Expression analyzes of early factors in midbrain differentiation programs

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A B S T R A C T

Mesodiencephalic dopaminergic (mdDA) neurons are born in the ventricular zone (VZ) of the midbrain between E10 and E12. Although these neurons all express specific DA markers like *Th* and *Pitx3*, they are subdivided into distinct subsets, each depending on a unique set of transcription factors and signaling cascades for their differentiation. How a neural progenitor commits to an mdDA neuronal cell-fate and how the specification into the different subsets is determined remains unclear.

To gain more insight into the development and specification of these neurons we have previously conducted a genome-wide expression analysis, in which dissected midbrain material (E10.5-E13.5) was compared to the adult mdDA region (Chakrabarty et al., 2012). In the present study, we have compared the genome-wide expression analysis including PITX3-GFP sorted (E12.5-E15.5) neurons to available expression data to search for genes specifically expressed in the midbrain during early stages of mdDA differentiation. We have divided these genes into 3 groups: (I) genes upregulated throughout differentiation (*Mest, NeuroD1,* and *Tcf12*), (II) genes upregulated during early stages of differentiation (*Hes5,* and *Tcf3*), and (III) genes upregulated during late stages of differentiation (*Enc1*).

Here, we show the expression profile of these genes in the embryonic midbrain during development and adult stage and compared that to the appearance of mdDA neurons via co-staining for TH. With this analysis we have identified 6 novel factors that may play a role during cell-fate commitment of neural progenitors or later during differentiation of the mdDA group of neurons.

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1. Introduction

The mesodiencephalic dopaminergic (mdDA) system is essentially involved in motivational behavior and movement control. During Parkinson's Disease (PD) the substantia nigra (SNc) is subject to degeneration, whereas the ventral tegmental area (VTA) remains relatively intact (Barzilai and Melamed, 2003). The specific degeneration of SNc neurons during PD is indicative for the existence of a different molecular make-up of groups of neurons within the mdDA neuronal population (Di Salvio et al., 2010; Smits et al., 2006, 2013; Veenvliet and Smidt, 2014).

MdDA neurons are thought to arise from neural progenitors located in the ventricular zone (VZ) of the midbrain from embryonic day (E)10.5-E14.5, with neuronal birth peaking between E11 and E12 (Bayer et al., 1995). These neural progenitors can form different neuronal cell-types present in the adult midbrain area. How the commitment of neural progenitors to an mdDA neuronal fate is regulated and how these neurons are further specified into the different subsets of the mdDA system remains a focus for study.

To gain more insight in the molecular components that may be involved in the early and late differentiation phase of mdDA neurons, we have previously conducted a genome-wide expression study on mdDA neurons at different embryonic stages (Chakrabarty et al., 2012). Dissected (E10.5-E13.5) midbrain material was compared to the adult midbrain, providing information about specific gene expression during embryonic midbrain development. Genes upregulated in relative early stages of differentiation (E10.5-E13.5) may play a role in the commitment of neural progenitors to an mdDA neuronal fate.

In this study we made use of the genome-wide expression study described above and the expression study comparing

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PITX3-GFP sorted (E12.5-E15.5) neurons to the adult mdDA system (Roessler et al., 2014) and compared these with known expression data (Allen Brain Atlas; http://brain-map.org) to investigate genes specifically expressed in the midbrain area and upregulated or expressed during mdDA neuronal differentiation. We classified these genes into 3 different groups: (I) genes expressed throughout differentiation, (II) genes expressed during early stages of differentiation, and (III) genes expressed during late stages of differentiation. Based on this list, we set up *in situ* hybridization experiments combined with TH immunohistochemistry to compare the gene-specific expression pattern relative to the emerging mdDA neuronal population. These experiments give an indication whether these factors could play a role in the specification and differentiation phase of developing mdDA neurons.

In this manuscript we describe the expression profile of 6 genes, of which *Mest, NeuroD1,* and *Tcf12* belong to group I, *Hes5,* and *Tcf3* to group II, and *Enc1* belongs to group III. The identified expression pattern indicates their potential role in developing mdDA neurons.

2. Results and discussion

2.1. Temporal and spatial expression of genes during mdDA neuronal development

To identify novel genes that are specifically expressed during mdDA neuronal development, our lab has previously conducted two genome-wide expression studies comparing E10.5-E13.5 dissected (Chakrabarty et al., 2012) and E12.5-E15.5 PITX3-GFP sorted midbrain material (Roessler et al., 2014) to an adult reference. The first study covers the peak of mdDA neuronal birth and gives information about genes that are upregulated during this process in and surrounding the mdDA neuronal population. The second transcriptome analyzes identifies genes that are upregulated within mdDA neurons when most neurons are born and contain PITX3, a marker for mdDA neurons.

After careful analysis of the available transcriptome analyzes, we have divided the gene expression patterns in 3 groups; group I genes are expressed throughout the development of mdDA neurons, group II genes are specifically upregulated in early stages of development and group III genes are upregulated during late stages of mdDA development. In this study we have selected 6 genes from group I-III that show a typical expression pattern in these genome-wide expression studies (Fig. 1A) and maybe good candidates for a role in mdDA neuronal development and specification. *Mest, NeuroD1,* and *Tcf12* are upregulated throughout development, indicating that they may serve a function in early and late mdDA neuronal differentiation (Fig. 1B, Group I). *Hes5,* and *Tcf3* are specifically upregulated in early stages of differentiation, indicating that these genes may play a role in early fate-specification of neural precursors in the ventricular zone (VZ) (Fig. 1B, Group II). *Enc1* is upregulated at late stages (Fig. 1B, Group III), which indicates that this gene may be involved in terminal programming of mdDA neuronal subsets.

