rats resulting in improved motor behavior (Kim *et al* 2002; Bjorklund *et al* 2002; Kriks *et al* 2011). In two of them, the authors point out that the dopamine replacement was beneficial itself, regardless of the complete differentiation of ESC into DA neurons and their integration (Kim *et al* 2002; Bjorklund *et al* 2002). However, in the study of Bjorklund *et al* the use of undifferentiated ESCs caused teratoma-like formation at the implantation site of 5 out of 25 rats, being an important adverse effect that cannot be left ignored (Bjorklund *et al* 2002). On the other hand, Kriks and colleagues obtained excellent survival, function and normal growth of implanted hESC derived DA neurons circumventing the earlier problems by using a floor-plate-based strategy with specification factors for DA neurons differentiation in three different species (Kriks *et al* 2011).

Human iPSCs lack the ethical concerns of hESCs. They can be generated from human fibroblasts using Oct 3/4, Sox2, Klf4, c-Myc (Yamanaka factors) as reprogramming factors to produce an embryonic stem cell-like state through viral transfection (Yamanaka et al 2006; Yamanaka et al 2007; Takahashi et al 2007). In these models, c-Myc and Klf4 work by modifying chromatin structure to facilitate Oct 3/4 and Sox2 activation, changing cell fate and establishing pluripotency through activation or suppression of other target genes, such as Fbx15 and Nanog (Yamanaka 2007). The cells created with this protocol are similar to human embryonic stem (hESC) cells regarding morphology, expression of surface antigens, genetic and epigenetic status, their ability to differentiate into the three germ layers as can be seen in the embryoid bodies they can form and the teratomas (Yamanaka *et al* 2007).

Nowadays iPSCs represent an interesting autologous source for the generation of DA neurons for cell replacement in PD patients avoiding graft rejection and immunosuppressive treatments. iPSC-derived DA neurons can be also useful for disease modeling studying the familiar forms of PD, preserving the genetic modifications that contribute to the disease and allowing the development of more specific treatments.

The aim of this review is to present an overview of the main protocols and methods currently used to generate DA neurons from human iPSC in order to find a general outline that might be helpful in future studies.

**Midbrain DA neuronal development**

In mammals, in the embryonic development of the midbrain two main clusters of DA neurons arise from the mesencephalic progenitors or precursors; SNc or VTA as mentioned earlier (Abeliovich *et al* 2007). The progenitors lay in the periventricular surface of a region called the floor-plate within the midbrain and they generate SNc mDA neurons in a series of proposed stages (Abeliovich *et al* 2007):

Firstly, the floor-plate organizer activates transcription-regulators initiating signaling pathways that instruct the neural stem cells to give rise to multiple cell types (Fig. 3a) (Denham et al 2012; Abeliovich *et al* 2007). Activation of the transcription factors Otx2 and Gbx2 start separating the midbrain from the hindbrain in humans and different species (Gat-Yablonski, 2011; Prakash et al 2004; Liu and Joyner, 2001; Joyner *et al* 2000). Also several downstream factors work as effectors to form the midbrain-hindbrain junction (MHJ), a neuroepithelial signalling center, and start neural specification. These include the homeobox transcription factors engrailed (En)-1 and -2, the Lim-domain transcription factor b (Lmx1b), winged helix/forkhead box A2:HNF3beta (Foxa2), and the paired-like homeodomain proteins Pax 2 and 5 (Fig. 3a) as shown in human embryonic stem cells (hESC)-derived DA progenitors (Yan et al 2005; Fasano et al 2010; Lee *et al* 2010; Vazin et al 2008).

Both, En1 and 2 work as signaling molecules in midbrain DA (mDA) neurons in human and are critical for their development and survival, being comprised to the neuroepithelium that later will give rise to the midbrain in mice (Yan et al 2005; Fasano et al 2010; Alves de Santos *et al* 2011; Simon *et al* 2001). En1 is also present in coexpression with Lmx1b hESC-derived mDA neurons (Fasano et al 2010; Chung et al 2011). Lmx1b is a progenitor marker required for the maintenance of a mature state of mDA neurons and is expressed since the development of the progenitors until the generation of fully mature mDA neurons (Smidt *et al* 2000).

