THE ROLE OF Ret RECEPTOR TYROSINE KINASE IN DOPAMINERGIC NEURON DEVELOPMENT

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Abstract—Glial cell line–derived neurotrophic factor (GDNF) is one of the most potent trophic factors identified for promoting survival and function of dopaminergic (DA) neurons in the midbrain. Ret, a member of the receptor tyrosine kinase (RTK) superfamily, mediates GDNF signaling. The role of Ret in the development of DA neurons is not clear however. Here we demonstrate the involvement of Ret in the DA neuron development both in vitro and in vivo. The dopamine transporter (DAT) gene was clearly induced in rat embryonic neural precursors that had been transfected with Ret. Temporary blockade of Ret expression in embryos using Ret antisense oligonucleotides (Ret-AS-ODN) in vivo led to reduced striatal DA content and a decrease of tyrosine hydroxylase (TH) positive fibers in the striatum. Additionally, some DA neurons in the substantia nigra (SN) underwent apoptotic cell death following the Ret-AS-ODN treatment. Taken together, the data suggest that normal function of Ret is required in vivo for the maturation of DA neurons, in particular for cell survival and fiber innervation. We further demonstrated Ret-induced expression of DAT in vitro. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Ret receptor tyrosine kinase, glial cell line–derived neurotrophic factor, dopamine transporter, antisense oligonucleotides, apoptosis.

Gial cell line–derived neurotrophic factor (GDFN), a distantly related member to the transforming growth factor-b (TGF-b) superfamily, was initially isolated and characterized as a potent neurotrophic factor specific for the survival and differentiation of midbrain dopaminergic (DA) neurons (Lin et al., 1993; Kotzbauer et al., 1996; Milbrandt et al., 1998). Subsequently it was shown that GDNF can also support the survival of a wide variety of CNS and peripherals nervous system (PNS) neuronal cell types, such as motor neurons and subsets of peripheral sensory, sympathetic and parasympathetic neurons (Henderson et al., 1994; Buj-Bello et al., 1995; Oppenheim et al., 1995; Trupp et al., 1995). Animal study has shown that GDNF protects mesencephalic DA neurons against assaults including axotomy, 6-hydroxydopamine- (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP) induced degeneration; GDNF was further shown to partially reverse effects of 6-OHDA- and MPTP-induced degeneration (Beck et al., 1995).

A multicomponent receptor for GDNF was identified as a complex consisting of a novel glycosylphosphatidylinositol-linked protein termed glial cell line–derived neurotrophic factor receptor-α (GDNFRα) and the receptor tyrosine kinase (RTK) Ret (Durbec et al., 1996; Treanor et al., 1996; Trupp et al., 1996). In this receptor complex, GDNFRα serves as the ligand-binding component and Ret serves as the signaling component. The resulting ligand-receptor interaction between GDNF, GDNFRα, and Ret triggers the autophosphorylation of tyrosine residues and the initiation of mechanisms underlying GDNF signal transduction (Massague, 1996).

The Ret proto-oncogene encodes a member of the RTK superfamily that is expressed during vertebrate embryogenesis in the developing excretory system, in the PNS and in motor and catecholaminergic neurons of the CNS, including ventral midbrain DA neurons (Takahashi and Cooper 1987; Takahashi et al., 1998; Avantaggiato et al., 1994; Durbec et al., 1996b; Marcos and Pachnis, 1996; Trupp et al., 1997; Young et al., 1998). Despite the widespread expression of Ret in the vertebrate nervous system, Ret mutations affect only a subset of PNS ganglia and loss of function mutations of Ret lead to congenital megacolon (Hirschsprung’s disease) in humans, a condition characterized by the absence of enteric ganglia from the terminal colon (Edery et al., 1994; Romeo et al., 1994). Mice homozygous for a targeted mutation of Ret die within 12–24 h of birth and lack the superior cervical ganglia (SCG) and enteric ganglia posterior to the proximal stomach (intestinal aganglionosis) (Schuchardt et al., 1994; Durbec et al., 1996b). The Ret-deficient mice have additional congenital problems beyond the nervous system including severe hypodysplasia or aplasia of the kidneys (Schuchardt et al., 1994, 1996; Robertson and Mason, 1995). However, DA neurons continue to be generated in Ret-deficient animals (Marcos and Pachnis, 1996). Detailed analyses of DA neuron numbers, cell maturation and the extent of target area innervation have not yet been reported. In mice lacking GDNF, DA neurons in the sub-
stria
tia nigra (SN) and the ventral tegmental area (VTA) appear to be present in normal numbers at birth (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). These results demonstrate that GDNF/Ret may not be necessary for the differentiation and prenatal development of DA neurons.

