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by

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General Abstract

Parkinson’s disease (PD) is a progressive neurological disorder characterized by motor deficits associated with nigrostriatal dopaminergic degeneration. Normal aging is the highest risk factor for developing PD. Current pharmacological and surgical treatments for PD are effective in improving symptoms, but do not prevent the progression of the disease. Neuregulins (NRGs) are pleiotrophic growth factors that are neurotrophic for dopamine cells in vitro and in vivo. In the present series of studies, the neuroprotective and neurorestorative roles of NRGs in the injured and aging nigrostriatal system were investigated.

In the first study, rats received 6-hydroxydopamine (6-OHDA) neurotoxin lesions to create an experimental model of PD, followed two weeks later by an infusion of the NRGs glial growth factor (GGF2) or neuregulin-2β (NRG-2β). The animals were assessed for cell survival and behavioral outcomes. The second study investigated age-related changes in NRG1 and its ErbB receptors in the chronologically aging rat mesostriatal system by Western blot and in situ hybridization. Animals were also infused with GGF2 in an aged model of PD to determine if NRG treatment was a potential therapy for the injured aged nigrostriatal system. The third study tested the hypothesis that reduction of ErbB4 receptor availability in the substantia nigra will exacerbate the detrimental effects of neurotoxin-induced damage upon the nigrostriatal system. ErbB4 expression was reduced in the substantia nigra using RNA interference followed by a striatal 6-OHDA lesion. The animals were then assessed for possible worsening of neurotoxin-induced motor dysfunction and dopaminergic cell death.
Results from the first study showed that infusion of either GGF2 or NRG-2β into the substantia nigra two weeks after striatal 6-OHDA injections significantly promoted functional recovery of motor function. In addition, both NRGs significantly increased the number of tyrosine hydroxylase (TH) neurons in the substantia nigra when compared to their appropriate controls. These findings demonstrate the neuroprotective and neurorestorative effects of NRGs administered after the establishment of experimental parkinsonism and identify NRG-2β as a novel neurotrophic agent for the dopaminergic nigrostriatal system. Results from the second study revealed a significant decrease in mRNA and protein for the functional NRG receptor ErbB4 and the dopaminergic biosynthetic enzyme TH in the substantia nigra of aging animals. The decrease in ErbB4 preceded that of TH. Reduced ErbB1 receptor protein was also observed in the ventral midbrain of aged rats. Despite the diminished availability of ErbB4 and ErbB1 receptors in the aging midbrain, supranigral GGF2 infusion was able to significantly protect against the morphological and behavioral deficits due to 6-OHDA lesion in an aging model of PD. These findings suggest that GGF2 administration may overcome the possible decline of NRG/ErbB trophic support for dopaminergic neurons in the injured aged midbrain. In the final study, knockdown of ErbB4 in vivo in the SNpc was achieved using a lentiviral vector-driven siRNA construct. This knockdown of ErbB4, however, did not exacerbate motor dysfunction or dopaminergic cell death in a model of experimental parkinsonism.

Overall, the results of this research demonstrate the neuroprotective and neurorestorative effects of NRGs in the injured and aged dopaminergic nigrostriatal system. Moreover, these findings indicate the potential therapeutic use of NRGs for the treatment of PD.
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Although there is not enough space to thank everyone, my heartfelt thanks goes out to everyone that has helped me during the time I have spent in graduate school. I would like to start by acknowledging my mentor Dr. Kim Seroogy. Kim you have taught me what it truly means to do good science and to be a scientist in every sense of the word. Your genuine love of scientific investigation is infectious. You encouraged me to be thoughtful and ask questions no matter what the subject matter. On a personal note you were always supportive of anything that was happening in my life, lab related or not. Thank you for all that you have done for me to prepare me to be a contributing member of the scientific community.

