Patterns of developmental mRNA expression of neurturin and GFRα2 in the rat striatum and substantia nigra do not suggest a role in the regulation of natural cell death in dopamine neurons

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Abstract

We examined the mRNA expression of neurturin (NTN) and its receptor GFRα2 in rat substantia nigra (SN) and striatum by northern analysis at ages ranging from postnatal day (PND) 2 to adult. NTN mRNA expression is developmentally regulated in striatum with a peak at PND10, but its expression in striatum is low, and less than that of SN. In SN, there is no developmental regulation. GFRα2 was expressed most highly during the first two postnatal weeks. Like NTN, GFRα2 mRNA was also more abundant in SN, at both PND2 and 14. Our results show that NTN expression is relatively low in the striatum, the target of dopamine (DA) neurons, and there is no apparent pattern of developmental regulation in SN. Thus these studies are not strongly supportive of a role for NTN in regulating natural cell death (NCD) in DA neurons, either as a target-derived or as a local paracrine factor.

Dopamine (DA) neurons of the substantia nigra (SN), like other neuronal populations, undergo a natural cell death (NCD) event during development [17,18,24]. This event is biphasic in rodents, with an initial peak at PND2 and a second at PND14. As envisioned by classic neurotrophic theory [4], the NCD event in these neurons is likely to be regulated by target interactions. In vitro studies have shown that the viability and differentiation of developing mesencephalic DA neurons are supported by striatal preparations [13,15,30]. We have shown in vivo that disruption of interactions between DA neurons and the striatal target, by target lesion [21], DA terminal lesion [22], or axotomy [10] leads to an augmentation of NCD. It is also likely, however, that other nontarget interactions may also regulate this event, such as a local paracrine or autocrine factors, and afferent projections [2]. However, none of the endogenous, physiologic factors that regulate this event for DA neurons have yet been conclusively identified.

The glial cell line-derived neurotrophic factor (GDNF) family members play a significant role as trophic factors, supporting viability of neurons during development. Considerable evidence now suggests that GDNF, in particular, may serve as a striatal target-derived neurotrophic factor for SN DA neurons. Its mRNA is highly expressed in striatum during the postnatal NCD event [6,8,9,28,29], and its receptor, GFRα1, and signaling kinase, Ret, are highly expressed in the SNpc [31,32]. We have shown that GDNF suppresses apoptotic cell death in DA neurons during the postnatal period both in vitro [7] and in vivo [25], and that intra-striatal injection of neutralizing antibodies to GDNF augments the NCD event in vivo [25]. We have also shown that sustained overexpression of GDNF in striatum in double transgenic mice increases the surviving number of DA neurons after the first phase of NCD (Kholodilov et al., submitted).
Neurturin (NTN), another member of the GDNF receptor ligand family of proteins, has been considered to possibly be a neurotrophic factor for DA neurons. It was originally purified and cloned by virtue of its ability to support the survival of sympathetic neurons in culture [20]. NTN exhibits its potent effects on the protection and restoration of midbrain DA neurons both in vitro and in vivo [1,16,20,23]. If NTN is a limiting, striatum-derived neurotrophic factor for
mesencephalic DA neurons, then it would be expected that, like GDNF, its expression should be relatively abundant in striatum, the expression of its receptor should be relatively abundant in SN, and expression should be regulated in phase with NCD events. We have therefore examined the time course, and the relative levels of striatal and SN expression of mRNAs for NTN, and its putative receptor GDNF family receptor (GFR)α2 [3,19,27].

Timed pregnant Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were used to obtain striatum and SN at PND2, 6, 10, 14, 28, and adult. The day of delivery was defined as PND1. SN and striatum tissue were obtained by microdissection as previously described [11]. The tissues were then frozen on dry ice and stored at −80 °C until RNA isolation. This protocol has been approved by the Institutional Animal Care and Use Committee at Columbia-Presbyterian Medical Center.

With a database search, we found three partial sequences for NTN. Two of them (GenBank accession no: AF184922 and AB032562) represent all of the coding sequence except the 5′-end. The third sequence, available through LabOnWeb (Compugen), represents the 5′-end of NTN cDNA. Based on these three known rat NTN sequence sources, we constructed a predicted rat NTN full coding sequence (646-bp) and designed primers. We were unable to obtain a complete product by RT PCR using RNA from several tissues. Therefore, we amplified three fragments of rat NTN cDNA (bps: 1–227, 206–385, and 365–671) and annealed them. PCR was then performed on the annealed fragments, and the full size coding region was successfully amplified. It was cloned into a pGEM-T vector and the sequence confirmed (GenBank accession no: AY190603).