Ret mRNA can be detected as early as embryonic day 11.5 (E11.5) in the ventral midbrain of mice (Walled et al., 2001), suggesting that Ret should have some effect on the development of DA neurons. Indeed, the Ret ligand GDNF is important for axonal sprouting as exogenously administered GDNF induces sprouting of DA neurons (Batchelor et al., 2000; Tomac et al., 1995). Moreover, a study examining transplantation of cells from GDNF-deficient mice into the adult striatum demonstrated that DA neurons from GDNF-deficient mice are severely impaired in their ability to survive and innervate host striatal tissue (Granholm et al., 2000). These results suggest that Ret affects both postnatal survival and innervation of DA neurons. Such postnatal functions cannot be resolved in knockout models since mice with null mutations for Ret die at an early postnatal age. In the present study we report the findings of experiments in culture cells as well as in animals treated with Ret antisense oligonucleotides (Ret-AS-ODN) in vivo; the aim of these experiments was to test the role of the Ret on the development of DA neurons both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Cell culture

Primary cultures of precursors from E13.5 mesencephalic tissue were prepared as described earlier (Storch et al., 2001). Timed-pregnant Sprague–Dawley rats (250–300 g) were purchased from the Experimental Animal Center at Beijing University Medical School (Beijing, China) and housed under standard laboratory conditions. All procedures for animals were performed in agreement with the SIBS Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee, Beijing Institutes for Biological Sciences. All efforts were made to minimize animal suffering and the number of animals used. Rats were killed and the midbrain from E13.5 rat embryos was dissociated and mechanically triturated. The cells were plated at a final density of $4 \times 10^5$ cells/cm$^2$ on poly-o-lysine-coated 24-well plates or flasks in completely serum-free N2 medium, consisting of 1:1 mixture of F12 and DMEM with 0.6% glucose, 100 U/ml penicillin and 0.5 $\mu$g/ml streptomycin. This media was supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml fibroblast growth factor (bFGF). Cells were incubated in a 95% air, 5% CO$_2$ atmosphere at 37 °C. Every three days, half of the medium was changed and growth factors were supplemented.

Retroviral vector construction and cell infection

To construct a retroviral vector that expresses Ret genes, the human Ret cDNA was released from pRC-CMV-HRet (a gift kindly provided by Dr. Masahide Takahashi, NU, Nagoya, Japan) by digestion with HindIII and ClaI. Ret cDNA was gel-purified and ligated into pLNCX2 (neo+; Clontech, Mountain View, CA, USA) retroviral vector to give the final pLNCX2-hRet expression vector. The construction was verified by restriction analysis. The PT67 packing cells were transfected with pLNCX2-hRet using Lipofect AMINE 2000 (Invitrogen, Carlsbad, CA, USA), followed by selection with G418 (300 $\mu$g/ml). The titers were measured with NIH 3T3 cells as target cells.

Cells were allowed to expand for 5 days before infection. The retrovirus supernatant containing Ret cDNA with 8 $\mu$g/ml polybrene was added to the cells. Twelve hours after infection, the supernant was removed and the cells were further incubated in the complete medium; 24 h later, the same infection procedure was repeated.