To examine the spatial and temporal expression pattern of these genes in the midbrain area and compare this to the TH-expressing neuronal population, we performed *in situ* hybridization of the different genes on different developmental stages together with TH immunohistochemistry.

2.2. Mest is mainly expressed in the ventricular zone (VZ) of the midbrain area and overlaps with TH-expressing neurons at E14.5 and in adult stages

Mest is a member of the α-β hydrolase protein family and known to be involved in adipocyte differentiation (Kadota et al., 2012; Lefebvre et al., 1998). It is expressed throughout development in the midbrain area (Fig. 2), consistent with the genome-wide expression studies. At E11.5 *Mest* is expressed at the isthmic organizer (IsO) and continues to the prosomere 3 (P3) area (Fig. 2A) (Anatomic maps in Fig. 2A and B were adapted from the Allen Brain Atlas [http://atlas.brain-map.org]). Although *Mest* appears to have a relatively broad expression throughout the midbrain, it is restricted to the VZ and subventricular zone/mantle zone (SVZ) of the floor plate (FP) and basal plate (BP) (Fig. 2B). At E12.5 expression of *Mest* shifts to the hindbrain in lateral parts of the embryonic brain, and to more caudal en medial regions in the midbrain, where it is detected to border the area of TH-expression (Fig. 2A–1 and B–1). At E14.5 the expression of *Mest* changes. In lateral areas a broad band of *Mest* expression in the intermediate stratum of the midbrain area partly overlaps with the most lateral parts of mdDA neurons (Fig. 2A). The expression in the VZ is strong, but reduced to a small band, and overlaps with TH-expressing neurons (Fig. 2A-2 and B-2) and some expression can be detected in the P2 and P3 areas. In the adult mdDA system, the expression of *Mest* is relatively low, but is still present in a few TH-expressing cells in the SNc area (Fig. 2B-3 black arrowheads).

As can be detected from the expression profile of *Mest* this gene is expressed during early and late stages of mdDA neuronal development. During early stages it is mainly expressed in the area of the midbrain that harbors DA neural progenitors, indicating that it may function in the differentiation of DA neural progenitors into mdDA neurons. However, at later stages it shows an overlap in expression with TH-expressing neurons of the mdDA neuronal population at specific areas in the midbrain, suggesting a possible role in subset specification.

2.3. NeuroD1 is specifically expressed in the mdDA area at E11.5-E14.5

NeuroD1 possibly plays different roles during brain development. Amongst other functions it is thought to be involved in (terminal) differentiation of neurons and later in neuronal survival (Cho and Tsai, 2004). Its role in mdDA development has not been extensively studied, although studies of Park et al. (2006) in rats by *in vitro* mdDA cultures suggest that *NeuroD1* is an important suppressor of *Nurr1*-induced neuronal differentiation. *NeuroD1* is expressed within and surrounding the midbrain area throughout development (Fig. 3). At E11.5 and E12.5 it is expressed in the midbrain area into the P3 area (Fig. 3A), which appears to be specific for the SVZ of the FP and BP of the midbrain (Fig. 3B) (for anatomical maps see Fig. 2A and B). At E12.5 its expression overlaps with caudal (Fig. 3A-1) and rostral (Fig. 3B-1) located TH-expressing neurons in the midbrain. At E14.5, *NeuroD1* expression overlaps with the TH-expressing neurons in the rostral part of the mdDA area in P2 and P3 (Fig. 3A-2) and just below the VZ in the SVZ (Fig. 3B-2). Surprisingly, although *NeuroD1* is mainly known to be an important factor in neurogenesis (Cho and Tsai, 2004), its expression, however weak, can still be detected in cells of the SNc of the adult mdDA system (Fig. 3B-3 black arrowheads).