Another important factor is Foxa2 which is expressed early in the floor-plate and basal plate around week 6 in human embryos induced by Sonic Hedgehog (SHH) soluble factor (Nelander et al 2009; Fasano et al 2010). It has been observed that its expression is necessary for midbrain-specific developmental genes and mDA neuron generation in mice, although its function is not fully understood in humans (Sasaki *et al* 1994; Lee *et al* 2010; Ferri et al 2007; Kittappa et al 2007). Similarly, Pax proteins are expressed in initial stages in mDA progenitors and are essential for normal development of mDA neurons (Favor et al 1996; Chalepakis et al 1993).

In addition, secreted proteins (soluble factors) as SHH, Fibroblast growth factor 8 (FGF8) and Wnt which are produced in the isthmic organizer, play important and vital roles in the development of mDA neurons in humans (Denham et al 2012; Fasano et al 2010; Yan et al 2005; review Verney et al 2011). SHH also induces a transcription cascade by encoding the action of the floor-plate (Fasano et al 2010). In a similar manner, FGF8 is essential for the early pattering of the midbrain and is necessary for the development of mDA neurons (Yan et al 2005). Finally, the Wnt family is crucial during human embryonic development since it regulates midbrain morphogenesis and mDA neurons differentiation (Hebsgaard et al 2009; Alavian *et al* 2011; Chung *et al* 2009; Ciani *et al* 2005).

In the second stage, dopaminergic precursors are generated after week 6 expressing additional transcriptional factors as Lim-domain transcription factor a (Lmx1a), Msh homeobox 1 (Msx1) and Neurogenin 2 (Ngn2) activated by SHH (Fig. 3b) (Nelander et al 2009). Lmx1a has been identified as a marker of dividing midbrain precursors which is necessary to trigger mDA neurons differentiation by activating downstream factors as Ngn2 proneural factor in postmitotic precursors in humans and mice (Nelander et al 2009; Andersson *et al* 2006a; Andersson *et al* 2006b). Additionally, the expression of Lmx1a induces Msx1 activity which also enhances neurogenesis (Andersson *et al* 2006b).

In the third stage, mDA human precursors escape the cell cycle around week 7 and start displaying early mDA markers as for instance the orphan nuclear receptor/transcription factor Nurr1 (Fig. 3c) (Nelander et al 2009). Importantly, this factor is necessary for mDA maturation and specification to induce TH expression in other species (Wallen *et al* 1999; Smiths *et al* 2003; Lee *et al* 2010).