**In vivo embryo microinjection**

The day on which spermatozoa were found in the vaginal smear was defined as day 0 of pregnancy. Surgery was performed on the morning of day 14 of gestation. Rats were deeply anesthetized with chloral hydrate (360 mg/kg, i.p.); 1.5 cm incision up symphysis pubis was surgically opened on the abdomen of the mother rats under sterile conditions and the uterus was gently lifted and oligodeoxynucleotides (ODNs) were injected into the amniotic cavity using a pulled glass needle (100 $\mu$m diameter) connected to an air-injection system. Injection volume was regulated to $\approx 1 \mu$l, giving a final ODN concentration of $\approx 30, \mu$M (Bavik et al., 1996). The uterus was then replaced into the abdominal cavity and the abdominal incision was sutured. After recovery from anesthesia, the rats were return to the cages with normal feeding for another 48 h. Embryonic brain tissue was subsequently obtained for RT-PCR, immunohistochemistry and high performance liquid chromatography (HPLC) testing.

Two sense and corresponding antisense phosphorothioate ODN probes, derived from the 5' upstream flanking regions of phosphotyrosinase kinase domains of Ret (sense 31-mer 5'-tta tag tag ta gta tag tag taa gta tag taa t-3', antisense 33-mer 5'-cct gcc cct ctc tga agg aag aag cgt gcc cgc-3') (Liu et al., 1996) were synthesized by Biologic Technology Co. Ltd., Shanghai, China. These antisense ODNs (AS-ODN) did not display significant homology with other known nucleotide sequences of mammalian genes available in the Gen EMBL data bank. One additional nonsense 31-mer phosphorothioated ODN was also synthesized with the sequences 5'-tta tag tag taa gta tag tag taa tag taa t-3' (Liu et al., 1996). All ODNs were dissolved in PBS and PBS was the vehicle used for control injections.

**RT-PCR**

Total RNA was isolated from cell culture or tissue using TRIZOL® reagent and cDNA was made using Superscript II reverse transcriptase system (Invitrogen) from 8 $\mu$g of total RNA. RT-PCR products were analyzed on an agarose gel containing ethidium bromide. DNA bands were photographed using a Kodak DC 290 Zoom Digital Science camera (Kodak, Rochester, NY, USA). The image was exported as a TIFF file and DNA bands were quantified using the Gel Doc 2000 video system. The quantification value of the band was designated by the absorbance (pixels). For quantitative PCR, cycle number and template quantity were determined to be in the linear range for each gene. Three independent PCR runs were performed for each experiment. The following primers were used to amplify target cDNA: Ret, 5'-gat cac gac gaa ctt cacc-3' and 5'-aga cct ggt tct cca tgg agt-3'; GFRα1, 5'-gat tgt ctg atg tcc gcc gag-3' and 5'-aatt cag tcc cga gtc ggc cag-3'; DAT, 5'-gag cca atg tct tca gtt ggc gc-3' and 5'-gga tcc atg gga ggt cca tgg-3'.

**Western blot analysis**

Embryonic brains were collected and homogenated in cell-lytic buffer (50 mM Tris–HCl (pH 7.5), 2 mM DTT, 2 mM EDTA, 1 mM EGTA, 150 mM NaCl and 1% SDS). Samples (40 $\mu$l/fane) were fractionated by SDS-PAGE in 5% acrylamide gels. The fractioned proteins were transferred to a nitrocellulose membrane. The membrane was cut in half and the top half was used for Ret immunoblotting while the bottom half was used for actin immunoblotting. The blot was blocked with 5% milk in tris-buffered saline with 0.1% TBS-T and probed overnight with primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies, the membranes were developed using developer (Roche Molecular Biochemicals, Mannheim, Germany).
The supernatant was collected and the contents were determined by homogenization, and centrifuged at 13,000 g for 10 min (DAB) and 0.01% H2O2 for 5 min. The antibody binding was visualized using Western blotting luminal reagent (Santa Cruz) according to the manufacturer’s instructions. In a darkroom, membranes were exposed to film for 15 min and developed according to routine protocols.