Based on its expression pattern, *NeuroD1,* similar to *Mest,* may have distinct functions during early and late mdDA development. During early development it is expressed in the area of the midbrain that harbors DA neural progenitors, suggesting that *NeuroD1* could play a role in the correct differentiation of neural progenitors into mdDA neurons, whereas in later stages its function could shift to a role in subset differentiation, based on the specific expression in the mdDA neurons of the SNc in the adult midbrain.
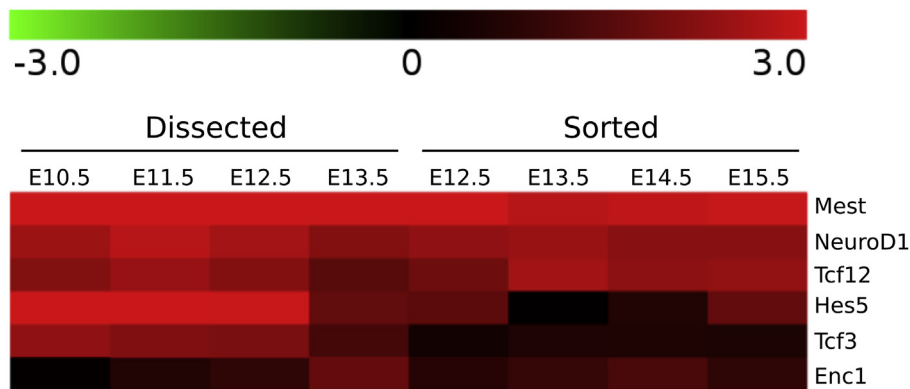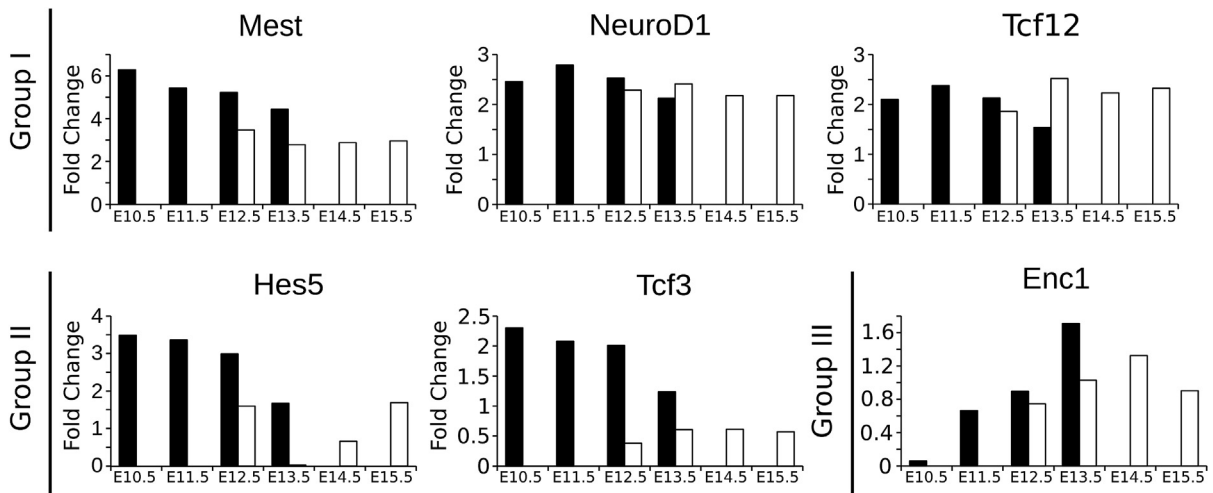
Fig. 1. Genome-wide expression study shows the temporal expression pattern of genes that are developmentally expressed compared to the adult midbrain.
A: Heat-map of the expression of 6 developmentally regulated genes from the genome-wide expression analysis performed on E10.5-E13.5 dissected midbrain material and E12.5-E15.5 PITX3-GFP sorted material.
B: Expression of individual genes from the genome-wide expression analysis; dissected midbrain material is represented by the black bars, PITX3-GFP sorted material by the white bars. The temporal expression patterns allows for categorization into, at least, three different groups. Group I (*Mest, NeuroD1,* and *Tcf12*) represents genes that are upregulated throughout development; Group II (*Hes5,* and *Tcf3*) contains genes that are upregulated in the early stages of development; Group III (*Enc1*) consists out of genes that are upregulated in late stages of development.

2.4. Tcf12 is specifically expressed in the midbrain at E11.5 until E14.5 and overlaps with medial mdDA neurons

Tcf12 is mainly known for its role in development of the immune system, where it is important for the differentiation of double positive CD4$^+$CD8$^+$ T-cells to single positive CD4$^+$ or CD8$^+$ T-cells (Barndt et al., 2000). In the brain it is thought to be involved in the expansion of precursor cells during neurogenesis (Uittenbogaard and Chiaramello, 2002). In the midbrain *Tcf12* is expressed from E11.5 onwards (Fig. 4). At E11.5, *Tcf12* is specifically expressed throughout the entire region that eventually harbors mdDA neurons (Fig. 4A and B). Its expression starts at the IsO and ends rostrally in the P3 area (Fig. 4A), where it comprises the VZ and SVZ of the entire FP region (Fig. 4B) (for anatomical maps see Fig. 2A and B). At E12.5 the expression of

Tcf12 overlaps with TH-expressing neurons in the most medial parts of the midbrain (Fig. 4A-1), which appears to be most prominent in caudal regions (Fig. 4B-1). At E14.5, the expression of *Tcf12* extensively overlaps with the medially located mdDA neurons (Fig. 4A-2) and remains exclusively expressed in the FP region of the midbrain (Fig. 4B-2). In adult stages *Tcf12* transcript could not be detected (Fig. 4B-3).