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<th>Description</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ACCtx</td>
<td>Anterior cingulate cortex</td>
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<tr>
<td>Art</td>
<td>Artemin</td>
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<tr>
<td>BN/F344</td>
<td>Brown Norway/Fisher 344 hybrid rat</td>
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<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate-putamen</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<tr>
<td>CE</td>
<td>Coefficient of effort</td>
</tr>
<tr>
<td>CDNF</td>
<td>Conserved dopamine neurotrophic factor</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus collosum</td>
</tr>
<tr>
<td>CTX</td>
<td>Cortex</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ErbB</td>
<td>Epidermal growth factor-like receptor</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<tr>
<td>GGF2</td>
<td>Glial growth factor 2</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus pars externa</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus pars interna</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
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<tr>
<td>MANF</td>
<td>Mesencephalic astrocyte-derived neurotrophic factor</td>
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<tr>
<td>MAO-B</td>
<td>Monoamine oxidase-B</td>
</tr>
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<td>MCtx</td>
<td>Motor cortex</td>
</tr>
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<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>Neuregulin</td>
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<td>Neuronal nuclei</td>
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<tr>
<td>NTN</td>
<td>Neurturin</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>PSP</td>
<td>Persephin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>RSC</td>
<td>Retrosplenial cortex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small inhibitory RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNI</td>
<td>Substantia nigra pars lateralis</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulate</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter 1

General Introduction and Background
Neurotrophic factors or growth factors are naturally occurring proteins that promote growth and survival and maintain the health of specific populations of neurons during development, in adulthood, and after injury. The use of growth factors to rescue or protect injured or dysfunctional neurons is a potential therapy for many neurological disorders. With a particular interest in neurodegenerative diseases like Parkinson’s disease (PD), we have begun to characterize the role of a novel family of neurotrophic factors for the dopaminergic nigrostriatal system called neuregulins (NRGs). The overall goal of this dissertation research is to determine if NRGs have potential as a neurotrophic factor therapy for the treatment of PD.

**Neuregulins**

Neuregulins are a family of homologous polypeptides that are encoded by the NRG1-4 genes which are structurally related growth and differentiation factors for tissues throughout the body. The signaling domain for NRGs is the epidermal growth factor-like domain, which is common among the NRGs (Burden and Yarden 1997; Gassmann and Lemke 1997; Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Falls 2003; Seroogy and Zhang 2006; see Figure 1). The most extensively studied NRG gene, NRG1, has at least 20 isoforms due to alternative splicing (Yarden and Sliwkowski 2001; Falls 2003; Seroogy and Zhang 2006; Hayes and Gullick 2008; Mei and Xiong 2008). Similarly the NRG2 gene has several splice variants while NRG3 and NRG 4 have not been extensively characterized (Carraway et al., 1997; Hayes and Gullick 2008). Like other neurotrophic factors, signaling occurs after binding to a high affinity receptor, for NRGs these receptors are from the epidermal growth factor-like family of receptors.
(receptor tyrosine kinases), the ErbB receptors. The ErbB receptors consist of ErbB1-4, with NRGs binding directly to both the ErbB3 and ErbB4 receptors (Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Falls 2003; see Figure 1). Upon binding of NRGs, signaling occurs through various combinations of ErbBs 1-4 via heterodimerization (or ErbB4 by homodimerization), activating a variety of downstream intracellular signaling pathways that can influence cellular processes including apoptosis, migration, growth, adhesion and differentiation (Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Falls 2003). Activation and signaling by NRGs through the ErbB receptors is further complicated by the differing functional abilities of the ErbB receptors to bind to NRGs and activate the downstream pathways. For example ErbB3 is deficient in kinase activity whereas ErbB2 exhibits the highest kinase activity but no known ligand. Thus, ErbB4 appears to be the most ‘functional’ NRG receptor (Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Falls 2003).