**Immunocytochemical staining**

Cultures were fixed with 4% paraformaldehyde, washed in PBS, and incubated at 4 °C overnight in dilution buffer with rabbit-anti-TH at a 1:5000 dilution (Sigma, St. Louis, MO, USA). After washing, cultures were incubated for 30 min in dilution buffer with secondary anti-rabbit IgG antibodies (Cy3 coupled IgG, 1:300, Sigma). Nuclei were counterstained with DAPI. Quantitative immunocytochemical data were shown as means ± standard error (S.E.) of cell counts obtained from 10 nonoverlapping 40× fields/condition from three separate experiments. Photographs were taken with Leica DC Viewer Digital Imaging Systems for Professional Microscopy (Leica, Wetzler, Germany). The number of positive cells was counted by using Leica Qwin Analysis software V2.8.

**Immunohistochemistry**

Brains were fixed in 4% paraformaldehyde for 3 days and then transferred to 30% sucrose in 0.1 M PBS overnight at 4 °C. Whole brains were successively cryosectioned coronally at 30 μm and every third section was selected for TH immunohistochemical staining. The sections were pre-treated with 1% H2O2 for 10 min, blocked with 5% fetal calf serum in PBS for 30 min and incubated with rabbit-anti-TH at a 1:5000 dilution (Sigma) at 4 °C overnight. After washing, sections were incubated for 2 h with secondary anti-rabbit IgG antibodies. The immunoreactivity was visualized by the avidin–biotin–peroxidase method (ABC kit, Vector, Burlingame, CA, USA) combined with 0.05% 3,3′-diaminobenzidine (DAB) and 0.01% H2O2 for 5 min.

**HPLC analysis**

The tissue of the striatum was isolated and suspended in 100 μl 0.1 N perchloric acid in 0.3 mM EDTA and 0.1% cysteine on ice, homogenized, and centrifuged at 13,000×g for 10 min at 4 °C. The supernatant was collected and the contents were determined by HPLC with electrochemical detection using a reverse phase column (Smith et al., 2003).

**Cell apoptosis assay**

Apoptotic cells were identified in sections of the subthalamic nucleus (SN) with TH immunohistochemical staining by TUNEL histochemistry using the In Situ Cell Death Detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. For double-labeling experiments, sections were incubated in 1% CoCl2 10 min before the DAB substrate was applied. This substrate produced a dark purple color which could be differentiated from the brown color obtained from TH immunohistochemistry.

**Stereology**

The total number of TH-positive neurons on one side of the SN was counted (n=4) throughout the entire extent of the SN. The SN was delineated according to the atlas (Alvarez-Bolado and Swanson, 1996). Cell counts were performed using a computer-assisted image analysis system. TH fiber density in the striatum was also calculated by Leica Qwin Analysis software V2.8. The striatum of each brain section was viewed at low power (10×), the region of TH-positive fibers was outlined and the area was measured. The area value was fixed at the same anatomic site in both control and experimental sections. The number of TH positive fiber/area was determined. Values are expressed as mean ± S.E. Differences among means were analyzed by using one- or two-way analysis of variance (ANOVA). The data were processed by SPSS for Windows software V11.5 (SPSS, Inc., Chicago, IL, USA).

**RESULTS**

Ret induces the expression of DAT in vitro

After 3–5 days in culture, the cultured cells formed a monolayer and cell aggregating were present (Fig. 1A). To characterize the nature of the cultured cells, several markers were tested. Immunofluorescent staining revealed that 89.5±6.70% of the cells expressed nestin (Fig. 1B, C), a marker for immature neural cells, indicating the precursor-like nature of these adherent cells. Ret was not expressed in those precursor cells (Fig. 1D) and this was confirmed by RT-PCR (Fig. 1G).

To study the function of Ret, retroviral infection was used to overexpress Ret in the precursor cells. The cells began to express DAT for 3 days after transfection before the expression of DAT was tested. RT-PCR results showed that Ret mRNA was present in the transfected cells (Fig. 1G) and Ret immunocytochemical staining revealed that ~42% of the cultured cells were successfully transfected (Fig. 1E, F). Ret immunoreactivity was apparently stronger in the nuclei than in the cytoplasm and membrane as shown in Fig. 1E. The reason is not clear.

