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Ephrinb3 induces mesostriatal dopaminergic projection to the striatum

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ABSTRACT

Dopaminergic neurons in midbrain are subdivided into three subsets, forming mesostriatal, mesocortical and mesolimbic projections, respectively. The molecular mechanism specifying mesostriatal projection, which is important for understanding the pathogenesis of Parkinson's disease, remains unclear. To probe the role of axon guide molecule Ephrinb3 in inducing mesostriatal projection, we labeled mesostriatal and mesocortical subset DA neurons with fluorescent microspheres, and purified these subpopulation cells with fluorescence-activated cell sorting (FACS). Moreover, real-time PCR was performed to address the expression of Ephrinb3 in mesostriatal DA neurons, and fluorescence *in situ* hybridization (FISH) was used to further verify the expression of Ephrinb3 in labeled neurons. The results showed that mesostriatal DA neurons were successfully isolated with retrograde labeling and FACS. Real-time PCR showed that the expression of Ephrinb3 was higher in mesostriatal DA neurons than in mesocortical neurons. Also, Ephrinb3 could be detected in labeled neurons with FISH. Our results indicate that Ephrinb3 is directly involved in the specificity of mesostriatal projection.

1. Introduction

Dopaminergic (DA) neurons produce neurotransmitter dopamine. Most DA neurons are located in the substantia nigra (SN) and the ventral tegmental area (VTA) of the midbrain in the mammalian central nervous system [1]. Early anatomical studies have demonstrated that midbrain DA neurons are composed of three neuronal subpopulations due to their different projections [2,3]. One group of DA neurons in SN forms mesostriatal projection, which was thought to exclusively innervate the caudate-putamen [4]. The other two groups of DA neurons within VTA give rise to mesocortical and mesolimbic projections, which mainly innervate prefrontal cortex and nucleus accumbens, respectively.

Accordingly, the functional studies showed that mesostriatal, mesocortical and mesolimbic projections mediate motor control, mood regulation and motivation (addiction), respectively [5–9]. Thus, the midbrain DA neurons are of different subtypes, sharing only the same neurotransmitter dopamine. Previous reports suggested that midbrain DA neurons receive region-specific signals that divide them into specific subsets distinguishable through molecular and physiological characteristics [10–13]. However, so far, little is known about midbrain DA neurons' specific connectivity in terms of function and neuroanatomy.

Previous advances in molecular biology have identified some genes and molecules involved in the generation, migration and differentiation of midbrain DA neurons [14]. For example, the transcription factor nurr1 was found to be essential for the maturation and differentiation of midbrain DA neurons [15]. In addition, it is also required for the expression of tyrosine hydroxylase [16]. Recently, the axonal guidance molecules have become increasingly important for their roles in the specificity and regulation of midbrain DA neurons. Identification of guidance factors and cues may address the mechanisms of axonal pathfinding of DA neurons [14]. Ephrins, the ligands of Ephs, are the largest family of receptor tyrosine kinases, and they play important roles in axonal guidance [17,18]. Ephrinb3 is a multi-functional factor that regulates cell apoptosis and augments T-cell signaling [19,20]. In particular, Kullander et al. [21] reported that Ephrin3 was a key axon guide molecule for regulating corticospinal tract projection. More recent study demonstrated that Ephrinb3 could mediate axon pruning and control axon pathfinding [22]. Ephrinb3 interacts with EphBs and EphAs, including EphB2 and EphA4 [23]. In the current studies, Ephb2 was expressed in the early ventral midbrain and controlled the morphological development of the ventral midbrain. Moreover, Ephrinb3 was expressed in ventral midbrain during development [24,25]. These results suggested that Ephrinb3 played an important role in midbrain DA neuron projection.

To explore this possibility, we labeled mesostriatal and mesocortical DA neurons with retrograde fluorescent microspheres, and purified labeled neurons with fluorescence-activated cell sorting (FACS). Then, real-time PCR was performed to detect the gene expression of Ephrinb3 in the sorted DA cells. Furthermore, the specific expression in DA subtypes neurons was confirmed with *in situ* hybridization.

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2. Materials and methods

2.1. Animals

CD-1 mice were used in the experiment. For retrograde fluorescence labeling, postnatal day 0 (P0) mice were used. All protocols involving animal handling were in accordance with the China Animal (Scientific) Procedures Act.

2.2. Fluorescence retrograde tracing

P0 mice were anesthetized on the ice for 5 min. Using a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), one hundred nanoliter fluorescent microspheres (lumafluor, Naples, FL) were injected into either the striatum or prefrontal cortex according to the following coordinates: striatum (with reference to bregma and skull): anterior (A) −0.3 mm, lateral (L) 1.2 mm and ventral (V) 1.5 mm; prefrontal cortex: A 1.0 mm, L 0.3 mm and V 1.0 mm. Glass pipettes with an outer diameter of 50–70 μm were used in the injections. Special care was taken during the injection and removal of the glass pipettes to minimize spread of tracer. The injected animals were used for FACS and TH immunostaining 24 h later for retrograde transport of the green microspheres. The animals used for *in situ* hybridization were injected with red microspheres.

2.3. Immunofluorescence staining

Twenty-four hours after injection with fluorescence microspheres, brains were removed from mice, fixed overnight at 4 °C in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose, and then sectioned in the coronal plane at 20 μm thickness. For immunofluorescence staining, sections were blocked in 0.3% BSA, 10% donkey serum, and 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) for 1 h at room temperature, then incubated in rabbit anti-TH primary antibody solution (diluted 1:2000, Sigma). A secondary reaction was done using Cy2-conjugated donkey anti-rabbit antibody (diluted 1:200, Jackson ImmunoResearch, West Grove, PA). Images were acquired using a Zeiss Axioscope microscope.