Known mostly for its role during development, NRG signaling plays an integral role in many processes including cell survival, migration, and differentiation of brain, breast, heart and other tissues (Burden and Yarden 1997; Gassmann and Lemke 1997; Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Falls 2003; Seroogy and Zhang 2006). This role for NRG signaling in development is exemplified in knockout (KO) animals. Mice with a pan-NRG1 KO exhibit defects in cardiac development and thus die during embryogenesis (Meyer and Birchmeier 1995). These mice also show abnormalities in neural crest cell populations including Schwann cells, cranial sensory neurons and sympathetic neurons (Meyer et al., 1997; Britsch et al., 1998). Furthermore
Figure 1: Diagrams depicting structure of NRGs and ErbB receptors. A: Schematic examples of the four NRG genes (NRG1-4); modified from Buonanno and Fischbach (2001). Common amongst the NRGs is an EGF-like domain which contains the biological activities of these proteins. NRG1 has at least 20 isoforms due to alternative splicing. The NRG2 gene also has several isoforms due to splice variants, whereas NRG3 and NRG4 are not well characterized. Expression of NRG4 is not found within the central nervous system. B: Diagram depicting ErbB receptor binding and dimerization; modified from Ozaki (2001). Notice that NRGs directly bind either ErbB3 or ErbB4, whereas ErbB2 is an orphan receptor that can heterodimerize with all other ErbB receptors. The EGF receptor (ErbB1) does not directly bind to NRGs but can act as a heterodimerization partner after NRG binding to ErbB3 or ErbB4. Note also that ErbB3 exhibits impaired kinase activity.
cell cultures from the spinal cord of NRG1 KO animals indicate an important role for
NRGs in oligodendocyte development (Vartanian et al., 1999). In mice with a reduced
expression of NRG1, abnormalities in the function of N-methyl-D-aspartic acid (NMDA)
receptors and increased behaviors associated with schizophrenia have been found (Mohn
et al., 1999; Gerlai et al., 2000; Stefansson et al., 2002; Chen et al., 2008). Although
NRGs do not directly bind ErbB2, mutant mice with ErbB2 knocked out display similar
phenotypes as NRG1 KO mice including embryonic lethality and neural crest
development abnormalities (Meyer and Birchmeier 1995; Lee et al., 1995). These
findings indicate the importance of ErbB2 as a heterodimerization partner for NRG
signaling in the CNS. Mice lacking the ErbB3 receptor die during embryogenesis due to
cardiac defects although at a later time point then either the ErbB2 or NRG1 KO mice
(Erikson et al., 1997; Riethmacher et al., 1997). In the nervous system, ErbB3 KO mice
exhibit a lack of Schwann cell precursors that results in a reduction of the sensory and
motor neurons of the dorsal root ganglia (Reithmacher et al., 1997). In ErbB1 (EGF
receptor) KO animals, there is neurodegeneration in several parts of the brain including
the frontal cortex, thalamus and olfactory bulb (Kornblum et al., 1998; Sibilia et al.,
1998). These neurodevelopmental deficiencies are not likely to be due to a lack of NRG
signaling since NRGs do not directly bind to ErbB1. ErbB4 knockout animals also 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 to be 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. Notwithstanding the latter study, the inability of the ErbB receptors to properly compensate for one another in these KO models indicate the importance of the multiple potential signaling pathways of NRGs through multiple ErbB receptors for normal development and maintenance of the CNS.

Neuregulins are distributed throughout the adult CNS including cortical areas, hippocampus, thalamus, hypothalamus, amygdala, and the ventral midbrain (Chen et al., 1994; Corfas et al., 1995; Law et al., 2004; Bernstein et al., 2006). ErbB receptors are also present in the adult CNS and are found in many areas of the brain including the nigrostriatal dopaminergic system (Steiner et al., 1999; Gerecke et al., 2001; Yurek and Seroogy 2001). Both mRNA and protein for all of the ErbB receptors are expressed within the striatum at differing levels (Steiner et al., 1999; Gerecke et al., 2001; see Figure 2). In the ventral midbrain, ErbB4 and ErbB1 are highly expressed in both the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (see Figure 3; Seroogy et al., 1994; Steiner et al., 1999; Gerecke et al., 2001; Zhang et al., 2004). Furthermore, ErbB4 and ErbB1 are localized to DA cells in the SNpc as evidenced by a reduction of expression after 6-hydroxydopamine (6-OHDA) lesions and through direct
colocalization with tyrosine hydroxylase (TH) mRNA (see Figure 3; Seroogy et al., 1994; Steiner et al., 1999; Yurek and Seroogy 2001; unpublished results). Both ErbB3 and ErbB2 are expressed at very low levels in the ventral midbrain, but they are not associated with dopaminergic cells (ErbB3 is found mainly in white matter) (Steiner et al., 1999; Gerecke et al., 2001). Although NRGs and ErbB receptors are present in the adult CNS, the role of NRGs remains to be fully elucidated in the adult brain.