Northern analysis was performed as previously described [11]. Briefly, RNA was isolated using Qiagen RNAeasy Mini

Fig. 1. Developmental time course of NTN mRNA expression in striatum and in SN. (A) Representative Northern analysis of NTN mRNA expression in striatum from PND2 to adult. Twenty micrograms of total RNA was loaded per each lane, and the blot was probed with a riboprobe generated from 306 bp cDNA of NTN. The blot revealed one major transcript, at 1.4 kb. (B) Developmental time course of NTN mRNA expression in striatum (N=16 for PND2 [N=2 per blot, 8 blots were examined]; N=8 for all other time points [N=1 per blot]). NTN mRNA expression is developmentally regulated in striatum. Higher levels of NTN expression were observed during the early developmental period, with the highest level in the PND10 (p<0.05). (C) Representative Northern analysis of NTN mRNA expression in SN from PND2 to adult. (D) Developmental time course of NTN mRNA expression in SN. The temporal pattern of NTN mRNA expression does not show a significant difference at any age, within the limits of the sampling interval used.

Fig. 2. Relative levels of NTN expression in striatum and SN at the two peaks of NCD in SN DA neurons. (A) Representative Northern analysis of NTN mRNA expression in striatum and SN at PND2 (N=2 for each region) and PND14 (N=2 for each region). (B) Quantitative analysis of NTN mRNA expression in striatum and SN at the two phases of NCD in SN (N=8 for each condition). NTN mRNA was more highly expressed in SN during both periods of maximal NCD in DA neurons (p<0.001).
kit. The RNA concentration of each sample was determined by measuring absorption at 260 nm on a GenQuant spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). Twenty micrograms of RNA was electrophoresed in 1.4% agarose-formaldehyde gel and transferred onto an Immobilon membrane (Millipore, Bedford, MA). To create probes for Northern analysis, oligonucleotide primers were designed based on our sequence for NTN (GenBank acces-
and then it progressively decreases after PND 14 (N examined); transcripts, at 3.1 and 4.0 kb (arrows). (D) Developmental time course of GFR the adult time points (Developmental time course of GFR interest between postnatal weeks 2 through 4. Similarly, decreased apparent level of expression of the mRNA of loading, is developmentally regulated[11], so that normal-
abla expression, often used as an indicator of total mRNA
lanes. We have previously shown that GAPDH mRNA of ethidium bromide stained gels to ensure equal loading of
We used quantitative analysis of total RNA and inspection of
vitro transcription system.

The hybridization was performed overnight at 68 °C in Ultrahyb buffer from Ambion (Austin, TX). The membrane was then exposed to phosphor imager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics, Indianapolis, IN). For the developmental studies, all developmental ages were represented by a single animal on each membrane, except PND2 which was represented by two. Eight independent hybridizations were performed, for an N=16 for PND2, and N=8 at all other developmental time points. Radioactive bands were expressed as a relative percentage of the radioactivity at PND2 on each membrane. We used quantitative analysis of total RNA and inspection of ethidium bromide stained gels to ensure equal loading of lanes. We have previously shown that GAPDH mRNA expression, often used as an indicator of total mRNA loading, is developmentally regulated [11], so that normalization by GAPDH mRNA levels results in a spurious, decreased apparent level of expression of the mRNA of interest between postnatal weeks 2 through 4. Similarly, α- and β-tubulin mRNA are developmentally regulated [5]. For these reasons, we did not normalize mRNA determinations by the mRNA for these genes. For the studies of the relative levels of expression in SN and striatum at the two peaks of NCD (PND2 and 14), two animals representing each region and each time point were represented on each blot. Four independent hybridizations were performed, for an N=8 for each region at PND2 and 14.

Northern blot analysis showed that NTN mRNA was detected in both striatum and SN as a single transcript of about 1.4 kb, somewhat larger than the 1.0 kb reported for mouse [20]. In striatum, NTN mRNA expression was developmentally regulated. The level was highest during the early developmental period; it began to increase after PND2 and reached its peak level at PND10, just preceding the second phase of NCD (Fig. 1A,B). Its expression thereafter rapidly declined, and the lowest levels were observed in the PND28 and adult at about 45% of levels observed at PND10. In SN, however, there was no develop-
mental regulation (Fig. 1C,D).

Separate experiments were performed to compare the relative levels of NTN mRNA expression in SN and striatum at the two peaks of NCD at PND2 and 14. Unlike what would be anticipated for a striatal target-derived neurotrophic factor, NTN mRNA was more highly expressed in SN, at more than twice the levels of expression observed in striatum, during both periods of maximal NCD in DA neurons at PND2 and 14 (Fig. 2A,B).

