Supplementary material

Ventral Mesencephalon-Enriched Genes that Regulate the Development of Dopaminergic Neurons in Vivo

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Supplementary Materials and Methods

Mouse lines and genotyping

Mice used in the present studies were maintained and handled according to the guidelines of the Society for Neuroscience (January 1985) and the Ethical Committee for the Use of Animals at the Shanghai Institutes for Biological Sciences, China. Ebf1 heterozygote mice were a generous gift from Dr. R Grosschedl, USA. Ngn 2 and Lmx1b heterozygous mice were generous gifts from Dr. F Guillemot, UK and Dr. RL Johnson, USA, respectively. These heterozygotes were maintained on a CD1/129 background and intercrossed to generate homozygous Ebf1, Ngn2, and Lmx1b mice for experiments. Timed pregnant females were obtained by overnight mating. The morning of detection of the vaginal plug was considered to be embryonic day (E) 0. Genotyping was performed as previously described (Lin and Grosschedl, 1995; Chen et al., 1998; Fode et al., 1998).

Immunohistochemistry and TUNEL staining

Cryosections (12-14 µm thick) were blocked for 1 h in 4% goat serum/0.5%Triton
X-100/phosphate buffered saline (PBS, pH7.4) followed by incubation at 4 °C overnight with one or more of the following primary antibodies: mouse anti-TH (1:500, Chemicon, Temecula, CA, USA), rabbit anti-TH (1:1000, Chemicon), rabbit anti-Ebf (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Nurr1 (1:100, Santa Cruz Biotechnology), mouse anti-βIII-tubulin (1:1000, Sigma, St. Louis, MO, USA), rabbit anti-Isl1 (1:500, Abcam, Cambridge, UK), rabbit anti-Lmx1b (1:2000, a gift from Dr. Y.Q. Ding, China), rabbit anti-Lmx1a (1: 1000, a gift from Dr. M German), rabbit anti-Pitx3 (1:500, a gift from Dr. MP Smidt, the Netherlands), rabbit anti-L1 (1:200, a gift from Dr. FG Rathjen, Germany) and rabbit anti-reelin antibodies (1:100, Chemicon). Secondary antibodies were diluted in 1% goat serum/0.1%Triton X-100/PBS. Sections were imaged using either a cooled CCD SPOT II (Diagnostic Instruments, MI, USA) on a microscope (BX51; Olympus, Japan) or a laser confocal microscope (Leica TCS SP2, Bensheim, Germany). Data were obtained and processed using Adobe Photoshop 7.0 software (Adobe Systems).

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining using the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instructions.

**Cell counting**

The number of TH⁺ and Pitx3⁺ cells was quantified in P0 *Ebf1⁻/⁻* mutants and their littermates. Four series of cryosections were collected from P0 pups and every third and fourth section (12 µm) was used for quantification of TH⁺, Pitx3⁺ and calbindin⁺ neurons, respectively as previously described (Sauer et al., 1995). The SN is divided into three parts: rostral,
intermediate and caudal. The part of the SN is seen with the medial terminal nucleus of the accessory optic tract (MTN), which separates the SN and VTA, was referred as the intermediate part of the VM. Therefore, the SN in the sections before and after the intermediate part that did not show the characteristic MTN structure was considered to be the rostral and caudal parts of the SN, respectively. The average number of TH$^+$ cells was calculated unilaterally for each animal. An estimation of the total number of Pitx3$^+$ cells in P0 pups was obtained by counting Pitx3$^+$ cells in every fourth section containing the SN and VTA. The average total number of TH$^+$ cells counted was calculated. All numbers were corrected for split cell counts using the Abercrombie formula (Abercrombie, 1946).
Legends for Supplementary figures

Figure S1. Microarray analysis of mouse ventral mesodiencephalon (VM). (A) Number of differentially expressed genes between eight pairwise comparisons among five time-points. Quantitative analysis showed that gene variation was mild between E10 and E11 as well as between E13 and E14. Only 20 genes showed more than two-fold change in their expression level between E10 and E11. Likewise, the number was much greater between E10 and E12 than that between E12 and E14 (576 vs. 110). (B) Hierarchical clustering of differentially expressed genes across all the samples from E10 to E14. Columns represent samples and rows represent genes. (C-D) Enlarged images of expression data from the selected areas in (B), showing a set of genes whose levels were either down-regulated (C) or up-regulated (D) with development. Profiles are shown in a color scale where green is low, red is high. Data shown were from three independent experiments. Some genes (bold in C and D) were selected for further study. (E-H) Expression profiles of cell-cycle related genes from microarray analysis from E10 to E14 ventral mesencephalon. Genes that are known to be involved in mitotic progression in G1 (E), G1/S (F), G2/M phases (G), and DNA replication (H). Values represent means ± SEM. *, P < 0.05, compared with E10, n=3.
**Figure S2.** Regional specific expression of another set of genes identified from the microarray analysis of the VM (A–P). *In situ* hybridization in sagittal sections of the mouse embryonic brain (E12) shows restricted expression of another set of 20 genes selected in the distinct layers of VM. The prototypical expression profile of each gene is shown in the right panel. The data represent means ± SEM of three independent experiments. Asterisks indicate statistical significance between two adjacent time points \((P < 0.05)\). Scale bar: 100 µm.

**Figure S3.** VM-enriched genes *Hmgb2* and *Fabp7* are not regulated by either *Ngn2* or *Lmx1b*. Expression of these genes in the VM are determined in E12 *Ngn2* or *Lmx1b* null mutant mice. Established mDA neuron markers *TH* and *Pitx3* serve as positive controls. (A) (a-a’) Deficiency of *Ngn2* results in depletion of TH. (b-b’) βIII-tubulin immunofluorescence in combination with Hoechst staining shows that ventral mesencephalic cells accumulate in layers I-III in the absence of *Ngn2*. (c-d’) *Hmgb2* and *Fabp7* expression is normal in *Ngn2*−/− mutants. (e-f’) show that TH and *Pitx3* are not expressed is the absence of *Lmx1b*. (g-h’) *Hmgb2* and *Fabp7* expression is normal in *Lmx1b*−/− mutants. (B) *Lmx1b* depletion does not alter *Ebf1-3* and *Lmx1a* expression. Data represent at least three independent experiments. Scale bars: 200 µm.
Supplementary References


