Published in final edited form as:

Neuroscience. 2009 September 29; 163(1): 482–489. doi:10.1016/j.neuroscience.2009.06.008.

DECREASED EXPRESSION OF ErbB4 AND TYROSINE HYDROXYLASE mRNA AND PROTEIN IN THE VENTRAL MIDBRAIN OF AGED RATS

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Abstract

Decreased availability or efficacy of neurotrophic factors may underlie an increased susceptibility of mesencephalic dopaminergic cells to age-related degeneration. Neuregulins (NRGs) are pleiotrophic growth factors for many cell types including mesencephalic dopamine cells in culture and *in vivo*. The functional NRG receptor ErbB4 is expressed by virtually all midbrain dopamine neurons. To determine if levels of the NRG receptor are maintained during aging in the dopaminergic ventral mesencephalon, expression of ErbB4 mRNA and protein was examined in young (3 months), middle-aged (18 months), and old (24–25 months) Brown Norway/Fischer 344 F1 rats. ErbB4 mRNA levels in the substantia nigra pars compacta (SNpc), but not the adjacent ventral tegmental area (VTA) or substantia nigra pars lateralis (SNl), were significantly reduced in the middle-aged and old animals when compared to young rats. Protein expression of ErbB4 in the ventral midbrain was significantly decreased in the old rats when compared to the young rats. Expression of tyrosine hydroxylase (TH) mRNA levels were significantly reduced in the old rats when compared to young animals in the SNpc, but not in the VTA or SNl. Tyrosine hydroxylase protein levels in the ventral midbrain were also decreased in the old animals when compared to the young animals. These data demonstrate a progressive decline of ErbB4 expression, coinciding with a loss of the dopamine-synthesizing enzyme TH, in the ventral midbrain of aged rats, particularly in the SNpc. These findings may implicate a role for diminished NRG/ErbB4 trophic support in dopamine-related neurodegenerative disorders of aging such as Parkinson's disease.

Keywords

neuregulins; aging; dopamine; substantia nigra; *in situ* hybridization; Parkinson's disease

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Section Editor: Dr. Miles Herkenham

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Neuregulins (NRGs) are a family of structurally related growth and differentiation factors. During development, NRGs play an integral role in many processes including cell survival, migration, and differentiation of both neuronal and non-neuronal cells (Burden and Yarden, 1997; Gassmann and Lemke, 1997; Buonanno and Fischbach, 2001; Yarden and Sliwkowski, 2001; Falls, 2003; Seroogy and Zhang, 2006). Signaling by NRGs occurs through binding to their epidermal growth factor-like receptors, the ErbB receptors (Buonanno and Fischbach, 2001; Yarden and Sliwkowski, 2001; Citri et al., 2003; Falls, 2003). Like NRGs, ErbB receptors are differentially expressed throughout development and play a role in cell growth, migration, differentiation, and myelination in many tissues and organs, including the nervous system (Carraway and Burden, 1995; Burden and Yarden, 1997; Gassmann and Lemke, 1997; Kornblum et al., 2000; Vaskovsky et al., 2000; Fox and Kornblum, 2005; Mechawar et al., 2007; Birchmeier, 2009).