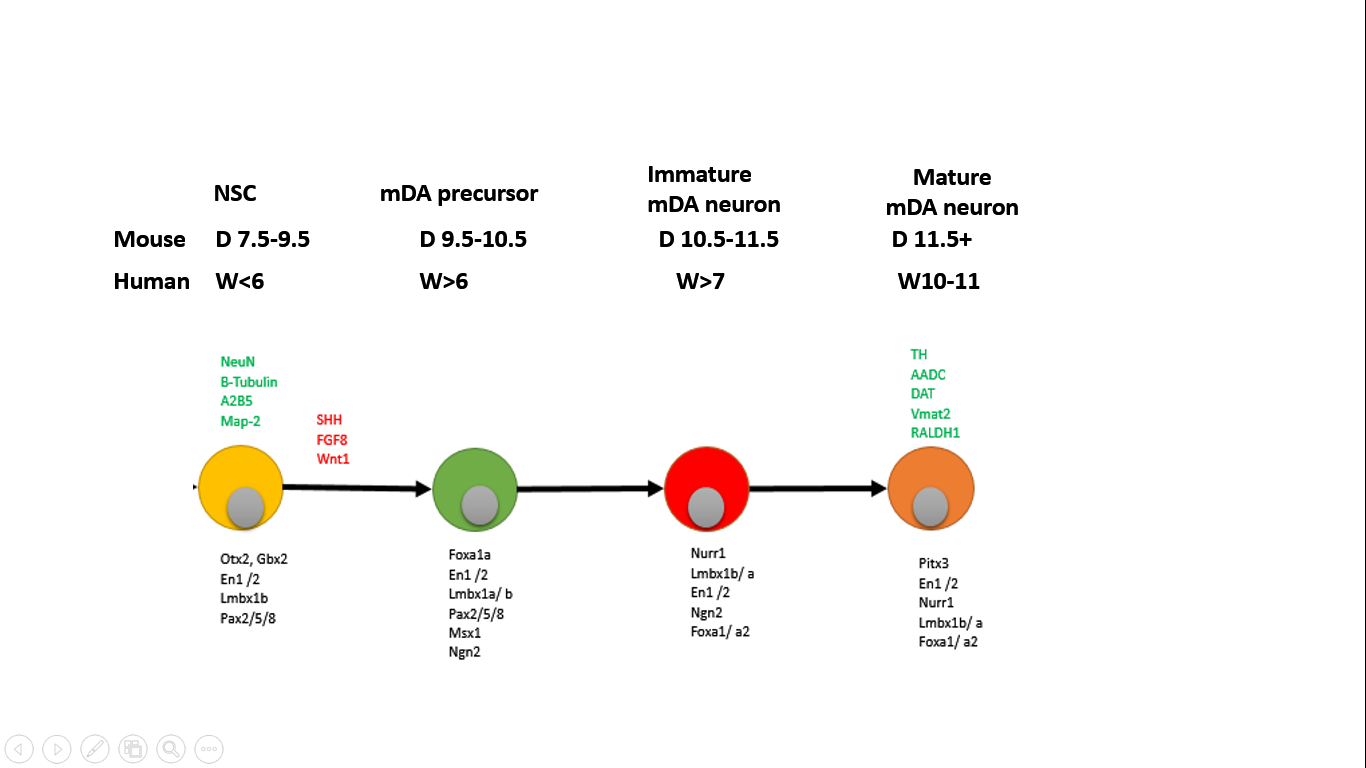


Figure 3: Proposed stages of the mDA development: a) neural stem cells (NSC) are generated from embryonic stem cells (ESC), expressing regulatory transcription factors (black) and presenting specific markers (green). b) By the influence of proteins (red) secreted at specific stages in the embryonic development, NSCs give rise to mDA precursors expressing additional transcription factors such as Fox1a and Msx1. c) Immature mDA neurons are generated expressing also Nurr1 onwards. d) Mature mDA neurons are generated and express Pixt3 transcription factor and present specific markers related with dopamine metabolism (TH, AADC, DAT, Vmat2 and RALDH1).

Finally, immature mDA become mature mDA neurons around weeks 10 to 11 presenting the characteristic phenotypical markers of enzymes as TH in the human embryo and retinaldehyde dehydrogenase isoform-1 (RADHL1), AADC and dopamine transporter (DAT) as well in hESC-derived mDA neurons, being specifically related to mDA populations (Fig. 3d) (Nelander et al 2009; Ang *et al* 2006; Wallen 1999; Smits *et al* 2003; Chung et al 2011; Roybon et al 2008). Another marker that is expressed upon maturation is Pitx3, its activity has been linked with TH expression in mDA SNc neurons (Nelander et al 2009). Afterwards, neurons take up their position in the future substantia nigra and start extending its axons (Freeman et al 1991; review Verney et al 2011).

In general, it has been reported that TH+ mDA neurons in human embryo development start appearing in the ventral mesencephalon around 6.5 weeks, migrating at 6.7 weeks and forming the first TH+ neurites at 9.0 weeks (Nelander et al 2009; Almqvist et al 1996; Freeman et al 1991; Verney et al 2001).

Better understanding of the factors involved in the human mDA development will help to generate functional human iPSC-derived DA-neurons mimicking the same human embryonic stages and induction factors in the IPSC differentiation protocols.

**Differentiating human pluripotent cells into DA neurons**

In earlier years, hESC have been used in experimental studies to develop mDA neurons (Chambers *et al* 2009; Chung *et al* 2011; Roybon *et al* 2008; Kriks *et al* 2011). In their differentiation protocols they have used different markers to identify the different intermediate cell stages from neural progenitor towards mature differentiated mDA neurons. For instance, PAX6, Otx2 and Corin (a floor plate marker) markers have been used to identify neural progenitor populations, and the coexpression of Foxa2, Lmbx1b to identify their dopaminergic fate (Chambers *et al* 2009; Chung et al 2011). Additionally, to identify mature mDA neurons, markers as TH, Pitx3 and Nurr1 were used (Chambers *et al* 2009; Chung et al 2011; Roybon *et al* 2008). It was observed that over-expressing Lmx1a in human neural progenitor cell lines, could enhance the TH+ neuron yield, an established marker of mDA neurons (Roybon *et al* 2008).

