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Expression analyzes of early factors in midbrain differentiation programs

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ABSTRACT

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.

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

Abbreviations: mdDA, mesodiencephalic dopaminergic; PD, Parkinson's Disease; SNc, substantia nigra; VTA, ventral tegmental area; VZ, ventricular zone; SVZ, subventricular zone/mantle zone; FP, floor plate; BP, basal plate; IsO, isthmic organizer; P1-3, prosomere 1–3; R1, rhombomere 1.

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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 suppressant 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.