Neuregulins and ErbB receptors are distributed throughout the adult central nervous system (CNS) including cortical areas, hippocampus, thalamus, hypothalamus, amygdala, and ventral midbrain (Chen et al., 1994; Corfas et al., 1995; Steiner et al., 1999; Gerecke et al., 2001; Yurek and Seroogy, 2001; Bruce et al., 2002; Law et al., 2004; Fox and Kornblum, 2005; Bernstein et al., 2006; Thompson et al., 2007). With respect to the ventral midbrain, ErbB4 is expressed in both the substantia nigra and ventral tegmental area (VTA) (Steiner et al., 1999; Gerecke et al., 2001). Moreover, ErbB4 mRNA is localized to dopaminergic neurons in the substantia nigra pars compacta (SNpc) as demonstrated by reduced expression following 6-hydroxydopamine (6-OHDA) lesions and through direct colocalization (Steiner et al., 1999; Yurek and Seroogy, 2001; Thuret et al., 2004; Abe et al., 2009; K.B. Seroogy, unpublished results). Although NRGs and ErbB receptors are present in the adult CNS, their functional roles remain to be fully elucidated, including within the midbrain dopaminergic cells. However, recent findings in fetal mesencephalic dopamine cell cultures demonstrate that the NRG-1 isoform glial growth factor 2 (GGF2) is both neurotrophic and neuroprotective in serum-free and 6-OHDA-challenged cultures (Zhang et al., 2004). Furthermore, when injected directly above the SNpc, GGF2 and another NRG isoform, NRG1- β 1, induce striatal dopamine overflow indicating functional effects of NRGs in the nigrostriatal system *in vivo* (Yurek et al., 2004; Seroogy and Zhang, 2006).

The nigrostriatal system is particularly vulnerable to age-related degeneration as indicated by functional and histological/neurochemical deficits. Some of the changes associated with aging in humans and non-human primates include reduced nigral volume and tyrosine hydroxylase (TH) cell number, and a decrease in striatal dopamine (Irwin et al., 1994; Emborg et al., 1998; Siddiqi et al., 1999; Stark and Pakkenberg, 2004; Collier et al., 2007). In rodent models of aging, some studies have reported decreased function behaviorally and neurochemically (e.g. Emerich et al., 1993; Yurek et al., 1998; Ling et al., 2000; Sanchez et al., 2008), whereas others have been unable to discern any age-related changes (e.g. McNeill and Koek, 1990; Stanford et al., 2002, 2003; Tamás et al., 2005). Nonetheless, taken together most evidence suggests that dysfunction of the nigrostriatal dopaminergic system is not only a consequence of diseases affecting the basal ganglia, but is also a result of the normal aging process. The neural mechanisms responsible for the loss of dopaminergic function during aging remain unknown.

Herein, we examine the effects of aging on the expression of ErbB4 and TH mRNA and protein in the ventral midbrain. The purpose of this study was to determine if there is an age-related decline in the ErbB4 receptor that would suggest a decrease in neurotrophic factor support to mesencephalic dopamine neurons. Diminished NRG/ErbB4 signaling could increase susceptibility of mesencephalic dopamine neurons to age-related degeneration and could have implications into the pathogenesis of Parkinson's disease.

Experimental procedures

Animals

Male Brown Norway/Fischer 344 F1 (BN/F344 F1) hybrid rats (NIA/Harlan Sprague-Dawley) were used in this study at ages 3 months, 18 months, and 24–25 months ($n=10$ for each age group). Adult rats weighed 300–650 g and were housed 2 per cage under normal conditions; 12 hr on/off light/dark cycles with free access to food and water. All procedures were conducted in compliance with the University of Cincinnati Institutional Animal Care and Use Committee.

In situ hybridization

Rats ($n=4$ for each age group) were anesthetized with an overdose of sodium pentobarbital and rapidly decapitated. Fresh-frozen, cryostat-cut, slide-mounted semi-adjacent sections through the rostrocaudal level of the ventral mesencephalon were hybridized with ^{35}S -labeled cRNA probes for detection and localization of ErbB4 and TH mRNAs using *in situ* hybridization, as previously described in detail (Seroogy and Herman, 1997; Numan and Seroogy, 1999; Numan et al., 2005). The TH cDNA plasmid (provided by J. Herman, University of Cincinnati) was contained in a pCR-TOPO vector and consisted of 366 bp. The ErbB4 plasmid (provided by H. Kornblum, UCLA) was contained in a pCR 2.1 vector and consisted of 1.8 kb (Kornblum et al., 2000). Sections were hybridized overnight at 60°C in hybridization solution with the ^{35}S -labeled probe at a concentration of 1×10^6 cpm/50 μl . After post-hybridization washes and ribonuclease A treatment, the slides were exposed to BioMax MR film (Kodak, Rochester NY) for 2 days (TH cRNA) or 13 days (ErbB4 cRNA) for generation of film autoradiographs. The films were developed with Kodak GPX developer and fixer (Kodak, Rochester NY).