The dopamine transporter (DAT) gene was not expressed in cultured primary precursors nor in cells treated with GDNF. Faint DAT mRNA was found in cells overexpressing Ret. However, strong expression of DAT was found in Ret-transfected cells that had been treated with GDNF (20 ng/ml) (Fig. 1G). These data suggest that the GDNF/Ret pathway plays a role in the maturation of DA neurons.

Ret does not induce TH positive cells in vitro

To investigate the effects of Ret on TH positive cells, we normalized the number of TH positive cells to the total number of cells, which was ascertained using DAPI, a nuclear stain. The percentage of TH positive cells in primary culture was 1.59±0.18% and 1.53±0.12% when treated with GDNF or nothing, and 1.82±0.21% (Fig. 2A) and 1.70±0.33% in Ret-transfected cells treated with GDNF or nothing (Fig. 2B). There were no significant differences in the number of TH positive cells in any treatment group (P>0.05). These data indicate that Ret does not induce TH positive cells in vitro.

Embryonic microinjection of Ret-AS-ODN can block Ret expression in vivo

To determine the appropriate dose of ODN and the time point of application to embryos, pre-experiment of Nurr1-AS-ODN transfection in vitro was done; 10 μM, 20 μM, and 40 μM.
40 μM, 80 μM and 120 μM ODN were used to observe the cell viability and the block of target protein. Forty-eight hours of application of 20–30 μM ODN was selected.

To investigate the role of Ret during the development of DA neurons in vivo, Ret-AS-ODN was injected into amniotic fluid; ~30 μM ODN was injected into amniotic fluid, 48 hours after microinjection, embryos were dissected from the uterus and embryonic development in vivo was assessed using multiple assays (Table 1). No malformations were observed in any embryo suggesting that the embryos developed normally and that the Ret-AS-ODN had no toxic effects on the embryos.

To verify that Ret-AS-OND could block embryonic Ret expression, total RNA was isolated from brain tissue in 12 embryos. RT-PCR revealed that microinjection of Ret-AS-ODN in vivo blocked Ret expression completely in nine of 12 cases (Fig. 3A). This was further confirmed by Western blot assays (Fig. 3B). In the remaining three of 12 cases, Ret expression was either partially blocked or unchanged. Injections of PBS, sense ODN and nonsense ODN were used as controls (n=6 for each group) to test the specificity of Ret-AS-ODN. RT-PCR revealed that Ret expression was not blocked in any control group indicating that this Ret temporarily “knockdown” model could be used.

Effects of Ret on cellular survival and fiber innervation of DA neurons and maintenance of striatal DA levels in vivo

To investigate the role of Ret on the development of DA neurons, TH immunohistochemical staining was performed on four cases where Ret-AS-ODN resulted in a total block-

Fig. 1. Ret induces the expression of DAT in vitro. Primary cultures of precursors from E13.5 mesencephalic cells were expanded for 5 days. Phase contrast photographs show monolayer and cell aggregating (A). Immunofluorescent staining shows nestin positive cells (B) and DAPI staining (C) with the same field as (B). Ret immunofluorescent staining showed that no Ret positive cells were found with the precursors (D), and ~42% of the cultured cells were Ret positive after Ret transfection (E), inset is the high magnification. A phase contrast photograph as the same field as (E) is shown in (F). RT-PCR results show that Ret and DAT are not expressed in the primary cultures with or without treatment of GDNF (G). However, DAT expression was clearly induced in the cells overexpressing Ret with treatment of GDNF (1. control, 2. control treated with GDNF, 3. cells overexpressing Ret, 4. cells overexpressing Ret treated with GDNF, 5. rat brain tissue from E17 used as a positive control). Scale bar=100 μm in A, D–F. Scale bar=50 μm in B–C and inset.
ade of Ret expression. TH immunohistochemistry revealed that TH positive cells were located in the SN and their projections in the striatum (Fig. 4). The total number of TH positive neurons was counted throughout the entire extent of the SN unilaterally and there was no significant difference in the average total number between the control and Ret-AS-ODN groups (3395 ± 35.9 vs. 3407 ± 42.6) (Table 2). TH density in the striatum was also calculated and density in the Ret-AS-ODN group was significantly lower than control (1809.33 ± 17.9 vs. 2980.19 ± 30.6; Fig. 5A–B and Table 2).

