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Organization of the human embryonic ventral mesencephalon

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ABSTRACT

The neurons in the ventral mesencephalon (VM) are organized into several nuclei consisting of distinct neuronal populations. These include the dopaminergic (DA) neurons of the substania nigra and ventral tegmental area, the oculomotor (OM) neurons that innervate the muscles controlling eye movement, and the reticular neurons of the red nucleus (RN) involved in motor control and coordination reviewed in Puelles (2007). The factors and genes that control the differentiation of the various neuronal populations in the VM have been extensively studied in the mouse and other model organisms but little is known about the progenitors and their protein expression in the developing human brain. In this study we analyze if key regulators identified in rodents are also expressed in the human VM during embryonic development. We report that BLBP and LMX1A mark the floor plate and that FOXA2 is expressed in both the floor plate and basal plate of the human VM. The proneural transcription factors NGN2 and MASH1 are expressed in the ventricular zone of the human VM within and lateral to the floor plate. The post-mitotic DA neurons express TH as well as NURR1 and PITX3. ISL1 and BRN3A can be used to detect the cells of OM and RN, respectively. We show that many key developmental control factors are expressed in a temporal and spatial manner in the human VM essentially corresponding to what has been observed in the mouse. This data therefore suggest similar roles for these factors also in human VM development and dopamine neurogenesis.

1. Results and discussion

In order to define characteristic protein expression in the different domains of the human mesencephalon and subsequently analyze the expression profile of important transcription factors involved in cell fate specification of different mesencephalic nuclei, we have collected sections spanning the entire rostro-caudal level of the mesencephalon from different stages of human embryonic development ranging from 5 to 8 weeks PC (post conception).

At 5.0 weeks PC we can not detect any cells expressing Tyrosine Hydroxylase (TH, the rate limiting enzyme in dopamine synthesis) positive cells (Fig. 1) although such early TH expression has previously been reported in one study (Almqvist et al., 1996). With our antibodies we detect the first TH expressing cells at 6.0 weeks PC (Fig. 4A). Between 6 and 8 weeks PC we see a large increase in TH positive cells, which matches previous reports of TH expression and dopamine production (Almqvist et al., 1996; Freeman et al., 1991; Verney et al., 2001). DA neurogenesis ceases at 10–11 weeks PC (Freeman et al., 1991), thus the human embryonic stages in the present study spans from the early onset of DA neurogenesis to mid DA neurogenesis. The appearance of the mesencephalon at onset and at mid DA neurogenesis are illustrated in Fig. 1. In terms of timing of DA neurogenesis, our human samples ranging from 5 to 8 weeks PC roughly equal to E9.5–E12 in mouse, where TH expressing cells can first be detected around E9.5–E10, peak production occurs at E11.5 and the generation of TH neurons is completed by E14 (Bayer et al., 1995).

To validate the specificity of the antibodies used in this study, we first tested a range of antibodies on human sections from different regions of the CNS. Based on the expression pattern in the various brain regions, we collected a list of 12 antibodies that labeled the correct cell populations (Table 1) and subsequently used them for our studies on the ventral mesencephalon.

1.1. LMX1A and BLBP expression defines the floor plate in the developing human mesencephalon

The neural tube (including the mesencephalon) can be subdivided into four longitudinal domains along the ventro-dorsal axis: floorplate, basal plate, alar plate and roof plate (Shimamura et al., 1995; Verney et al., 2001). The floor plate is a glia-rich structure