**Protocols to differentiate human iPSCs into mature midbrain DA neurons**

Likewise, there are several studies nowadays in which human iPSCs (hiPSC) were successfully differentiated into midbrain DA neurons (Table 1). However there is a need for consensus and standardization of the procedure to differentiate hiPSC into DA neurons including standard protocols for the accurate and detailed characterization of the hIPSC-derived DA neurons

In the early days of iPSC generation, viral transfection was used to induce the expression of the Yamanaka reprogramming factors representing a high risk of mutations and tumorigenesis. In the meantime, new methods have been developed (review see Roessler *et al* 2013; Soldner *et al* 2009). These new approaches made use of direct transfection of episomal vectors plasmids with the “Yamanaka factors”, also by using microRNAs, Cre-recombinase excisable viruses, direct protein delivery or synthesized mRNA to induce pluripotency (review Roessler *et al* 2013; Okita *et al* 2011; Soldner *et al* 2009; Rhee *et al* 2011). Nevertheless, these methods have low reprogramming efficiency and are technically demanding (Okita *et al* 2011).

Furthermore, a specific phenotype to define human mDA neurons is still needed. Importantly, FOXA2 and LMX1A have been pointed as robust markers for mDA, as observed in human ESC-derived mDA neurons (Kriks *et al* 2011; Cooper *et al* 2010; Doi *et al* 2014; Chung et al 2011; Roybon et al 2008). Likewise, L-type calcium channels and potassium channels type 2 (GIRK2) that provide pacemaker activity can also be pointed as specific markers of differentiated mDA neurons (Hartfield *et al* 2014; Cooper *et al* 2010). Last but not least, the expression of functional enzymes of mDA neurons, for instance, TH enzyme expression, and the neuron specific III-B-tubulin (TUJ) marker have been commonly used (Chambers *et al* 2009; Cooper *et al* 2010; Hartfield *et al* 2014; Kim *et al* 2011; Okita *et al* 2011; Rhee *et al* 2011; Soldner *et al* 2009; Wernig *et al* 2008). It is crucial to understand how the mDA markers change depending on the stage of maturation since it might be helpful to select them when generated from iPSC to reduce risk of tumorigenesis and even increasing the yield of mDA neurons.

In this review I will address a general outline of the methods and applications of human iPSC-derived midbrain DA neurons in the past years. They are, in general, protocols which are commonly used to generate mDA neurons, however there is still the need to fully characterize its yield (Table 1) (Yamanaka *et al* 2006; Roessler *et al* 2013).