The expression profile of *Tcf12* suggests that it may have a role in the transition from DA neuronal progenitor to differentiated DA neurons as it is mainly expressed in the area that harbors DA neural progenitors and is only slightly expressed in the area that contains differentiated mdDA neurons. Since the expression of *Tcf12* is lost in adult stages it is likely that its possible function is restricted to the specification and differentiation of mdDA neurons.
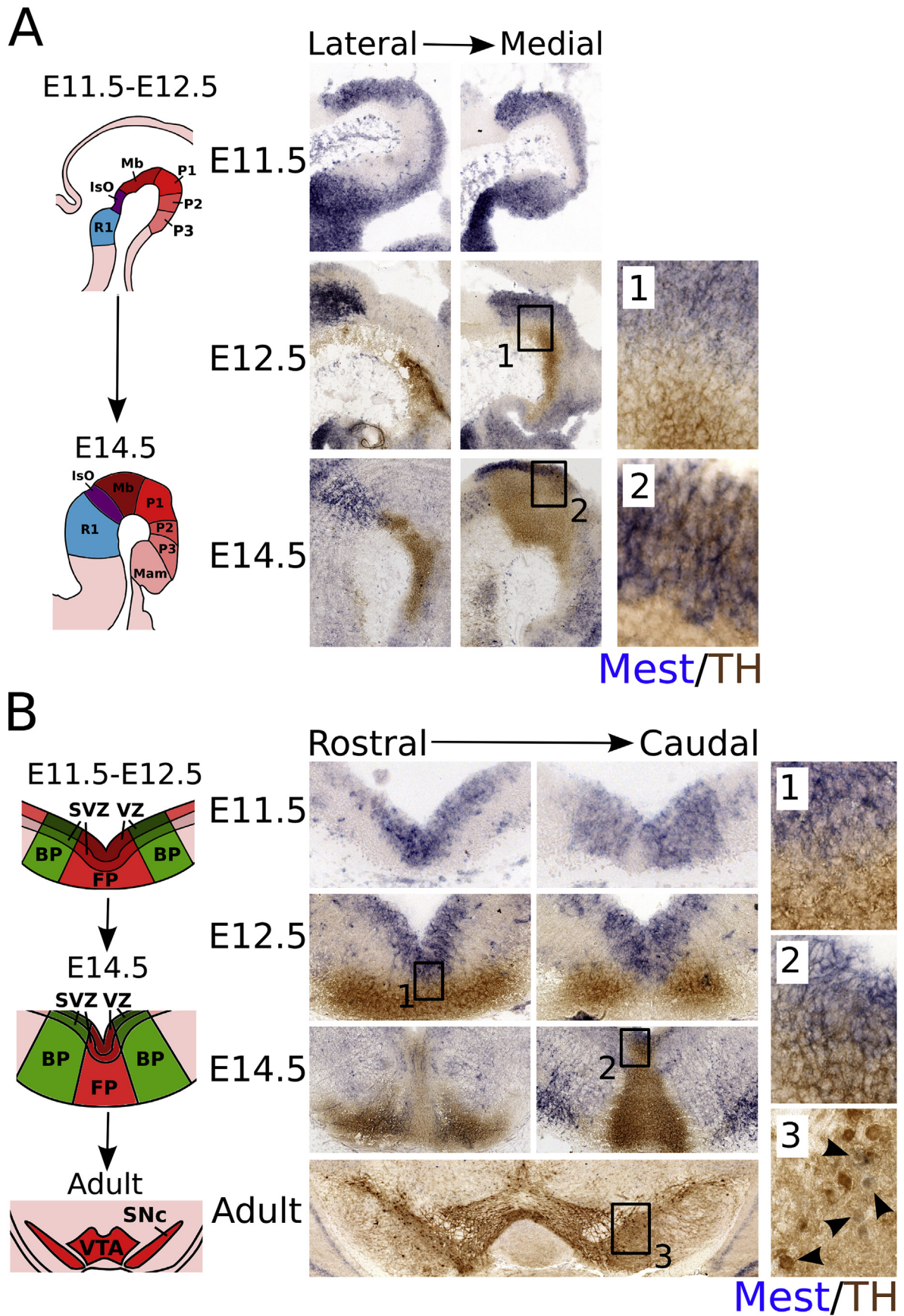
Fig. 2. *Mest* **is expressed throughout development in the mdDA region.**
A: Sagittal view of *Mest* expression throughout development. *Mest* (blue) is expressed from E11.5 onwards in the midbrain area. At E12.5 *Mest* borders and slightly overlaps with the
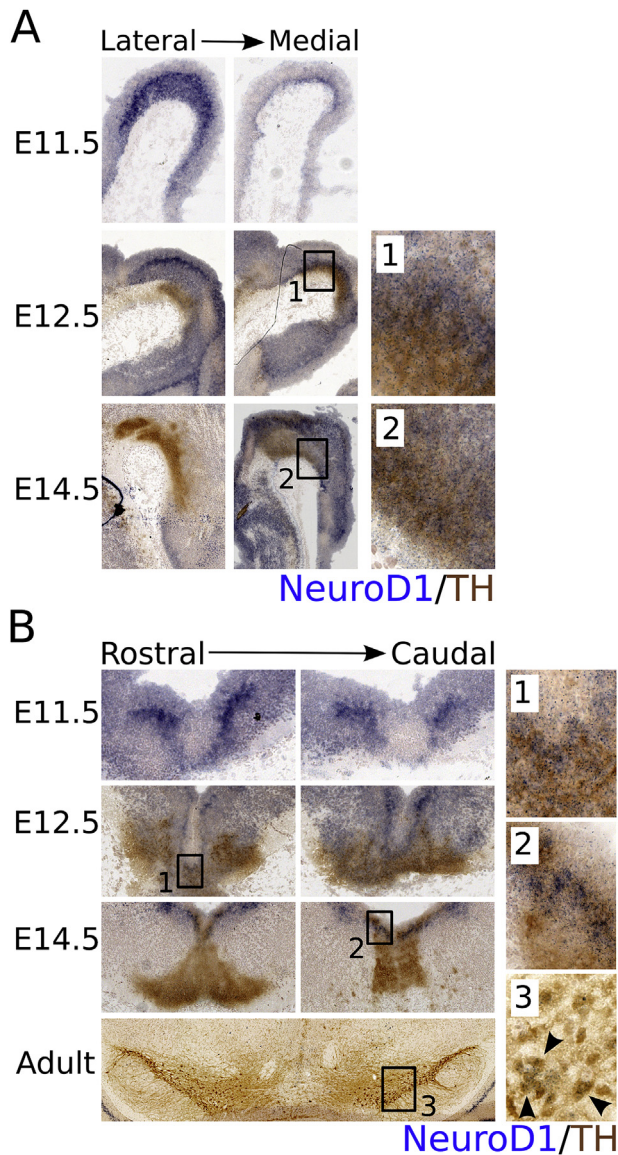
Fig. 3. *NeuroD1* expression is present throughout the midbrain and is detected in adult mdDA neurons of the SNc.

A: Sagittal view of *NeuroD1* expression throughout development. *NeuroD1* (blue) is specifically expressed in the midbrain area at E11.5, although its expression expands at later stages of development. At E12.5 it clearly overlaps with the rostral and caudal part of the TH-expressing neuronal population (brown) (**1**), which continues until E14.5 (**2**).

B: Coronal view of *NeuroD1* expression throughout development and in the adult. *NeuroD1* (blue) is expressed mainly in the SVZ of the future mdDA neuronal population at E11.5, and shows overlap in the rostral TH-expressing neuronal population (brown) at E12.5 (**1**). At E14.5 *NeuroD1* is still expressed in the SVZ of the midbrain, where it overlaps with the TH$^+$ population (**2**). In the adult *NeuroD1* is specifically expressed in neurons that are part of the SNc (**3** black arrowheads).