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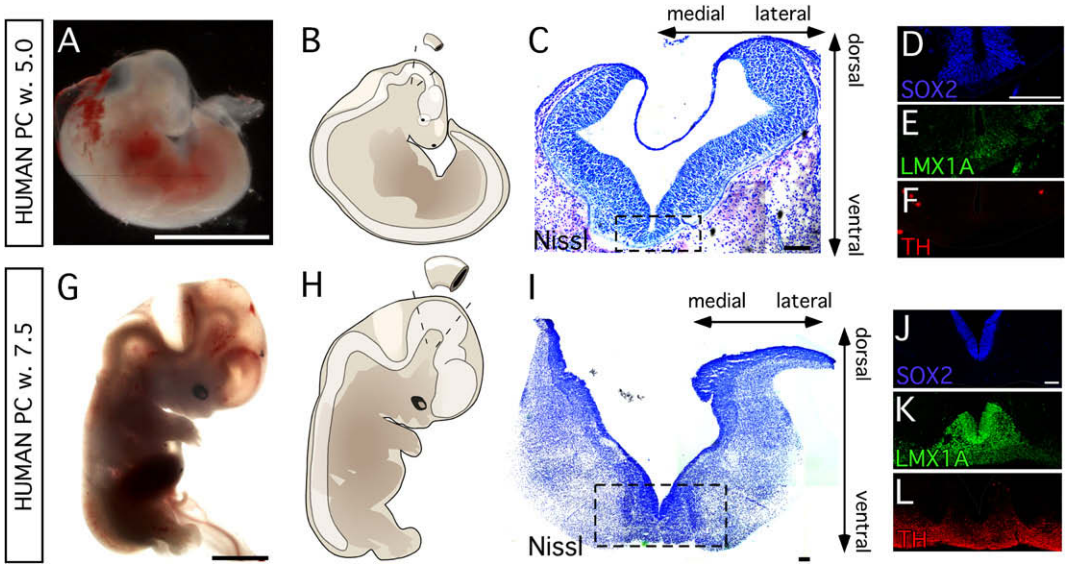

Fig. 1. The human mesencephalon at PC week 5.0 and 7.5. Bright field images of a human embryo at PC week 5.0 and 7.5 (A and G). These images were used to create schematic drawings of the embryo, making the neural tube and location of the mesencephalon easier to identify and also showing the mesencephalic area of the neural tube that was sub-dissected and used for sectioning* (B and H). The coronal sections obtained from the mesencephalon were subsequently stained with Nissl to show the structure of the mesencephalon at the stages analyzed. The ventral mesencephalon imaged in (D–F), (J–L) are marked with a black dotted square and arrows are used to point out the dorso-ventral vs. medial lateral axis (C and I). Immunohistochemical staining of SOX2 labeles the proliferative VZ in the VM sections (D and J), LMX1a, labels the mesDA neuron domain (E and K), and TH labels the post-mitotic DA neurons (F and L). *Note that the week 5.0 PC embryo was sectioned as a whole embryo without prior sub-dissection. Scale bars in A and G (5 mm) apply to A–B and G–H, respectively. Scale bars in C, I, D and J (100 μm) apply to C, I, D–F and J–L, respectively.

Table 1
Antibodies validated to work on human fetal brain sections.

Antibody	Species	Dilution	Company	Validated in
BLBP	Rabbit	1:5000	Chemicon	VM
BRN3A (Pou4f1)	Mouse	1:50	Santa Cruz	VM
FOXA2 (HNF3b)	Goat	1:600	Santa Cruz	VM, spinal cord, diencephalon
ISL1	Mouse	1:100	Hybridoma Bank (39–405)	VM, spinal cord
LMX1A	Rabbit	1:10,000	Gift from Dr. M. German	VM, spinal cord, dorsal midbrain, diencephalon
MASH1	Mouse	1:500	B.D. Pharmingen	VM, spinal cord
NGN2	Goat	1:100	Santa Cruz	VM, spinal cord
NURR1	Rabbit	1:1000	Santa Cruz	VM
PITX3	Rabbit	1:100	Gift from Dr. M.P. Smidt	VM
SOX2	Mouse	1:50	R&D	VM, diencephalon
TH	Rabbit	1:1000	Pel-Freez Biologicals	VM, diencephalon
TH	Mouse	1:1000	Chemicon	VM, diencephalon

that acts as a signaling center in most areas of the developing CNS. Originally, the floor plate was identified on the basis of morphological appearance, but now molecular markers that distinguish different sub-categories of floor plate cells with different functions have been identified reviewed in Placzek and Briscoe (2005). Several studies have recently established that the mesencephalic floor plate uniquely harbors neurogenic potential that is mediated by WNT signaling and that it is the floor plate cells that gives rise to the mesencephalic DA (mesDA) neurons (Bonilla et al., 2008; Hebsgaard et al., 2009; Joksimovic et al., 2009; Ono et al., 2007). These mesencephalic foor plate cells express LMX1A (a key transcription factor involved in mesDA neuron generation) as well as the common the floor plate marker FP4 (Hebsgaard et al., 2009; Ono et al., 2007). In the human mesencephalon midline radial glia have also been shown to express BLBP (Hebsgaard et al., 2009). Thus, combined expression of BLBP and LMX1A would reliably mark the floor plate in the developing human VM. In agreement, we found that BLBP is expressed in exactly the same ventral domain as LMX1A at all time points analyzed (Fig. 2A and B and data not shown) and consequently we use the expression of either of these two proteins to define the lateral border between the floor plate and the basal plate in the human mesencephalon.