Table 1: Recent studies using human induced pluripotent stem cells from fibroblasts to generate mDA neurons (abbreviations; EP: Electrophysiological).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Author | Reprogramming method | Protocol and characterization | Efficiency of hiPSC into mDA neurons | Markers |
| Wernig et al 2008 | Lentivirus | hiPS control cell lines (N8, N10, N14, O9 and O18) were differentiated using Okabe et al protocol and engrafted in 6OHDA rats after 8 weeks of maturation.  Characterization was made using mDA markers, EP properties, functional integration and DA release (data not shown). | Not mentioned. | VMAT2, PITX, TH, EN1, TUJ, PITX3, NURR1 |
| Chambers et al 2009 | Lentivirus | hiPS control cell lines (iPSc14 and iPSc27) were differentiated by dual SMAD signaling with Noggin and SB4431542 for 5 instead of 11 days, considering fully differentiated at day 19.  Characterization was made using mDA markers and gene expression without measuring DA release. | Not mentioned. However the neural induction was 82%. | TH, TUJ |
| Soldner et al 2009 | Lentivirus, later Cre-excised | hiPSC derived from somatic cells of patients with idiopathic PD and control patients were differentiated with either growth factors+SHH or MS5+Noggin- based  protocols as used by Perrier and Sonntag groups.    Characterization was made using mDA markers and genetic stability without measuring DA release. | Efficiency was measured using TH+/TUJ1+ cells (10%) | TH, TUJ1 |
| Hargus et al 2010 | Lentivirus, later Cre-excised | hiPSC of PD patients and control patients were differentiated and engrafted in 6OHDA rats after day 42 by using the stromal feeder cell-based protocol (Perrier et al. 2004; Sonntag et al 2007).  Characterization was made using mDA markers using NCAM as an isolating marker and functional integration without measuring DA release. | Efficiency of over 5000 TH+ neurons in grafts. | TH, GIRK2 |
| Cooper et al 2010 | Lentivirus, later Cre-excised | hiPSC from parental cell lunes derived from sporadic PD patients and control patients were differentiated by using Sonntag et al protocol until maturation at day 49.  Characterization was made using mDA markers, gene expression without measuring dopamine release. | Efficiency of 5.16 % ±2.6%. | TH, GIRK2, TUJ, FOXA2, PITX3, NURR1, EN1, AADC, DAT |
| Kriks et al 2011 | Sendai virus | hiPSC cell lines (2C6 and SeV6) and controls were engrafted in 6OHDA mice, rats and parkisonian monkeys after day 25 by using a modified dual SMAD inhibition floor plate -based mDA induction.  Characterization was made using gene expression profile, EP properties, functional integration and DA release (HPLC analysis). | Efficiency was measured using TH+ (80%), FOXA2 (80%), LMX1A (60%) and NURR1+ (50%) cells from total. | OTX2, FOXA2, NGN2, TH, NURR1, PITX3, DAT, GIRK2, NCAM |
| Okita et al 2011 | Episomal plasmid vectors | hiPSC derived from control patients were differentiated by using a SFEB method with dual SMAD inhibition protocol until maturation at day 29.    Characterization was made using mDA markers and gene expression without measuring dopamine production. | Not mentioned. | TUJ,  TH,  PAX6,  VMAT2 |
| Rhee et al 2011 | Direct protein delivery | hiPSC cell lines (IMR90-1 -4, Foreskin-1, SES8, Rv-hiPS 01-1, 02-3, piPSC-1, -2) were differentiated using Perrier et al protocol until maturation around weeks 7 to 11.  Characterization was made using mDA markers, EP properties, DA release (HPLC analysis) and functional integration. | Efficiency was measured using Tuj1+/TH+ cells (35-45% of total cells) from which >90% were mature DA neurons. | TUJ, TH, LMX1A, LMX1B, EN1, NURR1, GIRK2, NCAM, MAP2 |
| Kikuchi et al 2011 | Viral transfection | hiPSC cell lines (253G4) were differentiated and engrafted in PD monkey and NOD-SCID mice models using neuralbasal medium + growth factors protocol until maturation at day 28 and 42.  Characterization was made using mDA markers using NCAM as an isolating marker, RNA levels, DA release (HPLC analysis) and functional integration. | Efficiency was measured using Tuj1+/TH+ cells (3.14% ± 1.38% at day 28 and 85.46% ± 3.13% at day 42). | TUJ  TH  PAX6  NURR1  PITX3  GRIK2  VMAT2  DAT |
| Sanchez-Danes et al 2012 | Retroviral delivery | hiPSC derived from idiopathic PD, familial PD and age and sex matched controls were differentiated using a transduction with LMX1A and mDA pattering factors as protocol until maturation at days 30-75.  Characterization was made using mDA markers and gene/protein expression without measuring dopamine production. | Efficiency was measured using TH/TUJ1+ cells (9-29% independently of the presence or type of disease). | TUJ  TH  DAT  GIRK2  FOXA2 |
| Sundberg et al 2013 | Lentivirus | hiPSC cell lines (2135 and 1815) were differentiated and engrafted 6OHDA rats after day 30 using Kriks et al protocol and modifications of the same.  Characterization was made using mDA markers using NCAM and CD29 as isolating markers, EP properties and functional integration without measuring DA release. | Efficiency was measured using FOXA2+ cells (>50-70%), TH+ cells (>40-50%) and TH+/FOXA2+ (>40%) of total cells. | FOXA2, TH, LMX1A, GIRK2, EN1, PITX3, NURR1 |
| Badja et al 2014 | Lentivirus | hiPSC cell lines (not specified) were differentiated using medium based (mTeSR and neural induction medium) protocol until maturation in 20-40 days.  Characterization was made using mDA markers and EP properties without measuring DA release. | Efficiency was measured using TH+ cells (80%). | TH |
| Doi et al 2014 | Episomal plasmid vectors | hiPSC cell lines (836B3 and 404C2) were differentiated and engrafted in 6OHDA rats after day 42 using modified dual SMAD inhibition and floor plate induction protocols.  Characterization was made using mDA markers using CORIN as an isolating marker, gene expression profile, DA release (HPLC analysis), EP properties and functional integration. | Efficiency was measured using LMX1A+/FOXA2+/CORIN+ cells (75.5% ±8.2%). | FOXA2  LMX1A |
| Hartfield et al 2014 | Reprogramming plasmids | hiPSC cell lines (NHDF1 and 2) were differentiated using a medium based (mTeSR and neural induction medium) and dual SMAD inhibition protocol until maturation around 4 to 10 weeks.  Characterization was made using mDA markers, gene expression, karyotype analysis, DA release (HPLC analysis) and EP properties. | Efficiency was measured using TUJ1+ cells (39.5% ±3.5% and 41.7% ±2% from each cell line). | TH, GIRK2, TUJ, FOX2, PITX3, NURR1, EN1, AADC, DAT |

On the reviewed papers, two main protocols are followed with own adaptations by other research groups to generate hiPSC-derived mDA neurons. Firstly, the dual SMAD inhibition protocol used by Chambers and colleagues and secondly, the floor-plate based (FB) protocol used by Kriks group.