GFRα2 mRNA was detected in SN as a doublet of two mRNA species, approximately 3.1 and 4.0 kb, as previously reported [27]. However, in striatum, where levels of expression were much lower than in SN (see below), the doublet was more difficult to discern. In striatum, GFRα2 was developmentally regulated, with the highest levels during the first 2 weeks (Fig. 3A,B). In SN, there also was develop-
mental regulation, with high levels in the first two postnatal weeks, and the highest levels achieved during the first postnatal week (Fig. 3C,D). Thus, in SN, the GFRα2 receptor for NTN obtains the highest levels of expression prior to the peak level of expression for NTN in the striatal target.

In separate experiments to directly compare the relative abundance of GFRα2 in SN and striatum at PND2 and 14 on the same blot, we found that like NTN, GFRα2 mRNA was also more abundant in SN than striatum (at about 55% of levels observed in SN), at both PND2 and 14 (p<0.001) (Fig. 4).

NTN has been proposed to possibly serve as a target-
derived neurotrophic factor for SN DA neurons. This pos-
sibility has been suggested by its ability to support embry-
onic ventral mesencephalic neurons in culture [1,16], its mRNA expression in developing striatum [1,16], and its ability to both protect and restore adult SN DA neurons in lesion models [1,16,23]. The late developmental expression of NTN in striatum has been interpreted to mean that it may play a sequential role with GDNF, providing support during a later phase of development [1]. Our results are comparable to those of Akerud et al. [1] in that they show a highest level of NTN mRNA expression in striatum at PND10. However, unlike what would be expected for a target-derived neuro-

Fig. 3. Developmental time course of GFRα2 mRNA expression in striatum and in SN. (A) Representative Northern blot of GFRα2 mRNA expression in striatum. Twenty micrograms of total RNA was loaded per lane, and the blot was probed with a riboprobe generated from 597 bp cDNA of GFRα2. (B) Developmental time course of GFRα2 mRNA expression in striatum (N=16 for PND2 [N=2 per blot, 8 blots were examined]; N=8 for all other time points [N=1 per blot]). Note that GFRα2 is expressed during the first two postnatal weeks, with the highest level at PND10 and a significant decrease at PND28 and the adult time points (p<0.05). (C) Representative Northern analysis of GFRα2 mRNA expression in SN from PND2 to adult. The blot revealed two major transcripts, at 3.1 and 4.0 kb (arrows). (D) Developmental time course of GFRα2 mRNA expression in SN (N=16 for PND2 [N=2 per blot, 8 blots were examined]; N=7–8 for all other time points [N=1 per blot]). Note that expression of GFRα2 is abundant during the first postnatal week with a peak at PND6, and then it progressively decreases after PND 14 (p<0.01).
trophic factor, NTN was more abundantly expressed in SN than striatum during both of the major phases of NCD. Thus, in its regional expression pattern, NTN is quite unlike GDNF, which, as substantial evidence indicates, probably plays a role as a striatal target-derived neurotrophic factor during the first phase of NCD at PND2 in DA neurons (reviewed in Ref. [25]). During the first phase of NCD, GDNF mRNA levels in striatal target are at their peak [8] and are much more abundant than the levels in SN (unpublished observation). The higher level of expression of NTN mRNA in SN would be more compatible with a local autocrine or paracrine role in supporting DA neurons. However, against this possible role during development is our observation that there is no developmental regulation of SN expression.

Expression of GFRα2 mRNA in the ventral midbrain has previously been demonstrated by in situ hybridization in adult mice [12,16]. However, whether it is expressed during development has been less clear. Golden et al. [12] detected mRNA in the ventral midbrain of embryonic day 18 mice by in situ hybridization, but Widenfalk et al. [31] and Yu et al. [32] were unable to detect mRNA by in situ hybridization in SN at PND7 in mouse and rat, respectively. We have been able to detect GFRα2 transcripts by Northern analysis in microdissected ventral mesencephalon and find that it is developmentally regulated, with highest levels in the first postnatal week.

However, against the possibility that NTN acts as a neurotrophic factor for DA neurons through the GFRα2 receptor is the observation of Horger et al. [16] that GFRα2 expression does not co-localize with that of SN DA neurons identified by tyrosine hydroxylase immunohistochemistry. This study was performed in adult animals, and while the cellular pattern of expression may be different in the developmental setting, such a possibility at present is unknown. We therefore conclude that our studies, and those of others, of the patterns of mRNA expression for NTN and its putative receptor GFRα2 are not strongly supportive of a developmental neurotrophic role, either as a target-derived factor or as a local paracrine factor. This conclusion is supported by reports of homozygous null mice for NTN [14] and for GFRα2 [26], which show no effect on the mature number of SN DA neurons.

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References