1.2. The proneural transcription factors NGN2 and MASH1 are expressed in the VZ of the human VM

The proneural (basic helix-loop-helix) bHLH transcription factors Neurogenin-2 (Ngn2) and Mash1 are expressed in the proliferative region of the developing murine VM were they play an important role in regulating neurogenesis. Loss-of-function studies have shown that *Ngn2*, but not *Mash1*, is also required for correct fate specification and proper development of the mesDA neurons (Andersson et al., 2006; Kele et al., 2006). In the human developing VM, we found that NGN2 is expressed in the VZ as defined by Sox2 expression in parallel sections, (Fig. 3B and data not shown). Expression was detected in the VZ cells spanning the ventral midline directly overlying the domain of the newly formed post-mitotic DA neurons at all stages analyzed (Fig. 3B). The NGN2 expression also extends lateral to the dopamine domain (Fig. 3B) similar to what is observed in the mouse (Fig. 3A). High power confocal analysis, however, showed species variation in the distribution of NGN2 positive cells within the VZ: in the human embryonic brain NGN2 positive cells in the floor plate region can be detected throughout the entire thickness of the VZ (Fig. 3C–C″) similar to what is observed in the mouse (Kele et al., 2006;

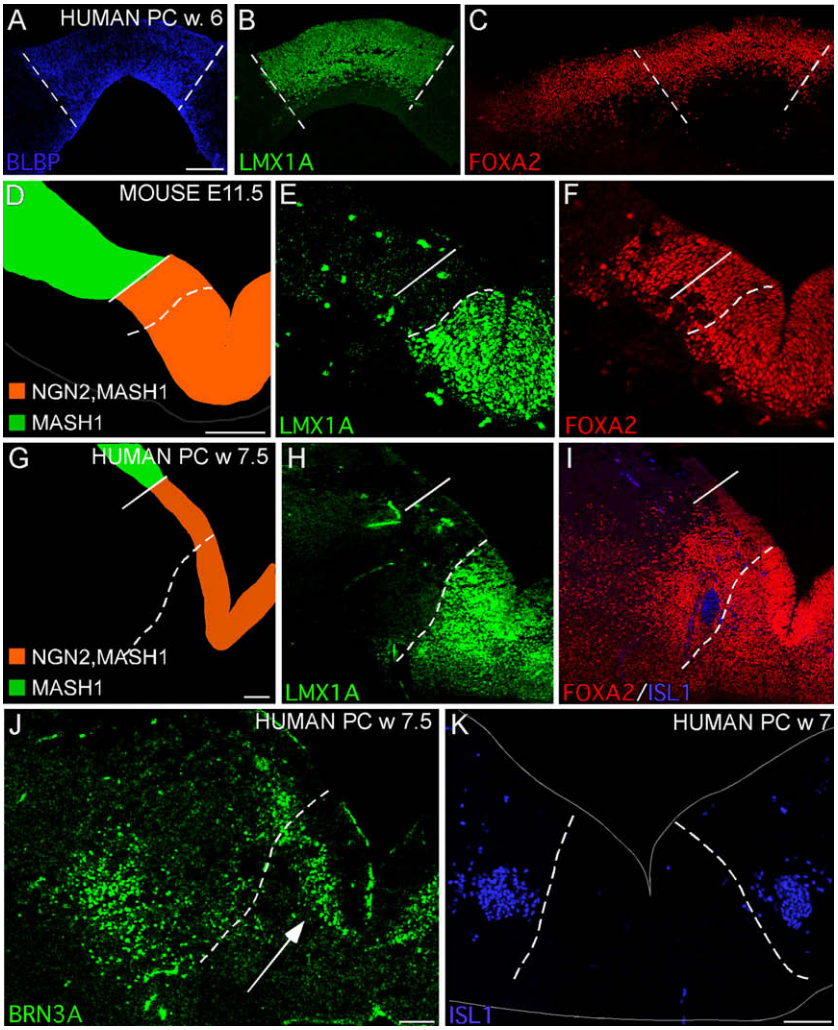

Fig. 2. Protein expression in the floor plate and basal plate of the human VM. BLBP (A) and LMX1A (B and H) expression delineate the floor plate in human VM and their expression was used to set the lateral border of the floor plate in the same or consecutive sections as marked with dotted line (A–K). FOXA2 is expressed within the floor plate and basal plate (C and F). Comparing the expression of NGN2/MASH1 double-positive domain with that of LMX1A and FOXA2, in mouse (D–F) and human (G–I) show that NGN2 expression extends laterally outside the LMX1A positive domain in both species (D–E, and G–H). FOXA2 expression in mouse extends lateral to the NGN2 positive region (D and F) whereas FOXA2 expression in human attenuates at the border of the NGN2 positive region (G and I). ISL1 (I and K) and BRN3A (J) are expressed within the FOXA2 positive basal plate (I–K). BRN3A positive cells are also found within the DA neuron domain (J, arrow). Scale bars in A, D, G, J and K (100 μm) apply to A–C, D–E, G–I, J and K, respectively.