DA levels in the striatum were tested by HPLC with five cases where Ret-AS-ODN completely blocked Ret expression. The results show that the average striatal level of DA was reduced to about 50% of the control group (Fig. 5E). Treatment Number Diameter of yolk sac (mm) Crown–rump length (mm) Length of head (mm)

Control 8 14.87 ± 1.34 13.13 ± 1.87 6.97 ± 2.11
PBS 6 14.04 ± 1.98 12.98 ± 2.31 5.92 ± 1.56
Nonosense-ODN 6 14.18 ± 3.21 12.87 ± 3.33 5.89 ± 1.32
Ret-sense-ODN 6 13.69 ± 2.67 13.01 ± 2.32 6.12 ± 2.22
Ret-AS-ODN 12 13.18 ± 3.48 12.86 ± 2.55 6.05 ± 2.57

Table 1. Effects of ODN treatments on embryos (mean ± S.E.)

DISCUSSION

The precursors isolated from E13.5 ventral midbrain cells should have the capacity to develop into DA neurons since 70% of DA neurons in the adult brain are located in this area (Nelson et al., 1996). In present study, Ret was not expressed during this developmental stage of precursors, as confirmed by immunohistochemistry and RT-PCR studies, although both TH and Ret begin to express from E11.5 in developing neurons (Wallen et al., 2001). So these precursors could be used as a tool to study role of Ret in the development of DA neurons in vitro. As differentiation of the mantle layer of the embryonic rat brain is just initiated at E13 (Kandel et al., 2000), a few TH-positive cells (1.53 ± 0.12%) could be found in our primary cultures. However, the DAT gene was not found in these cultured cells regardless of GDNF treatment. However, overexpression of Ret combined with GDNF treatment could induce DAT expression. It is known that the DAT gene transcript is detectable during the ontogeny of rat ventral mesencephalon at around E15 (Perrone-Capano et al., 1994). Once the DA axons have reached this nucleus at around E16 in the rat, the onset of DAT gene expression is rapidly followed by the appearance of high affinity DA uptake in the striatum (Fiszman et al., 1991). The expression of DAT therefore may indicate the maturation of DA neurons in the ventral mesencephalon and may demonstrate that Ret and its ligand GDNF play an important role in the maturation of DA neurons in vitro. Previous studies suggested that the expression of the DAT gene in DA neurons depends on direct target striatal cell interactions (Perrone-Capano and Di Porzio, 1996). In the present study, the cultured primary cells were heterogeneous and quite a few of the cells were likely to be glial progenitors. That is, 43.37 ± 7.25% of the cultured cells expressed GFAP, a marker for astrocytes (data not shown). After expansion for 10 days in vitro, it is possible that these cells could function as DA neuronal target cells to influence DA neurons. Furthermore, the addition of exogenous GDNF could promote DAT expression. Although the DAT gene could be tested in cells overexpressing Ret, there was no significant difference in the number of TH positive cells, suggesting that Ret may only be involved in the maturation rather than induction of DA neurons. The exact mechanism of Ret single pathway involved in this process needs further research work.