On the dual SMAD inhibition protocol, Noggin and SB431542 were used to destabilize Nanog and to inhibit activin/TGFB pathways on the first stage of differentiation towards mDA neurons for 5 days in culture. Later, after day 5, morphogenic factors as SHH and FGF8a were used in N2 media, being necessary to give neuronal identity inducing expression of FOXA2. Finally, to support maturation of mDA neurons also BDNF, ascorbic acid, TGFb1, cAMP and GDNF were used after culture day 9 (Fig. 4).

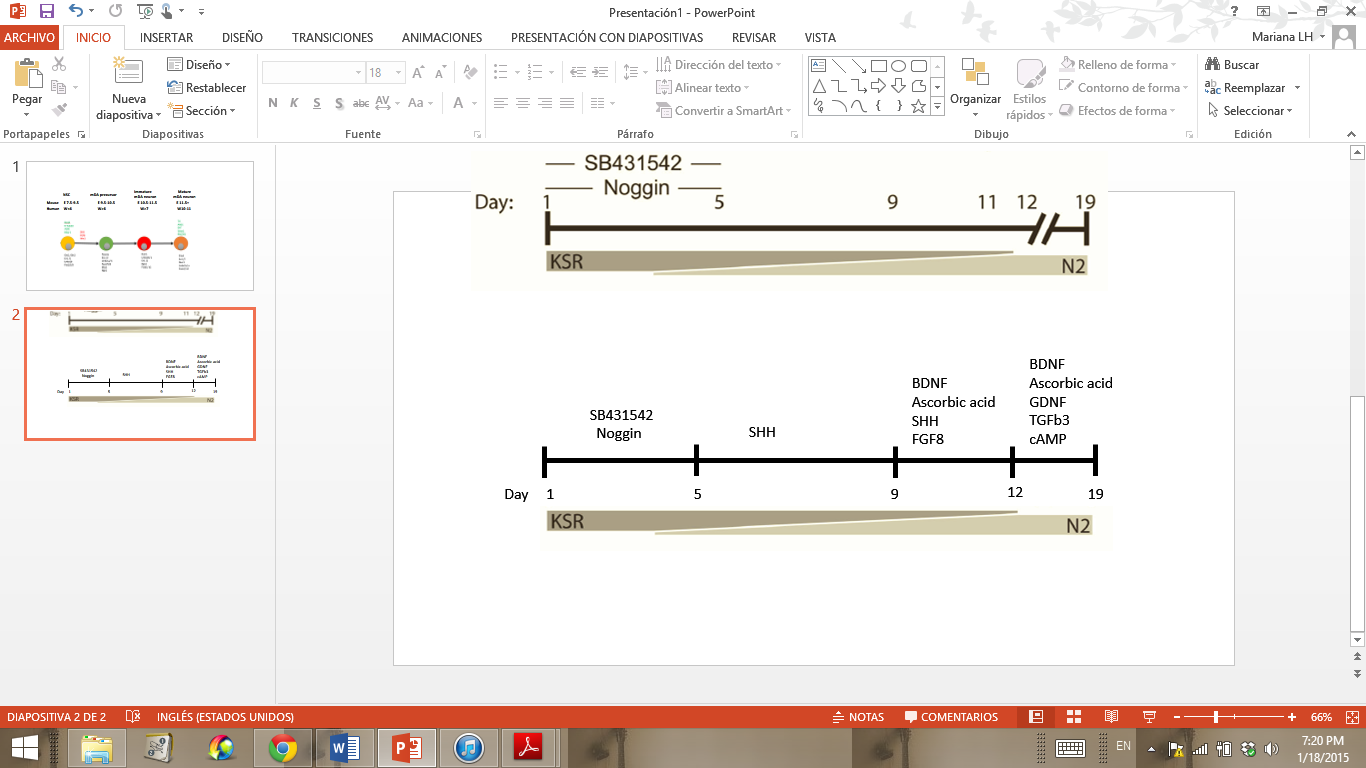


Figure 4: Protocol of Chambers group, neuronal pattering is started at day 1 where iPSC are in KSR medium (Knock-out serum replacement) with Noggin and SB431542 addition. From day 5 to 9 SHH is used followed by BDNF, ascorbic acid and FGF8 from day 9 to 12. Finally GDNF, TGFb3 and cAMP replace SHH and FGF8 to generate mature mDA neurons.