Thompson et al., 2006), whereas the majority of NGN2 positive cells lateral to the floor plate region are located at a basal position within the VZ (Fig. 3D and D′). This basal accumulation of NGN2 positive cells is not observed at any level in the mouse and the significance, if any, remains to be determined.

Within the VM, all NGN2 positive cells quantified by confocal microcopy at 7 weeks PC (100%, $n = 90$) also co-express MASH1 (Fig. 3E–E″). Like in the mouse, MASH1 expression also extends further lateral than NGN2 (Fig 3B) in the developing human VM (Fig. 3B). However, in contrast to the developing mouse brain at corresponding developmental stage, where MASH1 expression is markedly weaker within the medial NGN2 positive domain than in the lateral NGN2 negative domain (Fig. 3A) we found no difference in the level of MASH1 within or lateral to the NGN2 positive region in the developing human mesencephalon (Fig. 3B).

1.3. FOXA2 is expressed in the dopaminergic and non-dopaminergic domains of the VM

The forkhead/winged helix transcription factor FOXA2 is involved in both floor plate and basal plate specification in the developing mouse mesencaphalon (Ang and Rossant, 1994; Ferri et al., 2007; Ruiz i Altaba et al., 1995). In light of this, we performed a careful comparison of the expression of NGN2 with that of FOXA2 and LMX1A during human embryonic development at 7.5 weeks PC. The number, morphological appearance, amount of neurite extension and location of TH positive cell bodies at this stage as observed here as well as in previous studies (Almqvist et al., 1996; Freeman et al., 1991; Verney et al., 2001) suggests that this stage, in terms of dopamine neuron development, corresponds approximately to E11.5 in the mouse.

We observed that the NGN2/MASH1 double-positive domain attenuates approximately half way up to the alar-basal boundary (Fig. 2G, solid line marks NGN2/MASH1 border) extending beyond the border of the LMX1A expressing floor plate (Fig. 2G and H dotted line marks LMX1A and thus floor plate border). The lateral limit of the FOXA2 expression domain also extends beyond the floor plate and into the basal plate (Fig. 2I vs. H) at this stage as well as at earlier stages (Fig. 2C vs. B) and the attenuation of FOXA2 corresponds exactly to the lateral border of the NGN2/MASH1 double-positive domain. In the age-matched developmental stage of the murine VM (E11.5), FOXA2 is expressed within both the LMX1A