2.5. Hes5 is expressed in the VZ of the mid- and hindbrain, and slightly overlaps with TH$^+$ neurons at medial-dorsal positions

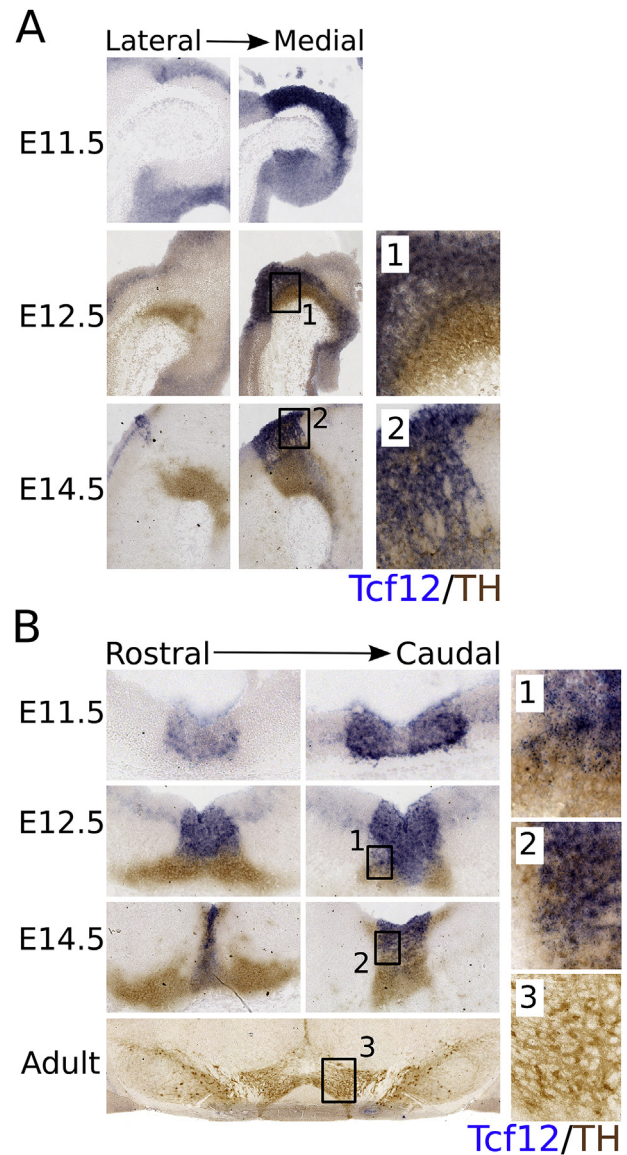*Hes5* and *Hes1* are Notch effectors and have complimentary

Fig. 4. *Tcf12* is specifically expressed in the midbrain area during development and overlaps with medially located TH-expressing neurons.

A: Sagittal view of *Tcf12* expression throughout development. *Tcf12* (blue) is expressed from E11.5 onwards in the medial midbrain area from the IsO up to the P2 area. At E12.5 expression of *Tcf12* overlaps with the medially localized TH-expressing neurons (brown) (**1**), which continues until E14.5 (**2**).

B: Coronal view of *Tcf12* expression throughout development and in the adult. Throughout development *Tcf12* (blue) is expressed in the VZ and SVZ of the FP region of the midbrain. Expression of *Tcf12* overlaps with the TH-expressing neuronal population (brown) in the medial part of the midbrain at E12.5 (**1**) and E14.5. (**2**). In the adult stage, expression of *Tcf12* was no longer detected (**3**).

expression patterns in the brain (Imayoshi et al., 2010). They are thought to control the timing of cell differentiation, which is important for the size and shape of the brain, but also for the structural integrity (Hatakeyama et al., 2004). *Hes5* is expressed in the VZ of the entire midbrain at E11.5-E14.5 (Fig. 5A and B) (for

TH-expressing population (brown) (**1**). At E14.5 most overlap can be detected in the VZ of the medial part of the midbrain (**2**).

Anatomic maps were adapted from the Allen Brain Atlas (http://atlas.brain-map.org).

B: Coronal view of *Mest* expression throughout development and in the adult. *Mest* (blue) is expressed throughout development in the FP and part of the BP of the midbrain area. Overlap with the TH$^+$ population (brown) is mainly detected in the medial part of the FP at E12.5 (**1**) and E14.5 (**2**). In the adult mdDA neuronal population some neurons of the SNc slightly express *Mest* (**3** black arrowheads).