2.4. Fluorescence-activated cell sorting

The midbrain were dissected 24 h after injection of fluorescent microspheres, then the cells of midbrain were dissociated using PPD solution (0.01% papain, 0.1% (w/v) neutral protease and 0.01% DNase I) in Hank's Balanced Salt Solution twice for 15 min at 37 °C with gentle triturating. Cells were resuspended in DMEM/F12 culture medium, and filtered through a 70-μm mesh. Subsequently, these cells were sorted on a FACS Vantage SE cell sorer (Becton Dickson, ABI PRISM 7900) for fluorescein-5-isothiocyanale (FITC) signals (Green fluorescent microspheres). Both mesostriatal and mesocortical DA neurons were selected. Cells were kept on ice pre- and post-sorting.

2.5. Quantitative real-time PCR

Real-time PCR was performed to detect the relative abundance of Ephrinb3 mRNA in mesocortical and mesostriatal DA subtype neurons. In brief, total RNA was extracted from the sorted DA neurons using Trizol reagent (Invitrogen, Karlsruhe, Germany) and then mRNA was reverse transcribed into cDNA using the Omniscript Reverse Transcription (Qiagen, Hilden, Germany). The cDNA was then subjected to real-time PCR on an ABI PRIS 7900 Sequence Detection System (Applied Biosystems, Germany). The PCR parameters were 40 cycles with 95 °C denaturation for 15 s, 60 °C annealing for 1 min and 72 °C extension for 50 s. The primers for Ephrinb3 were forward primer: 5′-TGCTGCTGTTAGGTTTTGCG-3′; reverse primer: 5′-CTGAGGAT AAAGCACGTAACCG-3′ (amplicon length, 113 bp). The primers for GAPDH (endogenous control) were obtained from Applied Biosystems. Samples were run in triplicate, and GAPDH expression levels were used for normalization. Calculation of relative abundances of Ephrinb3 mRNA was determined using the comparative C_T method after normalization to reference. Briefly, the difference (ΔC_T) between the C_T values of the target (Ephrinb3) and the reference (GAPDH) was calculated as following: $\Delta C_T = C_T(\text{target}) - C_T(\text{reference})$. And the comparative $\Delta\Delta C_T$ calculation involved finding the difference between the target's ΔC_T and the reference's ΔC_T. The last step in quantization was to transform these values to relative values as: comparative expression level $= 2^{\Delta\Delta C_T}$. In this experiment, the expression of Ephrinb3 was compared between mesostriatal and mesocortical groups.

2.6. In situ hybridization

In situ hybridization was performed as in a previous report [26] with modifications. Briefly, the mice were perfused with 4% PFA. The brains were cryoprotected in graded sucrose solutions and sectioned at 40 μm thickness. Free-floating sections were postfixed with 4% PFA for 10 min, washed three times in phosphate buffered saline (PBS) over 5 min. Subsequently, the sections were placed in prehybridization buffer (50% formamide, 5× SSC, 50 mg/ml tRNA, 1% SDS, 50 ml/ml heparin) and incubated at 60 °C for 1 h. ISH was performed using digoxigenin (DIG)-labeled riboprobe to mouse Ephrinb3 (GenBank Accession No. NM_007911) and FISH was performed using fluorescein-labeled RNA probe of mouse Ephrinb3. The probe concentrations for ISH and FISH were both 500 ng/ml. After incubation overnight, hybridization was continued by washing sections two times for 45 min at 60 °C in SolX (50% formamide, 2× SSC, 1% SDS), then rinsed three times for 15 min in TBST (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris–HCl, pH 7.5, 0.1% Tween 20) at room temperature. ISH signal was detected with an alkaline phosphatase-conjugated anti-DIG antibody and NBT_BCIP (Roche Diagnostics), and FISH signal was directly detected under a fluorescence microscope (Zeiss).

3. Results

3.1. Retrograde labeling mesostriatal and mesocortical DA neurons

To label mesostriatal and mesocortical DA subset neurons, the green fluorescence microspheres were injected into the striatum and the medial prefrontal cortex, respectively. Only the animals with the appropriate injection area were used in the experiments. Fig. 1A and C was the schematic diagram of tracer injection, Fig. 1B and D was the typical injection sites of striatum and prefrontal cortex. The injection sites were clear and remained restricted. Because the fluorescence microspheres were retrogradely transported to neuronal cell bodies, the mesostriatal and mesocortical DA cells could be labeled with the injected microspheres.

To examine the anatomical location of dopaminergic neurons, the traced midbrains were sectioned and then immunostained with TH primary antibody coupled with Cy3-conjugated secondary antibody. Labeled neurons were easily detected in the midbrain (Fig. 2A and C). When the tracer was injected into the striatum, fluorescent microspheres were detected to colocalize with TH-positive neurons, mainly in SN with a few in VTA. In contrast, injection of tracer into prefrontal cortex mainly labeled neurons in VTA. High-power images further illustrated the presence of retrogradely transported beads from either the mesostriatal or mesocortical neurons (Fig. 2B and D). Fluorescence was clearly detected in cell bodies and axons. The neurons labeled by microspheres were also positive for TH.