Western Blots

Rats ($n=6$ for each age) were euthanized by CO_2 asphyxiation for the protein analysis. The ventral midbrain was carefully dissected out of each brain in chilled artificial CSF (Harvard Apparatus, Holliston, MA), frozen on dry ice, and stored at -80°C . The Western blotting procedure was performed as described previously (Wolf et al., 1999), with slight modifications. To solubilize the proteins within the samples, the frozen ventral mesencephalic tissue was probe-sonicated in a buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin. The protein concentration of each sample was determined in triplicate using Bio-Rad's DC protein assay kit (Hercules, CA). The final volume of each sample was 25 μl and protein concentration was 3.86 $\mu\text{g}/\mu\text{l}$. For ErbB4 analysis 20 μl of each sample was loaded onto a 7.5% acrylamide gel. The remaining 5 μl of each sample was loaded onto a separate 7.5% acrylamide gel for TH. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) analysis was included as a loading control. Following SDS-polyacrylamide gel electrophoresis the proteins were transferred to nitrocellulose. The nitrocellulose membranes were blocked in 5% milk PBS/tween for 1 hour and then incubated at 4°C overnight in primary antibody [rabbit anti-ErbB4; 1:500 (Santa Cruz Biotechnology, Inc; Santa Cruz, CA); mouse anti-TH; 1:4000 (Chemicon; Temecula, CA); mouse anti-GAPDH; 2 $\mu\text{g/ml}$ (Ab-cam; Cambridge, MA)]. Following primary antibody removal, the membranes were washed in 2% milk PBS/tween (1 hour) and then incubated in the appropriate secondary antibody (anti-mouse IgG for TH and GAPDH; anti-rabbit IgG for ErbB4) diluted in 2% milk PBS/tween for an hour. The membranes were washed in PBS/tween for one hour. Antibody binding was visualized by enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ) and exposure to Hyperfilm ECL (GE Healthcare).

Analysis

For the semi-quantitative *in situ* hybridization analysis, the mean corrected gray levels were obtained for each age group to compare densities of hybridization. The level of mRNA

expression of ErbB4 or TH in the SNpc, SNI, VTA, or retrosplenial cortex was determined using optical density (OD) measurements with Scion Image software (National Institutes of Health). Figure 1 depicts the SNpc, SNI, and VTA as they were delineated for densitometric analysis in this study. Control measurements of the background OD were taken within each section (from the corpus callosum), and then subtracted from the OD value for each analyzed area to give a corrected value for each section. Values for the hybridization data are expressed as mean corrected grey levels $\pm$ the standard error of the mean (S.E.M.). Densitometric measurements were taken of each area from 6 sections per animal for each probe. The data were analyzed using one-way ANOVA, followed by the Newman-Keuls post-hoc test.

Western blot films were analyzed by densitometry using Scion Image software. The mean corrected grey levels were determined by measuring the OD of each band and subtracting background (an area in the same lane just below the band). The mean from the bands of young animals was set at 100% (control). Data are expressed as mean corrected grey level (percent control) $\pm$ S.E.M. The Western blot data were analyzed using one-way ANOVA followed by the Dunnett's multiple comparison post-hoc test.

Results

Expression of ErbB4 and TH mRNAs during aging

Representative autoradiographs of ErbB4 mRNA hybridization in rostral sections of the ventral midbrain are shown in Figure 2. Numerous areas throughout the brain contained cRNA-hybridizing cells including the SNpc, SNI, VTA, hippocampus and most neocortical regions. Decreased expression of ErbB4 mRNA was found in the SNpc of middle-aged and old BN/F344 animals when compared to the young rats ($F(2,9) = 27.24$, $p < 0.01$; Figure 3A). Although only rostral sections of the ventral midbrain are shown in Figure 2, the decrease in ErbB4 mRNA was evident throughout the entire rostrocaudal extent of the SNpc. There was no difference in expression levels between the middle-aged and old animals. Expression of ErbB4 mRNA did not differ significantly with aging in the adjacent VTA ($F(2,9) = 3.38$, $p > 0.05$) or SNI ($F(2,9) = 0.28$, $p > 0.05$) (Figure 3A). Thus, age-related reduction in ErbB4 mRNA is not evident in all dopaminergic cell populations and appears to be specific for the SNpc.