Using this protocol, Chamber and colleagues achieved an efficient differentiation of hESC and iPSC into mDA neurons around cultured day 19 with a neural induction of 82% (observed in HES5-GFP reporter line), also expressing markers as TH (the rate-limiting enzyme of catecholamine biosynthesis) and TUJ1. Remarkably, this is a shorter period of time than used earlier studies.

In the Kriks and colleagues paper, the dual SMAD inhibition is used along a novel FB protocol for mDA neurons induction and engraftment in three host parkisonian models. In here, iPSC and ES lines are selectively enriched to generate mDA neurons precursors with dual SMAD inhibition using LDN194189 and SB431542 since the first day. Additionally, SHH and Wnt are activated to obtain neural progenitors from culture day 3 to 11. On the one hand, activation of Wnt signalling is achieved by using a GSK3B inhibitor (CHIR99021) inducing coexpression of FOXA2/LMX1A and mDA neuron fate. On the other, purmophamine was used alone or in combination of recombinant SHH, to activate the signalling pathway of the latter. Maturation was carried out using Neurobasal/B27 medium supplemented with BDNF, GNDF, TGFβ3, cAMP, dibutytryl and ascorbic acid (Fig. 5).

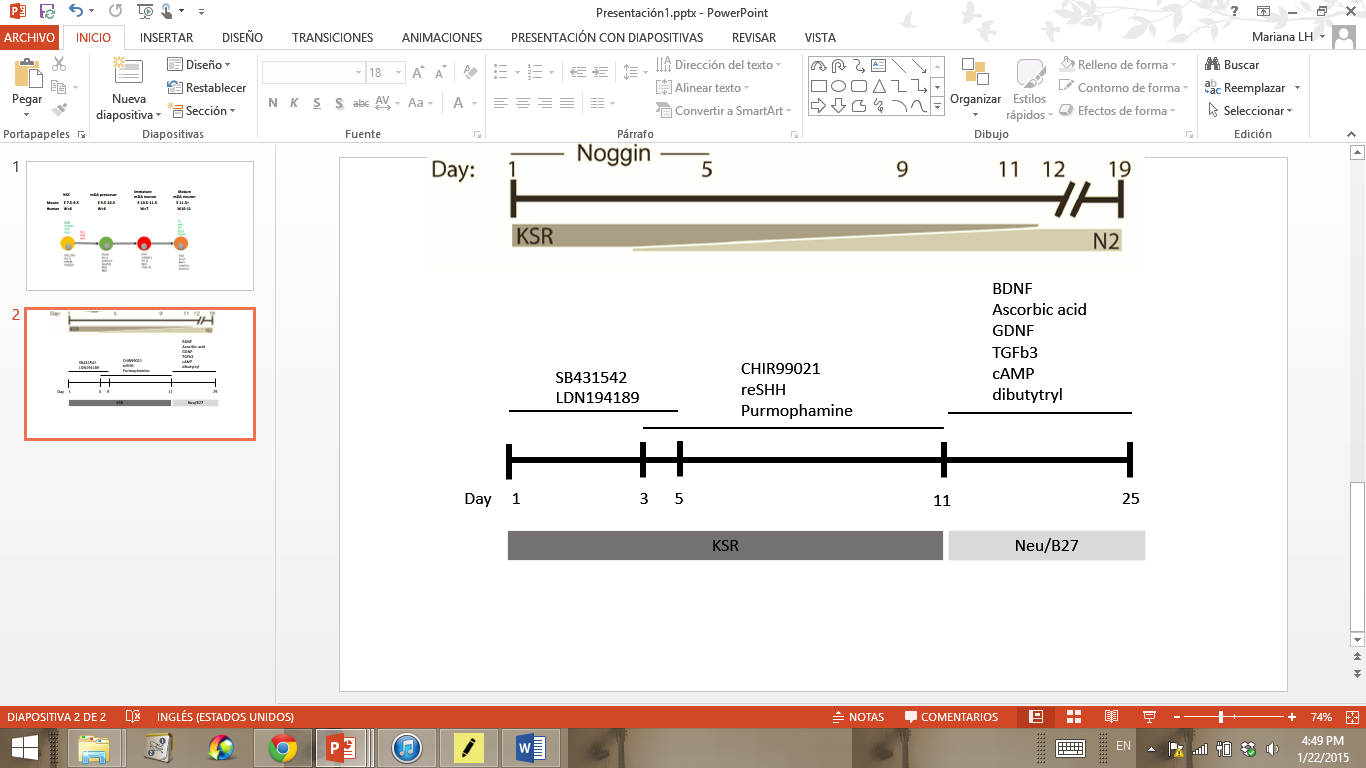


Figure 5: Protocol of Kriks and colleagues, neuronal pattering is started at day 1 where iPSC are in KSR medium (Knock-out serum replacement) with Noggin and LDN194189 addition until day 5. From day 3 to 11, Wnt is activated through CHIR99021 and recombinant SHH and Purmophamine are used to activate SHH. Finally, maturation is achieved by using BDNF, ascorbic acid, GDNF, TGFb3, cAMP and dibutytryl from day 11 to 25.

At day 25 several of the characteristic mature neuronal markers of midbrain DA neurons were expressed (Table 1). The mDA neurons probed to express mDA related genes (measured with global gene expression profiling), have an electrophysiological phenotype (measured with electrophysiological recordings) and dopamine release (measured with high-performance liquid chromatography) similar to natural conditions.

In addition, the generated mDA showed excellent survival when engrafted into two murine models, having robust survival of cells coexpressing TH+/FOXA2+ and TH+/NCAM+ and improving motor tests score of hosts after 5 months. Similarly, when grafted in brains of monkeys the graft core showed high survival of TH+ and FOXA2+, however the behavioral tests results are not mentioned in this case.

Moreover, the earlier protocols of Perrier and Sonntag research groups using a stromal feeder-based differentiation system, appears to have lower efficiency than dual SMAD inhibition and FB protocols when applied under different conditions in studies with iPSC (Perrier et al 2004; Sonntag et al 2007; Soldner et al 2009; Hargus et al 2010; Cooper et al 2010; Rhee et al 2011).