Anatomic maps were adapted from the Allen Brain Atlas (http://atlas.brain-map.org).
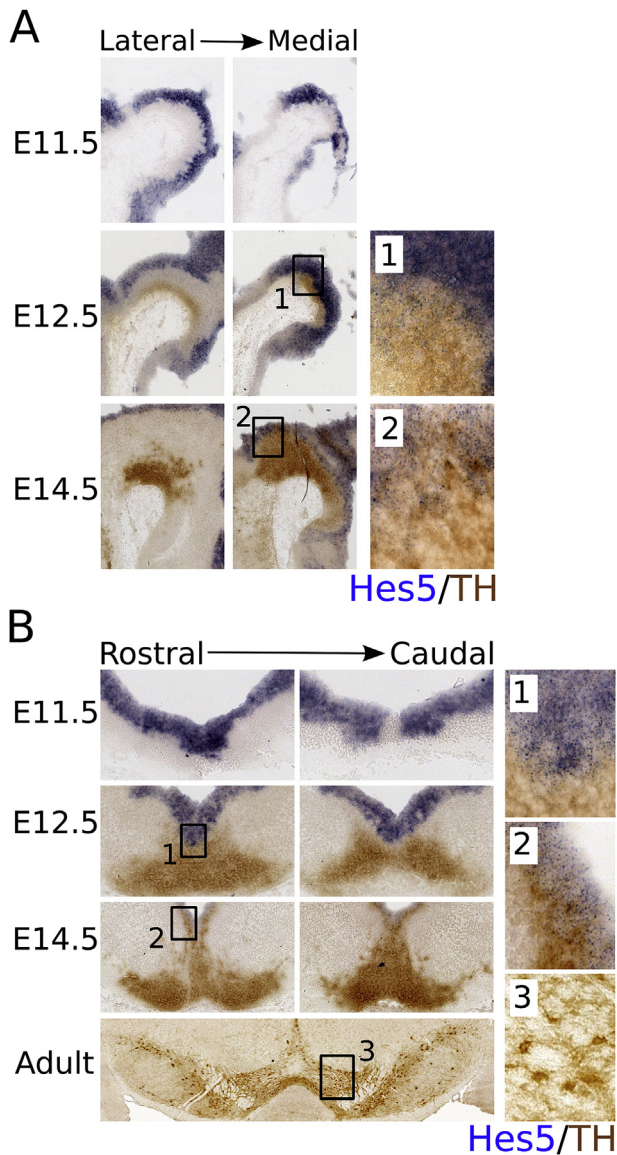
Fig. 5. Expression of *Hes5* is mainly present in the VZ of the midbrain area.
A: Sagittal view of *Hes5* expression throughout development. *Hes5* (blue) is expressed throughout the mid- and hindbrain, but not in the isthmic region. At E12.5 *Hes5* overlaps with the medially located TH-expressing neurons (brown) (**1**), which can be detected until E14.5 (**2**).
B: Coronal view of *Hes5* expression throughout development and in the adult. *Hes5* (blue) is specifically expressed in the VZ of the FP and the BP of the midbrain throughout development. At E12.5 (**1**) and E14.5 (**2**) *Hes5* expression overlaps with few TH⁺ neurons (brown) in the VZ of the midbrain. Its expression is not detected in the adult mdDA neuronal population (**3**).

anatomical maps see Fig. 2A and B). Its expression extends from the hindbrain rhombomere 1 (R1) into P3, but it is excluded from the IsO (Fig. 5A). This expression profile can be detected throughout development. *Hes5* is expressed throughout the VZ of the embryonic midbrain (Fig. 5B). However, it does show some overlap with TH⁺ neurons at the most medial area at both E12.5 (Fig. 5A-1) and E14.5 (Fig. 5A-2), which appears to be specific for the FP region (Fig. 5B-1 and B-2). In the adult midbrain *Hes5* expression could not be detected (Fig. 5B-3).

Since *Hes5* is mainly expressed in the area of the midbrain that contains DA neural progenitors it is possible that it could play a role in the differentiation of a neural progenitor to an mdDA neuron

and/or is important in increasing and maintaining the stem-cell pool in the midbrain.

2.6. Tcf3 is expressed in the VZ and SVZ of the midbrain at E11.5 until E14.5 and is lost in adult stages

Tcf3 belongs, together with *Tcf12*, to the E-box binding protein family, and is mainly known for its role in development of the immune system (Jones and Zhuang, 2011). In the brain it is thought to be involved in neural stem cell differentiation (Fischer et al., 2014). Although *Tcf3* is a family-member of *Tcf12*, their expression profiles in the midbrain are different. *Tcf3* is expressed in the hindbrain and extends into the P3 areas of the midbrain (Fig. 6A). Its expression is present in the VZ of the FP and BP of the midbrain (Fig. 6B) (for anatomical maps see Fig. 2A and B). *Tcf3* expression shows some overlap with TH-expressing neurons in the most medial parts of the embryonic midbrain at E12.5 (Fig. 6A-1) and E14.5 (Fig. 6A-2). This overlap is mainly detected in caudal areas and specific for the FP region of the midbrain (Fig. 6B-1 and 2). *Tcf3* transcript is not detected in the adult midbrain region (Fig. 6B-3), suggesting that *Tcf3*, if it has a function in mdDA neuronal development, could only play a role in the differentiation of neuronal progenitors to mdDA neurons and/or in the proliferation of neuronal stem cells as it only shows a strong expression during development in the area of the midbrain that contains DA neural progenitors.

2.7. Enc1 is specifically expressed in the SVZ of the midbrain-P3 area and overlaps with the rostral group of mdDA neurons

Enc1 is a kelch-related actin binding protein, that is mainly expressed in neurons and in undifferentiated adipocyte progenitors. It is thought to be important in the regulation of cytoskeletal changes during neuronal and adipocyte differentiation (Hernandez et al., 1997; Zhao et al., 2000).

Enc1 expression starts at E11.5 and remains expressed in the adult midbrain (Fig. 7). It is expressed in the midbrain-P3 area from E11.5-E14.5 (Fig. 7A), where it appears to be mainly present in the SVZ of the midbrain area (Fig. 7B) (for anatomical maps see Fig. 2A and B). At E12.5 it clearly overlaps with a rostral part of TH-expressing neurons in the P2-P3 area (Fig. 7A-1), which is specific for the FP region of the midbrain (Fig. 7B-1). This specific expression in the rostral TH⁺ neuronal population is still present at E14.5 (Fig. 7A-2). At this stage *Enc1* also overlaps with more medially located TH-expressing neurons in the VZ (Fig. 7B-2). In the adult *Enc1* is, although less prominently, expressed in mdDA neurons of the SNc (Fig. 7B-3).

The expression pattern of *Enc1* suggests that it may be involved in late differentiation of DA neuronal progenitors towards fully differentiated mdDA neurons, since it is clearly expressed in the SVZ at all stages, which contains neurons that started differentiating. Furthermore, it could be involved in the programming of the mdDA rostral subset, since it is expressed rostrally from E11.5 and in the adult stage.