To determine if this age-related decrease in ErbB4 mRNA expression was present in non-dopaminergic areas of the brain, the retrosplenial cortex, present at the same level (and in the same section) as the ventral midbrain, was analyzed across the aging spectrum. Representative autoradiographs are shown in Figure 2. There were no significant differences among any of the age groups in the retrosplenial cortex ($F(2,9) = 0.48$, $p > 0.05$; Figure 3A). Taken together with the VTA and SNI data, these results suggest that the reduction in ErbB4 mRNA in the aged brain is not global, but rather is restricted to the SNpc.

Semi-adjacent sections through the ventral mesencephalon were hybridized for TH mRNA detection. Representative autoradiographs of TH cRNA hybridization in the rostral ventral midbrain are shown in Figure 2. Labeling for TH mRNA was analyzed for changes in expression during aging and is also useful in delimiting the area of the SNpc, SNI, and VTA (Figure 1). Expression levels for TH mRNA in the SNpc were significantly reduced between the young and the old animals ($F(2,9) = 8.45$, $p < 0.01$; Figure 3B). Although no significant changes in TH mRNA during aging were found in the VTA, a trend toward a decrease in expression was noted in this structure ($F(2,9) = 1.84$, $p = 0.12$; Figure 3B). There were no significant changes in TH mRNA levels among any of the age groups in the SNI ($F(2,9) = 1.08$, $p > 0.05$; Figure 3B).

Expression of ErbB4 and TH protein during aging

Changes in mRNA do not always translate into equivalent changes in protein, thus we assessed expression of ErbB4 and TH protein in each age group using Western blot analysis. Figure 4A shows representative autoradiographs of Western blots from young, middle-aged, and old animals for ErbB4, TH, and GAPDH (housekeeping protein used as a loading control). The expression of ErbB4 protein (migrating at 185 kDa) was significantly decreased between the young and the old rats, and a strong trend was found toward a decrease in the middle-aged animals when compared to the young animals ($F(2,15) = 5.80$, $p < 0.05$ for old versus young rats, $p = 0.054$ for middle-age versus young rats; Figure 4B). Expression of TH protein (migrating at 60 kDa) in the ventral midbrain during aging was also examined. There was a significant decrease in TH protein between the young and old rats ($F(2,15) = 4.75$, $p < 0.05$; Figure 4B). No differences in the protein levels of GAPDH were observed in any of the animals regardless of age ($F(2,15) = 0.53$, $p > 0.05$; Figure 4B).

Discussion

In the present study we demonstrate an age-related reduction of both message and protein for the ErbB4 receptor and the dopaminergic biosynthetic enzyme TH in the SNpc of BN/F344 rats. The decrease in ErbB4 mRNA was evident in the middle-aged animals and was sustained with advanced age, whereas the significant decline in ErbB4 protein only appeared in the old rats. Tyrosine hydroxylase exhibited decreases in mRNA and protein in the old animals when compared to young animals.