RT-PCR and Western blot analyses revealed that Ret expression was completely blocked 48 h after microinjection of Ret-AS-ODN, indicating that the Ret-AS-ODN provides a reliable depletion. It is known that a facilitation of
uptake mechanisms, intracellular trafficking and nuclease resistance of phosphorothioate modified oligodeoxynucleotides (P-ODN) might facilitate the use of these compounds for experimental and therapeutic purposes. However, toxicity to the host cells occurs when the highest doses are administered (Gamper et al., 1993). In this study, six of the 5'-most and 3'-most phosphodiester bonds were replaced by phosphorothioate bonds, leaving 21 unmodified bonds in the center of the ODN with the all synthesized ODN used in the experiment (Kofron et al., 1997). All of the embryos developed without malformations suggesting that our temporary Ret knockdown model could be employed to study the role of Ret in vivo.

The levels of DAT gene transcripts and the number of uptake sites are selectively increased in rodent E13 mesencephalic DA neurons in vitro after adding E16 striatal cells in co-culture (Perrone-Capano and Di Porzio 1996). In the striatum, DA is first detected at E16, suggesting that DA nigral fibers reach their target tissue at this embryonic age (Fiszman et al., 1991; McCaffery and Drager 1994). These results suggest that mesencephalic DA neurons respond to specific target influences within a restricted developmental window from E13 to E16, which is consistent with the period of fiber innervation to the striatum from the DA neurons. At this time, GDNF mRNA begins to be present at a low level in the mesencephalon (Choi-Lundberg and Bohn, 1995). Using Cy3-labeled p53-PT-ODN, Tepel et al. (2004) found that cellular uptake of ODN was achieved in vivo within 3 h after i.p. injection. Although P-ODN can increase the stability of ODNs, they are still degraded by nucleases within 48 h (Latham et al., 1994). In our in vitro experiments, the target protein was completely blocked in cultured cells 24 h after AS-ODN was applied, and this protein appeared again at low levels 72 h after application of AS-ODN. Therefore, we chose E14 as the first day to begin blockade of Ret expression by Ret-AS-ODN and 48 h later, brain tissue was obtained to examine changes in DA neurons.

In the present study, immunochemical staining showed that TH positive fiber density in the striatum was decreased in embryos with inhibition of Ret, indicating that Ret may play a role in DA fiber innervation. It is known that during neuronal development, the direction of axonal target innervation is determined by intracellular signals and guidance in the environment from the point of origin to the appropriate targets including soluble, membrane-bound, and extracellular matrix molecules (Goodman 1996). Ledda et al. (2002) recently reported that target-derived GFRα1 can also act in trans as an axonal guidance molecule for neurons. A relative reduction of GFRα1 in developing axons allows exogenous GFRα1 to capture GDNF and utilize it for recognition by axonal Ret receptors. This exogenous GFRα1 potentiates neurite outgrowth and acts as a long-range directional cue by creating positional information for Ret-expressing axons in the presence of GDNF by activation of cyclin-dependent kinase 5 (Cdk5). Ret was expressed in DA neurons as early as E13.5, so GDNF and its receptor complex may act as cues guiding DA axons to their target, the striatum. The reduced number of TH positive fibers in the striatum after Ret inhibition in our in vivo experiments further confirmed that Ret influences maturation of DA neurons.

During the course of development, most neuronal populations undergo a regressive event, termed natural, developmental, or physiologic cell death (Cowan et al., 1984; Oppenheim, 1991). Like most neuronal populations, DA neurons in the SN do not seem to escape this pruning process. The genesis of nigral DA neurons is completed between E11 and E17 in the rat. This initial pool of DA neurons in the SN is subjected to a regressive event that begins just before birth and proceeds during the first 3 weeks of life (Janec and Burke, 1993; Oo and Burke, 1997). The number of DA neurons in the SN undergoing apoptosis is highest at postnatal day (PND) 2 and drops rapidly, reaching a low at PND 10. A transient resurgence in the number of apoptotic cells follows, peaking at PND 14. At PND 32 and older, apoptotic neurons are no longer seen in the SN (Jackson-Lewis et al., 2000). In the present study, TUNEL positive cells could be found in TH positive cells with Ret inhibition in the SN, while no apoptotic cells
were present in the control, indicating that this apoptosis was not caused physiologically. It has already been shown that GDNF can promote the survival, proliferation and differentiation of DA neurons as an autocrine or paracrine molecule target from the striatum and is transported retrogradely to the DA cell bodies in the mesencephalon (Tomac et al., 1995). The blockade of Ret leads to apoptosis in DA neurons, suggesting that Ret plays an important role in the maintenance of survival of DA neurons. In the present study, it is understandable therefore that the loss of striatal DA content was accompanied by the loss of TH positive fibers and the apoptosis of DA neurons.