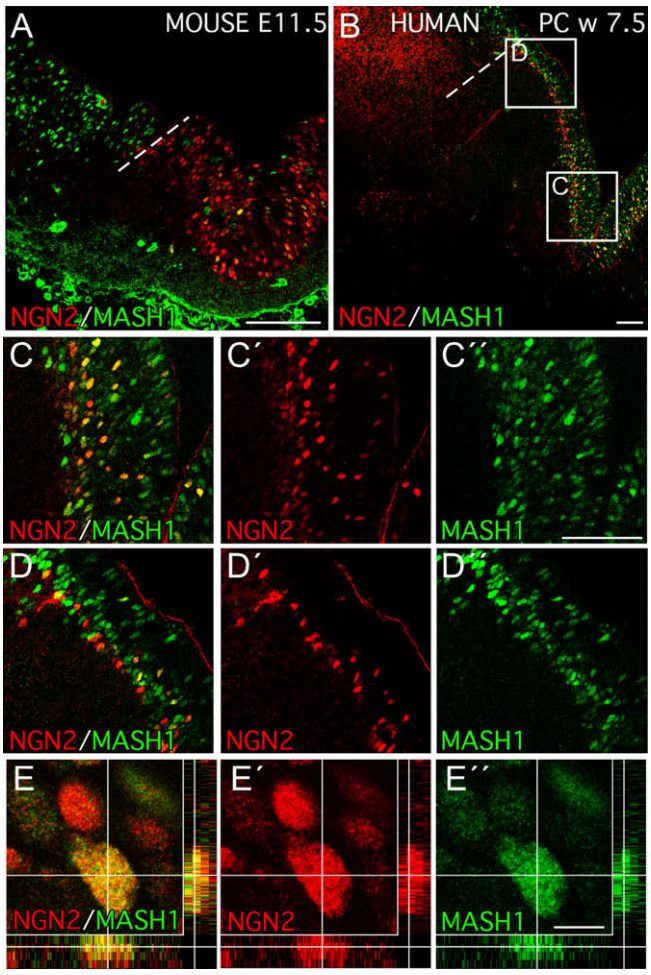

Fig. 3. NGN2 and MASH1 are expressed in the VZ of the human VM. NGN2 and MASH1 are expressed in the VZ of the developing mouse (A) and human mesencephalon (B). In the medial part of the human VM, NGN2 positive cells are distributed throughout the entire thickness of the VZ (C–C″), whereas the majority of the cells at a more lateral position are found at a basal position within the VZ (D–D″). The NGN2 positive cells also co-express MASH1 (E–E″). The fluorescence observed outside the VZ in the lateral part of the VM was confirmed to be unspecific background using confocal microscopy. Scale bars in A, B and C″ (100 μm) apply to A, B, C–D″, respectively. Scale bars in E (10 μm) apply to E–E″.

and the NGN2/MASH1 double-positive domain but in this case FOXA2 expression also extends laterally to both of these domains (Fig. 2D–F). Thus, FOXA2 is expressed in the floor plate and basal plate of the human VM, but in contrast to mouse, the human FOXA2 expression attenuates already at the same lateral position as NGN2 instead of extending further lateral to this domain as observed in mouse at corresponding (E11.5) developmental age. It cannot be ruled out that this difference in FOXA2 expression is due to a temporal asynchrony between the two species. We do not have the possibility to study the lateral border of FOXA2 in relation to MASH1/NGN2 and LMX1A in later human embryos but when performing the same analysis on earlier mouse embryos (E10) the borders where exactly the same as those observed at E11.5 (not shown) supporting our data that the lateral extent of FOXA2 expression varies between mouse and human.

1.4. The mesDA neurons in the ventral tegmental area and the substantia nigra

We next studied the expression of key proteins and transcription factors of mesDA neuron development in human embryos ranging from onset to mid DA neurogenesis (Fig. 4). At 6 weeks PC, post-mitotic mesDA neurons detected by TH and PITX3 have just started to appear in the region below the VZ (Fig. 4A and B). At later stages, corresponding to high production of mesDA neurons (7–8 weeks PC), the mesDA neurons are found in the mantle zone in a spatial location similar to that observed in the mouse (Fig. 4C and D) with the ventral tegmental area and the substantia nigra starting to appear as clearly distinguishable structures. At this stage, NURR1 is co-expressed in all post-mitotic TH expressing neurons in the mantle zone (Fig. 4E, F–F″). PITX3 is expressed in all the TH expressing cells (Fig 4G, I–I″), but as also reported for the mouse (Maxwell et al., 2005), a subset of the PITX3-expressing neurons in the lateral domain do not co-express TH and vice versa (Fig. 4H, H–H″).

1.5. The basal plate nuclei

In addition to DA neurons, glutamatergic and cholinergic neurons of the red nucleus (RN) and oculomotor (OM) are born within the FOXA2 positive domain. The neurons/precursors of these two nuclei express the transcription factors Islet-1 (ISL1) and Brain-3a (BRN3A, also known as Pouf4 (Agarwala et al., 2001; Wallen et al., 1999)), and are located laterally to the DA neuron domain in distinctly clustered nuclei in the basal plate of the developing murine VM (Puelles, 2007).

The FOXA2 expression in the human mesencephalon shares its lateral border with that of NGN2/MASH1double-positive cells and thus extends further laterally than the LMX1A positive floor plate domain (Fig. 2G–I) and consequently also extends into the basal plate.