The presence of multiple NRG genes and several splice variants from those genes indicate differential signaling properties of these NRG isoforms. This was evident early in the study of NRG biology in which four NRG1 isoforms were discovered nearly simultaneously with different biological functions (reviewed in Falls 2003). For the studies in this dissertation, current evidence indicating differing spatial, temporal, and cellular distributions of NRG2 versus NRG1 proteins is of interest. Specifically, NRG1 expression is higher during development and decreases in postnatal animals, whereas NRG2 expression increases after development (Longart et al., 2004; Talmage and Role, 2004). In the adult brain, NRG1 and NRG2 proteins appear to be expressed in complimentary areas of the brain (e.g. NRG1 in the hippocampal regions CA1 and CA3, while NRG2 in the dentate gyrus) (Longart et al., 2004; Talmage and Role, 2004). Moreover, NRG2 proteins are expressed almost exclusively within dendrites, whereas NRG1 proteins have more widespread expression and can be found within axons, dendrites, and somata (Longart et al., 2004; Talmage and Role, 2004). Most importantly for potential differences in signaling, NRG2 and NRG1 proteins differ in the preferred ErbB receptor combinations they activate (Carraway et al., 1997; Nakano et al., 2000; Sweeney et al., 2001). Evidence indicates that NRG2 isoforms may be more effective
than NRG1 proteins at activating ErbB4/ErbB1 receptor combinations (Carraway et al., 1997). Thus, for the ventral midbrain where ErbB4 and ErbB1 are the most highly expressed of the ErbB receptors NRG2 proteins could be activate signaling through ErbB receptors more effectively than NRG1 proteins.

Administration of NRGs to several neuronal cell culture systems in vitro is neurotrophic and neuroprotective (Vaskovsky et al., 2000; Gerecke et al., 2004; Zhang et al., 2004; Di Segni et al., 2006). For example, activation of cells with NRG through ErbB4 receptors stimulates neurite outgrowth in PC12-ErbB4 (cells induced to express ErbB4) (Vaskovsky et al., 2000). Inducing ErbB4 expression in PC12 cells also protects the cells from 1-methyl-4-phenylpyridinium (MPP+)‐induced toxicity (Di Segni et al., 2006). For hippocampal cell cultures, NRG-1β increases the arborization and primary outgrowth of neurites (Gerecke et al., 2004). In cerebellar granule cell cultures NRG treatment also increases neurite outgrowth and migration (Rio et al., 1997; Rieff et al., 1999). Administration of GGF2 promotes Schwann cell migration and indirectly enhances sympathetic neuron survival and outgrowth (Mahanthappa et al., 1996). The neuroprotective and neurotrophic effects of NRGs on dopaminergic cells in culture will be discussed in more detail in a later section.

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 (MS) and peripheral neuropathy
Figure 2

**A**

Autoradiograms depicting ErbB1-4 mRNA within the striatum of normal Sprague-Dawley rats. Note that while there is high level punctate expression of ErbB3 and ErbB4 in the striatum, ErbB1 and ErbB2 are expressed ubiquitously at low levels throughout the striatum.

**B**

Western blot autoradiograms depicting ErbB1-4 protein expression in the rat striatum from 3 separate animals.