3. Conclusion

Here, we have shown the expression profile of 6 developmentally regulated genes expressed throughout the midbrain area, which were selected based on genome-wide expression study performed previously (Chakrabarty et al., 2012; Roessler et al., 2014). These data allowed us to divide genes expressed during development into different groups: group I represents the genes that are upregulated throughout differentiation; group II contains genes that are upregulated at early stages of differentiation and
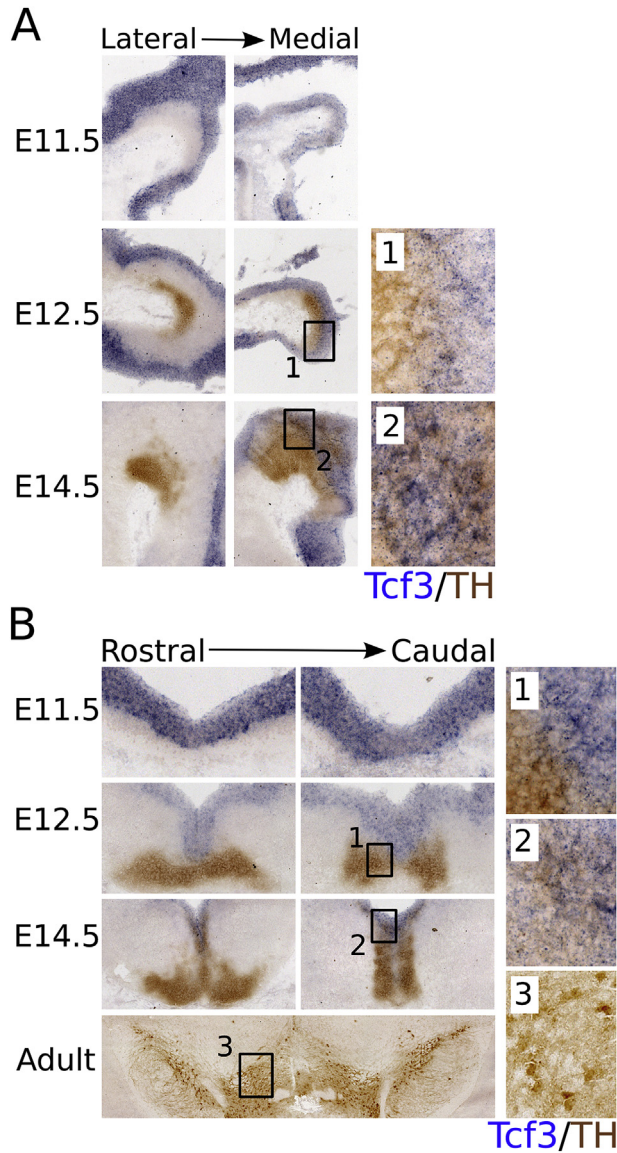
Fig. 6. *Tcf3* is very broadly expressed throughout the mid- and hindbrain, but is confined to the VZ.

A: Sagittal view of *Tcf3* expression throughout development. *Tcf3* (blue) expression is present throughout the midbrain area, but is specific for the VZ. At E12.5 the expression of *Tcf3* overlaps with the TH-expressing population (brown) in the medial part of the midbrain (**1**), which can also be detected at E14.5 (**2**).

B: Coronal view of *Tcf3* expression throughout development and in the adult. *Tcf3* transcript (blue) is present throughout the VZ of the FP and BP in the embryonic midbrain. At E12.5 (**1**) and E14.5 (**B**) its expression overlaps with medially located TH-expressing neurons (brown). In the adult mdDA neuronal population expression of *Tcf3* is no longer detected (**3**).

Fig. 7. *Enc1* is expressed throughout development and overlaps mainly with rostrally located TH$^+$ neurons and in the SNc in the adult mdDA neuronal population.

A: Sagittal view of *Enc1* expression throughout development. *Enc1* (blue) is expressed throughout the midbrain area during development. At E12.5 its expression specifically overlaps with the rostrally located group of TH-expressing neurons (brown) (**1**), which can be detected until E14.5 (**2**).

B: Coronal view of *Enc1* expression throughout development and in the adult. *Enc1* (blue) shows a specific expression in the SVZ of the FP and BP of the embryonic midbrain throughout development. At E12.5 the expression of *Enc1* overlaps with rostrally located TH-expressing neurons (brown) (**1**), whereas some TH-expressing neurons in SVZ can be detected at E14.5 (**2**). In the adult, *Enc1* is detected in part of the TH$^+$ neurons of the SNc (**3** black arrowheads).

genes of group III are upregulated during late stages of differentiation. The genome-wide expression study together with combined *in situ* hybridization and TH immunohistochemistry experiments provides insight in the spatial and temporal expression patterns of genes that may be involved in the development and specification of mdDA neurons.