Neuregulins and their ErbB receptors are critical for the development of the CNS and the peripheral nervous system (PNS) (reviewed in Burden and Yarden, 1997; Gassmann and Lemke, 1997; Esper et al., 2006). The importance of NRG signaling through the ErbB4 receptor is emphasized in studies of mutant mice. ErbB4 knockout animals result in embryonic lethality with cardiac and hindbrain defects (Gassmann et al., 1995). Mice rescued from embryonic lethality with cardiac ErbB4 replacement have abnormal neural crest cell migration as well as an increased number of large interneurons in the cerebellum (Tidcombe et al., 2003; Golding et al., 2004). Conditional nervous system ErbB4 knockout mice exhibit behavioral disturbances including lower spontaneous motor activity and reduced grip strength (Golub et al., 2004). Other conditional ErbB4 mutants demonstrate aberrant neuroblast migration from the subventricular zone and deficits in differentiation of olfactory interneurons (Anton et al., 2004). Interestingly, Thuret et al. (2004) were unable to ascertain any behavioral or morphological disturbances in the mesencephalic dopaminergic system in brain-specific ErbB4 null mice. This lack of phenotype is thought due to a strong compensatory response (Thuret et al., 2004). It should also be noted that aged ErbB4 mutant mice were not examined in the Thuret et al. study. After development, the function of NRG/ErbB signaling is less well understood, but has been implicated in the maintenance of neuronal connections and in many disease states including schizophrenia, stroke, multiple sclerosis and peripheral neuropathy (Cannella et al., 1999; Parker et al., 2002; Talmage and Role, 2004; Norton et al., 2006; reviewed in Esper et al., 2006). Furthermore, NRGs *in vitro* are neurotrophic and neuroprotective in several neuronal culture systems (Vaskovsky et al., 2000; Gerecke et al., 2004; Zhang et al., 2004; Di Segni et al., 2006).

With respect to the nigrostriatal dopaminergic system, ErbB4 is highly expressed in the SN and VTA where it is present within almost all of the dopaminergic cells (Steiner et al., 1999; Yurek and Seroogy, 2001; Abe et al., 2009; K.B. Seroogy unpublished observations). Given the sparse expression of ErbB2 and ErbB3 in the SNpc, it is likely that the known trophic and functional effects of NRGs on mesencephalic dopaminergic cells *in vitro* and *in vivo* (Yurek et al., 2004; Zhang et al., 2004) are mediated through ErbB4 receptors. Thus, NRGs signaling specifically via ErbB4 receptor activation could be important not only in the survival and

functioning of developing mesencephalic dopamine cells, but also in the maintenance and functioning of midbrain dopamine cells in the adult and aging brain. The current study suggests that a decrease in NRG/ErbB4 support via loss of ErbB4 receptors occurs in aged animals and is specific for the ventral midbrain. This progressive loss of NRG/ErbB4 activation may have important functional implications for the mesostriatal dopaminergic system.

It is unlikely that compensatory changes in expression of ErbB3, the only other ErbB receptor that directly binds NRGs, could replace the diminishing influence of ErbB4 in the aging SNpc. Few ErbB3-expressing cells are located in the adult SNpc and those present are not colocalized to dopaminergic neurons (Steiner et al., 1999; Abe et al., 2009). Moreover, although we did not analyze ErbB3 expression in these BN/F344 rats, we have observed no changes in ErbB3 mRNA levels in the midbrain of aged Sprague Dawley rats (J.W. Dickerson and K.B. Seroogy, unpublished results).

It has been theorized that a loss of neurotrophic support underlies the vulnerability of the nigrostriatal dopaminergic system to age-related loss and/or to degeneration in disorders like Parkinson's disease (Hefti and Weiner, 1986; Hymen et al., 1991; Unsicker 1994; Temlett et al., 1996). Neurotrophic factors (including NRGs) are known to be neuroprotective to cultured dopamine cells against insults with 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (e.g. Hyman et al., 1991, 1994; Collier and Sortwell, 1999; Yurek and Seroogy, 2001; Zhang et al., 2004). Neurotrophic factors also protect dopaminergic neurons from degeneration and improve functional outcomes in animal models of PD, albeit with differing levels of effectiveness (e.g. Altar et al., 1994a, b; Tomac et al., 1995; Kearns et al., 1995; Gash et al., 1996; Rosenblad et al., 1998; Collier and Sortwell, 1999; Klein et al., 1999; Kordower et al., 2000; Fox et al., 2001; Yurek and Seroogy, 2001; Sun et al., 2005). Thus select neurotrophic factors (including NRGs) are important in the development, maintenance, function, and protection of the nigrostriatal dopaminergic system.