**Figure 2**: Expression of ErbB receptors in the striatum. **A**: Autoradiograms depicting ErbB1-4 mRNA within the striatum of normal Sprague-Dawley rats. Note that while there is high level punctate expression of ErbB3 and ErbB4 in the striatum, ErbB1 and ErbB2 are expressed ubiquitously at low levels throughout the striatum. **B**: Western blot autoradiograms depicting ErbB1-4 protein expression in the rat striatum from 3 separate animals.
Figure 3: ErbB mRNA expression in the ventral midbrain. Both ErbB4 and ErbB1 are highly expressed within the dopaminergic ventral midbrain. Neurotoxic lesioning of the nigrostriatal pathway with 6-OHDA significantly decreases the signal for ErbB4 and ErbB1 (and TH expression) but not ErbB3. This indicates that ErbB4 and ErbB1 are located directly on dopaminergic cells within the substantia nigra.
(Cannella et al., 1999; Parker et al., 2002; Talmage and Role 2004; Norton et al., 2006; reviewed in Esper et al., 2006). Recent interest in NRG has increased due to the discovery of the NRG1 gene as a susceptibility gene for the development of schizophrenia (Stefansson et al., 2002, 2003a,b; Li et al., 2004; Britsch 2007; Mei and Xiong 2008; LeStrat et al., 2009). This is supported by mouse models of reduced NRG1 expression which display schizophrenia-like behaviors (although the exact mechanism remains to be determined) (Mohn et al., 1999; Stefansson et al., 2002; Golub et al., 2004). Experimental models of ischemic stroke lead to increased expression of NRG1 (Parker et al., 2002). Exogenous administration of NRG1 in rat models of ischemia significantly reduce ischemic damage (Parker et al., 2002; Shyu et al., 2004; Xu et al., 2004, 2005).

Treatment with NRGs of mice with experimental autoimmune encephalitis decreased the severity of the disease and promoted the remyelination in the CNS lesions (Cannella et al., 1998; Marchionni et al., 1999). In active and chronic MS lesions there is a significant reduction of astrocyte-derived NRG expression (Viehover et al., 2001). For animal models of peripheral neuropathy NRG1 treatment significantly improved nerve regeneration through its effects on Schwann cells (Verge et al., 1996; Munson and McMahon 1997; Munson et al., 1997; Bennett et al., 1998). Schwann cells also respond to nerve damage and loss of axonal contact by producing NRG1 (Carroll et al., 1997; Oka et al., 2000). Taken together, these studies described above illustrate the presence and plasticity of NRGs and ErbB receptors in the normal and diseased brain and indicate a potential role for NRG signaling in the maintenance and/or regeneration of the adult brain.
**Parkinson’s Disease**

Parkinson’s disease is a progressive neurological disease first described by James Parkinson in ‘An essay on the shaking palsy’ in 1817. Now almost two centuries since the description of this disorder, we not only have no cure, but are not certain of the exact pathogenesis of idiopathic PD. It is the second most common neurodegenerative disease, affecting 1% of the population over 60, with aging as the largest risk factor for the development of PD (de Lau et al., 2006; Olanow et al., 2009; Chen 2010). Current therapies are designed to address the primary symptoms of PD but do not slow down the continued progression of this neurodegenerative disorder. Thus the development of therapies designed to slow or stop the progression of PD, while also reversing the symptoms of this disease, would greatly improve the quality of life for PD patients. Furthermore, such therapies would reduce the economic burden of care for PD patients, currently estimated at 10.8 billion dollars (Chen 2010).

The most notable symptoms for the diagnosis of PD are the presence of one or more of the primary motor symptoms including resting tremor, postural instability, rigidity, and bradykinesia. Control of these voluntary motor movements lies within the basal ganglia circuitry. The pathophysiological feature linked to the development of these motor symptoms is the loss of dopaminergic neurons in the SNpc (Kapp 1992). The resulting loss of dopamine, and, thus, dopaminergic tone, from the SNpc to the target striatum (the nigrostriatal pathway) in the basal ganglia leads to a disruption of the normal functioning within the circuit. In the normally functioning basal ganglia there is a balance between the signals that promote movement (the direct pathway) and those that suppress movement (the indirect pathway) (see Figure 4; Alexander et al., 1986; Bartels...
and Leenders 2009). When released into the striatum from the SNpc, dopamine serves to excite D1 receptors in the direct pathway and inhibit D2 receptors in the indirect pathway (Gerfen et al., 1995; Bartels and Leenders 2009). The excitation of D1 receptors in the striatum stimulates inhibitory neurons, and leads to a direct inhibitory effect on the γ-aminobutyric acid (GABAergic) neurons of the globus pallidus pars interna (GPi) and substantia nigra pars reticulata (SNr) (Bartels and Leenders 2009; Gerfen 2010). The binding of dopamine to D2 receptors in the striatum inhibits the projections to the globus pallidus pars externa (GPe) (Bartels and Leenders 2009; Gerfen 2010). This results in a disinhibition of the subthalamic nucleus (STN) and a net excitatory effect of the projections from the STN to the GPi/SNr (Bartels and Leenders 2009; Gerfen 2010).