We have identified at least 3 genes that, based on their expression pattern, could serve a function early in mdDA differentiation; *Tcf12* belonging to group I, *Hes5* belonging to group II, and *Tcf3* also belonging to group II. These genes are only expressed during development and show a specific expression in the VZ of the midbrain. *Mest* and *NeuroD1*, both belonging to group II, show a
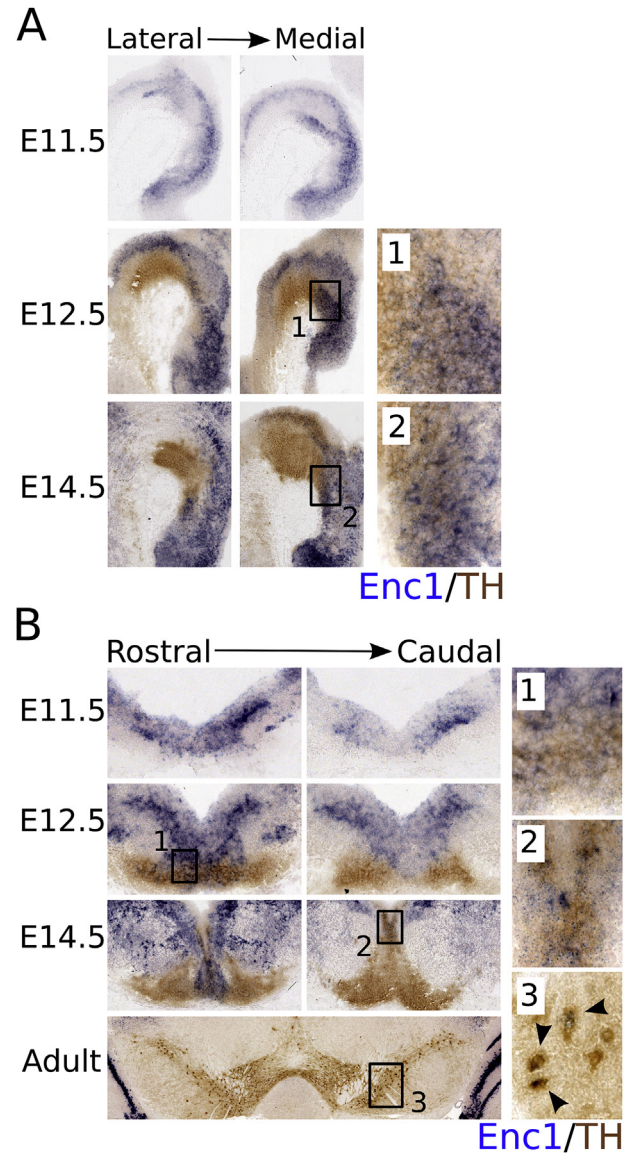
high expression during development compared to the adult reference, although they were also expressed in the adult mdDA neuronal population. These data indicate that these genes could serve a dual function, one in mdDA neuronal development and one in maintenance and survival of mdDA neurons. Finally, we identified *Enc1*, belonging to group III, as a novel subset-specific gene. This transcript showed a specific expression in the rostral part of the developing midbrain from E11.5 onwards and remains expressed in part of the neurons of the adult SNc, indicating that this gene could play a role in SNc development and maintenance.

Taken together, this study provides novel leads towards the programming details of mdDA neurons. Especially genes that remain expressed in a subset of the adult mdDA neuronal pool could be of high interest as they may play a role in the specific vulnerability of the SNc in neurodevelopmental disorders as PD.

4. Experimental procedures

4.1. Ethics statement

All animal studies are performed in accordance with local animal welfare regulations, as this project has been approved by the animal experimental committee (Dier Ethische commissie, Universiteit van Amsterdam, DEC-UvA, and Dier Ethische commissie), and international guidelines.

4.2. Animals

Embryos were generated by crossing C57BL/6 mice. Pregnant [embryonic day 0.5 (E0.5) is defined as the morning of plug formation] and adult mice were sacrificed by cervical dislocation. Embryos and brains were collected in $1\times$ PBS and immediately frozen on dry-ice and stored at $-80\,^{\circ}$C. Cryosections were sliced at 16 μm, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored at $-80\,^{\circ}$C until further use.

4.3. In situ hybridization and combined TH immunohistochemistry

In situ hybridization with digoxigenin (DIG)-labeled probes was performed as described previously (Smidt et al., 2004). Fresh frozen sections were fixed in 4% PFA for 30 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Probe hybridization was carried out at 68 °C O/N with a probe concentration of 0.4 ng/μl in a hybridization solution containing 50% deionized formamide, $5\times$ SSC, $5\times$ Denhardt's solution, 250 μg/ml tRNA Baker's yeast, and 500 μg/ml sonicated salmon sperm DNA. The following day slides were washed in $0.2\times$ SSC for 2 h at 68 °C followed by blocking with 10% HIFCS in buffer 1 (100 mM TricHCl, pH = 7.4 and 150 mM NaCl) for 1 h at RT. DIG-labeled probes were detected by incubating with alkalin-phosphatase-labeled anti-DIG antibody (Roche, Mannheim), using NBT/BCIP as a substrate.

DIG in situ hybridization was performed with the following probes: 649 bp Mest fragment bp 648–1298 of mouse cDNA, 859 bp NeuroD1 fragment bp 36–895 of mouse cDNA, 965 bp Tcf12 fragment bp 1027–1992 of mouse cDNA, 178 bp Hes5 fragment bp 154–331 of mouse cDNA, 920 bp Tcf3 fragment bp 1024–1945 of mouse cDNA, and 660 bp Enc1 fragment bp 1113–1772 of mouse cDNA. E11.5 in situ hybridization was not followed by TH-DAB IHC, these slides were washed 2×5 min in $T_{10}E_5$, dehydrated with ethanol, and embedded in Entellan.

After DIG in situ hybridization, E12.5, E14.5, and adult sections were immunostained for TH. Slides were incubated in 0.3% H_2O_2 in Tris-buffered saline (TBS) for 30 min at RT. Thereafter, blocking was performed with 4% HIFCS in TBS. Slides were incubated O/N with primary antibody Rb-TH (Pelfreeze, 1:1000) in TBS. The following day slides were incubated for 1 h with goat-anti-rabbit biotinylated secondary antibody (Vector, 1:1000) in TBS, followed by incubation with avidine-biotin-peroxidase reagents (ABC elite kit, Vector Laboratories 1:1000) in TBS. The slides were stained with DAB (3,3′-diamino-benzidine) for a maximum of 10 min. Slides were dehydrated with ethanol and embedded with Entellan.

Competing interests

The authors declare that no competing interests exist.

Acknowledgements

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