Table 2. Total number of TH positive cells in the SN and TH fiber density in the striatum (mean±SE)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
<th>Control</th>
<th>Ret-AS-ODN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH positive cells in SN</td>
<td>4</td>
<td>3395±35.9</td>
<td>3407±42.6</td>
</tr>
<tr>
<td>TH fiber density in striatum</td>
<td>4</td>
<td>2980.19±30.6</td>
<td>1809.33±17.9*</td>
</tr>
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* P<0.05 as compared with control groups.
Since the principle of TUNEL staining is to label the end of cleaved DNA, reflecting the very early stages of apoptosis, the fact that our experiment focused on a very short period (48 h) may have led to inadequate time for complete morphological changes to occur. With more time, greater levels of apoptosis of DA neurons may have been achieved and the number of DA neurons should have been decreased.

AS-ODN is a useful tool in achieving specific inhibition of targeted gene expression (Stein et al., 1991). AS-ODN combined with in vivo microinjection in the embryos provides a model to study specific genes that may be involved during development. One caveat is the rapid degradation of the ODN, resulting in the need for repeated applications. Future studies should therefore employ siRNA techniques since they provide longer periods of gene inhibition.

Although several genetic studies have established the critical roles of GDNF, GFRα-1 and Ret in the development of the mammalian enteric nervous system (ENS) (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998), the exact role of Ret in the development of DA neurons is not clear. In this study, we demonstrated for the first time that temporary knockdown of Ret expression during a critical period of embryonic development leads to the decrease of TH positive fibers in the striatum and the content of DA, as well as the apoptosis of DA neurons in the SN. Through in vivo and in vitro experiments, the data strongly suggest that Ret is responsible for the later maturation of DA neurons, including their survival and fiber projections during development of the mesencephalon. Although our experiment is confined to the embryonic tissue, we speculate that the DA neurons would continue to lose due to apoptosis. So the death of Ret knockout mice is the result of a complicated reason, such as lack of ganglia and DA neurons. This gene temporary knockdown model by AS-ODN microinjection in vivo could be used to study postnatal functions of Ret in the future.

CONCLUSION

Here we report on studies that address the in vitro and in vivo roles of the Ret on the development of DA neurons. The DAT gene was clearly induced in the rat embryonic neural precursors transfected with Ret. Temporary block of the expression of Ret in embryos led to low striatal DA content and the decrease of TH positive fibers in the striatum. Additionally, a few TUNEL positive cells could be identified with the TH positive cells. Taken together, these findings suggest that normal function of the Ret is required in vivo for the maturation of DA neurons; Ret appears to be particularly important for DA neuron survival and fiber in-

![Fig. 5. Effect of Ret on the maturation of DA neurons in vivo. TH positive fiber density in the Ret-AS-ODN group (B) was reduced compared with control (A). (A and B are from same anatomic site, n=4.) The average level of DA in the striatum of Ret-AS-ODN-treated brains was reduced to half of control (E) (* P<0.05, n=5, values expressed as mean±S.E.). Some TUNEL positive cells were found in TH positive cells in the Ret-AS-ODN-treated group with dark purple color located in the nucleus (D, arrow), while no TUNEL positive cells was found in the control group (C). Scale bar=50 μm in A–B, and 200 μm in C–D.](Image)
nervation. Additionally, Ret can induce the expression of DAT at least in vitro.

Acknowledgments—This work was supported by the National Natural Science Foundation of China (Nos. 30270433 and 30430280) and the Beijing Committee of Science and Technology (No. Z0005187040311).

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(Taken from the abstract of a scientific paper)