This balance between the excitatory projections from the STN and inhibitory signals from the direct pathway act to balance the degree of inhibition from the GPi/SNr GABAergic projections that are exerted on the thalamus, which projects to motor areas of the cerebral cortex (Bartels and Leenders 2009; Gerfen 2010).

In PD the loss of dopaminergic input into the circuit disrupts this delicate balance. In the direct pathway there is a loss of inhibition of the GPi/SNr (Albin et al., 1989; DeLong 1990; Bartels and Leenders 2009; Gerfen 2010). The loss of dopamine in the indirect pathway interrupts the disinhibition of the GPe leading to a net excitatory output by the STN onto the GPi/SNr (Albin et al., 1989; DeLong 1990; Bartels and Leenders 2009; Gerfen 2010). This now dysfunctional circuit increases the inhibition from the GPi/SNr to the thalamus leading to a diminution of the excitatory output from the thalamus to the cortex (see Figure 5) (Albin et al., 1989; DeLong 1990; Bartels and Leenders 2009; Gerfen 2010).
**Figure 4**: Circuitry in the normally functioning basal ganglia and associated connections.

The red arrows indicate inhibitory connections, whereas the blue arrows indicate excitatory connections. Normal dopaminergic tone from the SNpc (in black) leads to balanced activation of the D1 and D2 receptors in the striatum. The release of dopamine in the striatum results in excitation of D1 receptors on inhibitory neurons of the striatum. This results in appropriate activation of the direct pathway (inhibition of the GP/SNr). Dopamine binding to D2 receptors in the striatum leads to inhibition of GABAAergic neurons in the striatum (part of the indirect pathway). The inhibitory striatal neurons of
the indirect pathway project to the GP, where they inhibit the output from the GP to the STN. The inhibitory input from the GP to the STN controls the degree of excitation from the STN onto the GP/SNr. When the direct and indirect pathways are balanced, the net effect is a properly controlled amount of inhibitory output from the basal ganglia to the thalamus. Thus, the excitatory output from the thalamus to the cortex is able to properly stimulate voluntary movement. STN = subthalamic nucleus, GP = globus pallidus, CPu = caudate putamen, SNr = substantia nigra pars reticulate, SNpc = substantia nigra pars compacta, CTX = cortex. Modified from Paxinos and Watson (2007).
Figure 5: Circuitry in the dopamine-depleted basal ganglia and associated connections. The thickness or thinness of the lines indicate increased or decreased neurotransmitter release. The red arrows indicate inhibitory connections and the blue arrows indicate excitatory projections. Loss of dopamine release (thin black line/arrow) into the striatum leads to a loss of inhibition onto D2 receptors and loss of excitation onto D1 receptors. The loss of excitation by dopamine of the D1 receptors results in a loss of the inhibition from the striatum to the GP/SNr in the direct pathway. In the indirect pathway there is a loss of inhibition on the striatal GABAergic neurons. This leads to an increase in the
inhibition from the striatum to the GP and a loss of inhibition from the GP to the STN. The loss of inhibition on the STN increases the excitatory output from the STN to the GP/SNr. Combining the decrease in inhibition from the direct pathway with the increased excitation from the STN effectively increases the inhibitory output from the basal ganglia onto the thalamus. Increased inhibition of the thalamus decreases the excitatory output from the thalamus to the cortex. STN = subthalamic nucleus, GP = globus pallidus, CPu = caudate putamen, SNr = substantia nigra pars reticulate, SNpc = substantia nigra pars compacta, CTX = cortex. Modified from Paxinos and Watson (2007).
Dopamine replacement therapy with the dopamine precursor levodopa is currently the standard of care for the treatment of PD. Unfortunately due to the progressive nature of PD, eventually the dopamine replacement becomes either ineffective or has debilitating side effects including dyskinesias. The actual cause of the degeneration of dopaminergic cells in the SNpc is still unknown although a combination of genetic and environmental factors likely plays a role. Thus, there is a pressing need for better therapies to treat this dysfunctional circuitry as well as to prevent and/or reverse the dopaminergic cell loss in this disease.