When analyzing the expression of BRN3A, which in the murine VM is expressed in precursors and neurons of the RN, we found that BRN3A in the developing human VM shows a similar expression pattern, with positive cells situated in the basal plate within the FOXA2 positive domain lateral to the LMX1A expressing floor plate domain (Fig. 2J). However, the BRN3A positive population appears slightly more diffuse and the usual compacted shape of the nucleus was less obvious. Scattered BRN3A expressing cells could also be detected extending into the DA neuron domain (Fig. 2J, arrow).

In the rostral part of the developing human VM, the ISL1 positive OM neurons were positioned in a clearly defined and distinct cluster below the NGN2 positive part of the VZ outside the LMX1A positive domain at the same lateral position in the basal plate as the cells of the RN (Fig. 2K). Although located within the FOXA2 positive domain, the ISL1 expressing cells within the human VM where found to be FOXA2 negative (Fig. 2I and Fig. 5A) corresponding to previous findings in mouse (Ferri et al., 2007).

1.6. DA and non-DA neuron precursors intermingle in the caudal VM

When analyzing ISL1 expression in all rostro-caudal levels of the human VM, we could also observe scattered ISL1 positive cells within the DA neuron domain in caudal sections of the VM (Fig. 5A and B). The finding of both ISL1 and BRN3A positive cells within the LMX1A expressing DA domain in the human VM is not observed in mouse and could be explained by a tangential migration of these neuronal subtypes into the human DA neuron domain or that a small fraction of OM and RN neurons are born within this domain. Alternatively, a subpopulation of human DA neuron precursors may transiently express ISL1 and BRN3A during development. That the scattered ISL1 expressing cells within the DA neuron domain did not express FOXA2 (Fig. 5C) and further investigations showed that they were also TH negative (Fig. 5D), suggests that they represent precursors for cholinergic neurons that intermingle with the precursors for mesDA neurons at caudal regions of the human VM. To confirm that this is the case, and rule out the alternative

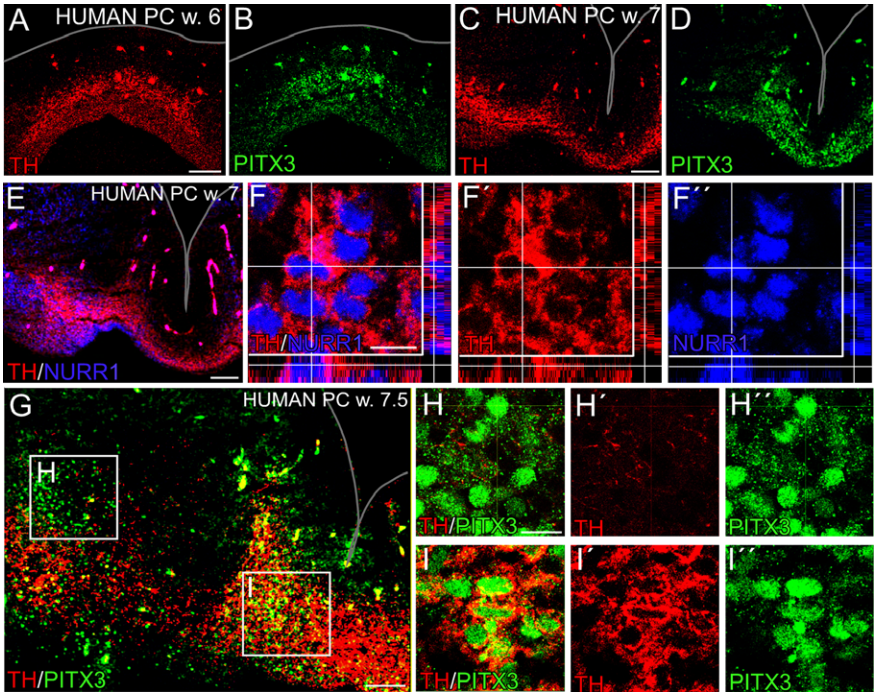

Fig. 4. The mesDA domain. Post-mitotic mesDA neurons started to appear at 6 weeks PC as detected by TH (A) and PITX3 (B). At mid mesDA neurogensis, the mesDA neurons (C and D) become organized into what will become the ventral tegmental area and substantia nigra. The TH expressing neurons co-express NURR1 (E–F″) and PITX3 (G, I–I‴). A subset of the PITX3 positive mesDA neurons did not co-express TH (D, H–H‴). Scale bars in A, C, E and G (100 μm) apply to A–B, C–D, E and G, respectively. Scale bars in F and H (10 μm) apply to F–F″ and H–I″, respectively.