There have been only a few studies indicating a decrease in mesostriatal neurotrophic support with age in rats. In Sprague Dawley rats, a decrease in BDNF protein was detected in several areas of the aged brain including a non-significant decrease of 18% in the SN (Croll et al., 1998). A reduction in the BDNF high affinity receptor trkB was also found in the SN of these same rats (Croll et al., 1998). Another study reported a significant 52% reduction of BDNF in the striatum of aged Sprague Dawley rats (Katoh-Semba et al., 1998). Aged F344BN rats exhibited a significant reduction of GDNF protein in the striatum and ventral midbrain (Yurek and Fletcher-Turner, 2001). The current study demonstrates a 25% decrease in ErbB4 message and a 34% decrease in ErbB4 protein, both significant reductions, by 24 months of age in BN/F344 hybrid rats.

Changes in neurotrophic factor support in the nigrostriatal dopaminergic system have also been observed in aged animals following injury or other manipulations. Young animals that receive a neurotoxic lesion of the SNpc have increased expression of BDNF and GDNF protein in the striatum (Zhou et al., 1996; Ling et al., 2000; Yurek and Fletcher-Turner, 2000, 2001; Collier et al., 2005). This compensatory striatal increase of BDNF and GDNF in response to neurotoxin administration in young rats, however, is attenuated in aged rats (Yurek and Fletcher-Turner, 2000, 2001). In addition, GDNF is less effective at preventing 6-OHDA-induced degeneration in old versus young rats, indicating a decreased responsiveness to neurotrophic factors in the aged brain (Fox et al., 2001). Aged animals also display a decreased viability of dopaminergic cell grafts when compared to younger animals, possibly due to a diminished trophic factor environment (Collier et al., 1999; Sortwell et al., 2001). Evidence for this is supported by BDNF and GDNF infusions or Schwann cell cogafts, which improve graft survival and outgrowth (Hudson et al., 1995; Sinclair et al., 1996; Yurek et al., 1996; Yurek 1998; Collier et al., 1999). Thus, chronological aging leads to decreased neurotrophic support through the

direct loss of neurotrophic factor signaling, the attenuation of compensatory mechanisms, or diminished responsiveness to neurotrophic factor therapy. These deficiencies, alone or in combination, may increase the susceptibility of midbrain dopaminergic cells to neurodegeneration. In comparisons of Parkinson's disease patients to healthy controls, decreases of GDNF, BDNF, and ciliary neurotrophic factor (CNTF) have been found in the nigrostriatal system, whereas there are little or no changes in neurotrophin-3 (NT-3), NT-4 or nerve growth factor (NGF) (Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000; Seigel and Chauhan, 2000; Chauhan et al., 2001). Although these studies may indicate a role for neurotrophic factors in the diseased-state brain, decreased neurotrophic factor support in the naturally aging brain remains to be fully elucidated.

The animals used in the present study are BN/F344 hybrid rats. These rats are recommended based on National Institute on Aging (NIA) data indicating a normal range of age-related pathologies in these animals including lower incidence of renal pathology and no specific tumor susceptibility (Lipman et al., 1996). Although these hybrid rats exhibit motor deficits, which occur starting at 18 months of age (Yurek et al., 1998), changes in the SNpc have not been reported in the animals. In this study we observe a decrease in ErbB4 mRNA coinciding with the timing of deficits in behavior reported in Yurek et al. (1998), but prior to reduction in TH mRNA and protein indicating the possibility that a loss of NRG/ErbB4 occurs prior to alterations within dopaminergic neurons in the SNpc. Future studies using precise stereological counting methods would be needed to assess any age-related dopaminergic cell loss occurring in the SNpc of BN/F344 animals. Additional studies also need to investigate modulation of other neurotrophic factors, including NRG ligands, in the nigrostriatal dopaminergic system of BN/F344 animals to better understand the changing neurotrophic factor environment of the aging animal.