Summed up, immunocytochemistry on these various studies (Table 1) shows loss of pluripotent genes after differentiation, a strong expression of markers observed in mDA neurons (Chambers et al 2009; Cooper et al 2010; Doi et al 2014; Sundberg et al 2013; Hartfield et al 2014; Badja et al 2014). Also, in broader characterization studies, these cells expressed mature mDA neurons markers as ADDC, GIRK2, DAT, VMAT2 and PITX3, having normal electrophysiological properties and being able to excrete dopamine as well (Doi et al 2014; Hartfield et al 2014). Full characterization is necessary to generate mDA neurons with a more specific phenotype that resembles the native ones.

**Conclusions**

There are several techniques to generate midbrain DA neurons from human IPSCs, Firstly it is important to mention that none of the differentiation procedures gave a 100% efficiency, meaning that the resulting cell suspension is contaminated with other cell types. To that purpose, iPSC-derived midbrain DA neurons can be purified using specific cell surface markers (CD markers) (Sundberg *et al* 2013; Doi et al 2014). Also it has been reported that using CORIN antibody is efficient to isolate progenitor cells, nonetheless it has low expression not making suitable to great scale production (Sundberg *et al* 2013). Therefore, more studies are required with different CD markers, representing a risk-safe alternative and might help unravel the specific phenotype of midbrain DA neurons (Sundberg *et al* 2013; Payne *et al* 2014).

Using IPS-cells derived from, in particular familiar forms of Parkinson patients is important and useful to investigate the mechanisms that cause a certain gene mutation to lead to the specific degeneration of DA neurons in these patients (Cooper *et al* 2010; review Roessler *et al* 2013). With respect to the use of IPSC-derived DA neurons for clinical transplantation, timing is an important factor; earlier it was proposed that DA neurons from the middle stage of maturation presenting NURR1+ were suitable for transplantation, however other studies propose DA neurons in premature stages as the best for treatment of PD (Doi *et al* 2014). Even more, Sundberg *et al* uses later stages of maturation for transplants, leaving questions to be answered in future studies considering the similarity of gene expression in human IPSC-derived DA neurons with true primary human DA neurons related to their stage of differentiation.

Importantly, genetic and epigenetic screening to characterize iPSC-derived DA neurons is crucial to be able consider them fully stable and differentiated, not only the markers expression and physiological features (Yamanaka *et al* 2007; Payne *et al* 2014).

There is still a lot to be done to be able to use iPSC-derived cells into the clinic, however in the meantime, these can also be used as models to understand underling mechanisms of this challenging disease.

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