possibility that ISL1 is transiently expressed by early mesDA neuron precursors in the human VM before the onset of TH expression, we performed a double staining for ISL1 and NURR1. However, we could not detect any cells co-expressing ISL1 and NURR1 in any of the embryos analyzed (Fig. 5E–H). Thus, we conclude that ISL1 is not expressed by human mesDA neuron precursors but instead that some ISL1 positive neurons intermingle with the DA neuron precursors at caudal levels of the human VM.

2. Experimental procedure

2.1. Human and mouse embryonic tissue source

Human tissue was obtained from PC week 5–8 old legal terminated embryos with approval of the Swedish national board of health and welfare in accordance with existing guidelines including informed consent from women seeking abortions. The gestational age of each embryo was determined by crown-to-rump length (CRL) measured at the time of dissection when the quality of the embryo allowed for this and otherwise estimated by ultrasound measurements prior to abortion. The external features of the embryo were also carefully monitored to confirm that CRL estimated an appropriate embryonic stage. With exception of the 5.0-week-old embryo, which was fixed and sectioned as a whole embryo, recovered nervous tissue was first micro-dissected and the entire mesencephalic tube collected for fixation (see Fig. 1). To confirm the correctness of the sections level and orientation we performed a careful analysis of SOX2, TH, FOXA2 and LMX1A expression to ensure that the correct area always was imaged. A total of six embryos (5.0, 6.0, 7.0, 7.5 (2), and 8.0 weeks PC) were used for this study.

The mouse embryos were harvested at E10.0–E11.5 (NMRI, Charles River) in approval with local ethical guidelines and approved animal care protocols. For staging mouse embryos, the morning of the vaginal plug corresponds to embryonic day 0.5 (E0.5).

2.2. Immunohistochemistry and structural staining

Histological analysis of human and mouse VM was performed by immunohistochemistry. Human tissue and mouse embryos were fixed in 4% paraform aldehyde (PFA) over night and cryoprotected in 30% sucrose before frozen in O.C.T Tissue-Tek (Sakura FineTek, Europe BF). The sections were boiled in 10 mM citrate buffer (NGN2 and BRN3A only) and pre-incubated at RT for 30 min in blocking-solution containing 5% normal serum/0.25% Triton X-100 (Amresco)/0, 02 M KPBS or 1% milk/10% normal serum/1 mg/ml Bovine serum albumin (BSA)/0,02 M KPBS (PITX3 and BRN3A only). The sections were thereafter incubated with primary antibodies diluted in blocking solution at 4 °C over night followed by pre-incubation for 30 min and 2 h incubation with a fluorophore-conjugated (Molecular Probes or Jackson Laboratories) or biotinylated (Vector Labs.) secondary antibody at RT. Biotinylated secondary antibodies were followed by 1 h incubation with fluorophore-conjugated Streptavidin for 2 h at RT. Primary antibodies used were: mouse anti-TH (1:2000, Chemicon), rabbit anti-LMX1A (1:10,000, M. German), mouse anti-SOX2 (1:50, R&D), mouse anti-BRN3A (1:50, Santa Cruz), goat anti-FOXA2 (1.600, Santa Cruz), rabbit anti-NURR1 (1:1000, Santa Cruz) mouse anti-ISL1 (1:100, Hybridoma Bank), mouse anti-MASH1 (1:500, B.D. Pharmingen), goat anti-NGN2 (1:100, Santa Cruz), rabbit anti-PITX3 (1:2000, M.P. Smidt) (Table 1).

Nissl stainings were performed on fixed mesencephalic sections from PC week 5.0 and 7.5 human embryos. Sections were stained in 0.5% Cresyl violet (Sigma) solution for 1 min and quickly rinsed in distilled water followed by 2 × 2 min in 95% ethyl alcohol and 2 × 2 min in absolute alcohol. Thereafter, sections were cleared with Xylen for 2 × 2 min and cover-slipped with DPX (Merck).