In conclusion, we have found decreases in ErbB4 mRNA and protein occurring with advancing age. The aging-induced decrease in ErbB4 mRNA commences prior to indications of nigral dopaminergic changes as measured by TH mRNA and protein analyses. Thus, these findings raise the possibility that a progressive loss of NRG/ErbB4 signaling may contribute to the natural age-related decline of nigrostriatal dopaminergic function and possibly to the pathogenesis of age-related neurodegenerative disorders such as Parkinson's disease.

Acknowledgments

This work was supported by NIH grant NS39128 to K.B.S. and a Scottish Rite Foundation Fellowship to J.W.D.

Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BN/F344	Brown Norway X Fischer 344 hybrid rat
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSF	

	cerebral spinal fluid
DEPC	diethylpyrocarbonate
ECL	enhanced chemiluminescence
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GGF2	glial growth factor 2
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF	nerve growth factor
NRG	neuregulin
NT-3	neurotrophin-3
NT-4	neurotrophin-4
OD	optical density
PBS	phosphate-buffered saline
PD	Parkinson's disease
PNS	peripheral nervous system
RSCTX	retrosplenial cortex
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNpc	substantia nigra pars compacta

SNI	substantia nigra pars lateralis
SSC	standard saline citrate
TH	tyrosine hydroxylase
MTA	ventral tegmental area
6-OHDA	6-hydroxydopamine

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Figure 1.

Reverse image film autoradiogram showing a section through the mid-rostrocaudal level of the ventral midbrain hybridized with TH cRNA. The areas of the ventral tegmental area (VTA), substantia nigra pars compacta (SNpc), and substantia nigra pars lateralis (SNI) are outlined as delineated for densitometric analysis in this study. Scale bar = 500 μ m.

Figure 2.

Reverse image film autoradiograms showing sections at rostral levels of the SNpc hybridized with either ErbB4 cRNA (A–C) or TH cRNA in semi-adjacent sections (D–F). Note the progressive decrease in expression of ErbB4 mRNA in the SNpc (arrows) among the young (A), middle-aged (B), and old (C) animals. In the same sections, no apparent loss of ErbB4 mRNA labeling is observed in the adjacent VTA, SNl, or in cortical regions (retrosplenial cortex, RSCTX). TH mRNA expression is also reduced in the old animals (F) when compared to the young animals (D) in the SNpc but not in the SNl or VTA. Scale bar = 1000 μ m.

Figure 3.

Densitometric analysis of ErbB4 and TH cRNA hybridization during aging. **A:** Quantification of hybridization signal revealed a decrease in expression of ErbB4 mRNA in the SNpc of the middle-aged and old animals when compared to the young animals. Other areas within the same sections including the VTA, SNI and RSCTX exhibited no significant changes due to aging. **B:** Expression of TH mRNA was significantly decreased in the SNpc of old animals versus young animals, but no changes were found in the VTA or SNI. Data are expressed as mean corrected grey level $\pm$ S.E.M. *p<0.01 compared to the 3-month (young) age group.

Figure 4.

A: Representative autoradiograms from Western blots of the ventral midbrain for two different sets of animals at 3 (young), 18 (middle-aged) and 24 (old) months of age. Bands indicating protein expression of ErbB4 (migrating at 185 kD), TH (migrating at 60 kD), and GAPDH (migrating at 36 kD) are shown. Note the apparent progressive reduction in levels of ErbB4 and TH during aging. **B:** Quantification of the blots revealed a significant decrease in protein expression between young and old animals for ErbB4 in the ventral midbrain. There was also a strong trend toward a decrease for ErbB4 protein in the middle-aged animals when compared to the young animals. TH protein expression was reduced in the ventral midbrain of old animals when compared to young animals. Quantification of the housekeeping protein GAPDH

revealed no differences among the age groups. Data are expressed as mean corrected grey level (represented as percent control) $\pm$ S.E.M. * $p < 0.05$, ^ $p = 0.054$ compared to the 3-month (young) age group.