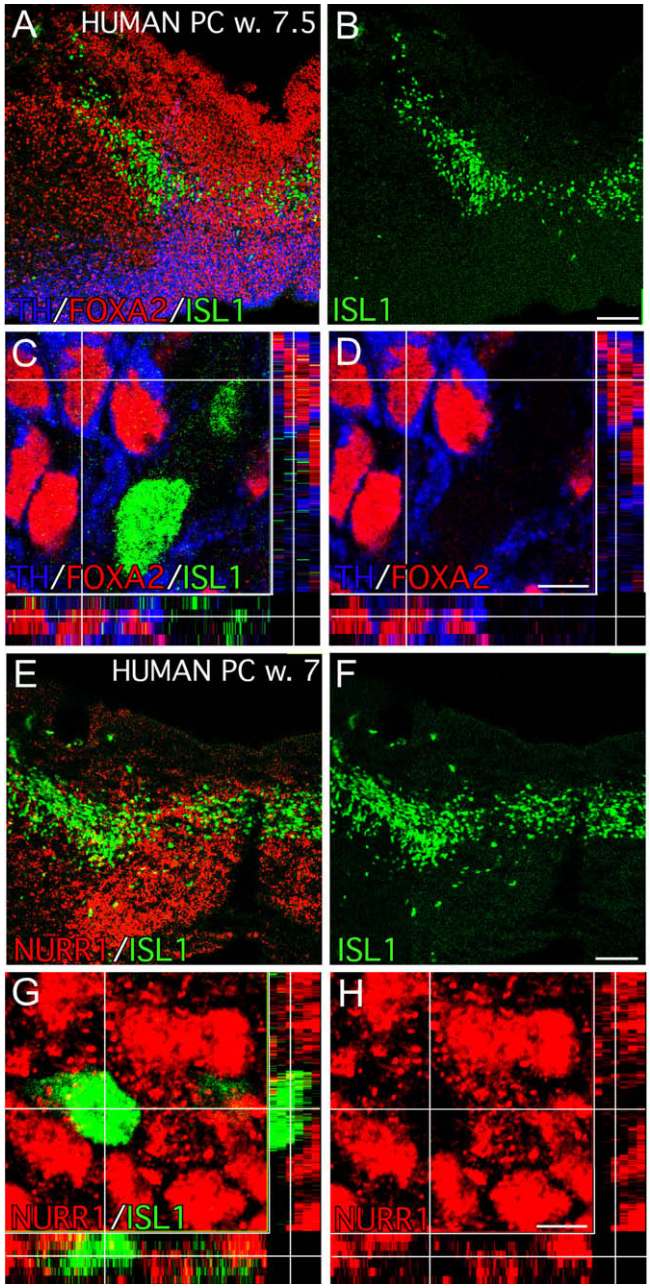

Fig. 5. DA and non-DA neuron precursors intermingle in the floor plate. Scattered ISL1 positive cells can be found in caudal sections within the DA neuron domain (A–B, and E–F), but do not co-express FOXA2 (C), TH (C and D) or NURR1 (G and H). Scale bars in B, F (100 μm) apply to A–B, E–F, respectively. Scale bars in D, H (10 μm) apply to C–D, G–H, respectively.

2.3. Microscopy

All immunohistochemical stainings were analyzed by confocal microscope (Leica) at 20× or 63× resolution. Double staining was confirmed by conduction of high-magnified confocal Z-stacks. All figures were assembled in Canvas software.

2.4. In vivo quantification

To estimate the proportion of NGN2 positive cells co-expressing MASH1 within the human DA neuron domain (delineated using LMX1A positive staining in consecutive sections). Confocal images were taken at 63× resolution along the ventricular zone at PC week 7.5 and the number of double-positive cells were quantified.

3. Concluding remark

The prospect of using DA neuron precursors for cell replacement therapy in Parkinson's disease has put emphasis on developing methods for efficient differentiation of human stem or progenitor cells into DA neurons, bearing the characteristics of mesDA neurons. For this purpose, it is important to gain knowledge of how these neurons develop in the human embryonic brain, and we have therefore examined the expression patterns of some central markers expressed at different stages of murine VM and mesDA neuron development in human tissue. We show that transcription factors with key functions in murine VM development are also expressed in a similar temporal and spatial pattern in the human VM and thus are likely to play similar roles also in human mesDA neurogenesis. The set of antibodies used in this study reliably label the correct cell populations in the human VM and thus this library of markers can now be used to accurately characterize the DA neuron precursors and mature DA neurons generated *in vitro* which is of highest interest when optimizing protocols for human mesDA neuron differentiation from human stem cell cultures.

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