**Rodent models of PD**

There are several *in vivo* rodent models of PD using toxins or genetic manipulation to induce the dopaminergic cell death or Lewy body pathology normally associated with PD patients. The genetic models of PD are most commonly mouse models and involve either the deletion of genes important in the development of dopaminergic cells or the introduction of mutations found in familial forms of PD. The studies in this dissertation employ the use of the striatal 6-OHDA model of PD, which is one of the most prevalent rat model of PD.

The neurotoxin 6-OHDA is commonly used to produce rat models of PD. Originally isolated in 1959, 6-OHDA is a hydroxylated analogue of dopamine that is taken up by catecholamine transporters (Senoh and Witkop 1959; Breese and Traylor 1971). Once inside the neurons, 6-OHDA collects in the cell bodies and is readily oxidized, creating reactive oxygen species which are highly toxic and induces cell death (Saner and Thoenen 1971; reviewed in Blum et al., 2001; Blandini et al., 2008; Meredith...
et al., 2008). Accumulation of 6-OHDA can also occur in the mitochondria, where it inhibits complex I, further aiding in the induction of cell death (Schober 2004; Blandini et al., 2008). Intracranial administration of 6-OHDA is used because the neurotoxin does not readily cross the blood-brain-barrier. Sites of intraparenchymal injection typically include the striatum, medial forebrain bundle (MFB), or directly into the SN. The concentration and location of the 6-OHDA injection determine the time-course, size, and whether striatal dopaminergic fibers or the dopaminergic cells in the SN degenerate first (Meredith et al., 2008). Injections directly into the nigra result in very rapid dopaminergic cell death followed by loss of TH+ dopaminergic cell fibers in the striatum (Jeon et al., 1995; Meredith et al., 2008). When injected into the MFB, there is rapid loss of THir fibers in the striatum followed within 42 hours by dopaminergic cell death in the SN (Zahm, 1991; Meredith et al., 2008). Injection into the striatum leads to a more progressive lesion as the 6-OHDA is taken up by the dopaminergic terminals in the striatum and transported back to the cell bodies of origin in the SN (Przedborski et al., 1995; Meredith et al., 2008). The cell death in the ventral midbrain continues to progress for at least 4-6 weeks (Meredith et al., 2008). 6-OHDA lesions are very effective at modeling the dopaminergic cell death and motor dysfunction associated with PD, but does not model any of the extra-nigral pathology or inclusions normally associated with PD (Dauer and Przedborski, 2003; Meredith et al., 2008).

Another dopamine specific neurotoxin is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Discovered accidentally by a chemistry graduate student attempting to make a synthetic opioid, MPTP induced PD symptoms that were responsive to levadopa treatment (Ballard et al., 1985; Burns et al 1985; Langston 1985; Fahn 1996).
One of the advantages of MPTP is that it readily crosses the blood-brain-barrier. Once in the brain, MPTP is taken up by glial cells and converted into MPP+ by monoamine oxidase-B (MAO-B) (Schober 2004; Blandini et al., 2008). Once converted to MPP+, it can be taken up through the dopamine transporter and exert toxic effects upon the dopaminergic cells. Specifically MPP+ can collect in the cytoplasm or mitochondria and induce cell death by inhibiting complex I, creating a deficit in ATP, or increasing production of reactive oxygen species (Schober 2004; Smeyne and Jackson-Lewis 2005). Unfortunately MPTP susceptibility varies among mouse strains and is ineffective for rats due to their lack of MAO-B (Sedelis et al., 2000). However, intracranial injection of MPP+ can be an effective dopaminergic neurotoxin for rats (Yazdani et al., 2006). The degree of dopamine depletion and cell death that occurs in the mouse MPTP model can be variable depending on the injection paradigm. It is apparent that MPTP injections kill the dopamine cells very quickly and can continue to cause cell death with repeated injections (Bezard et al., 1997; Meredith et al., 2008). In this model inclusions and extranigral pathology have been demonstrated (Wallace et al., 1984; Hallman et al., 1985; Meredith et al., 2002). However, it is not considered to be a good model of motor dysfunction, since it does not correlate with the degree of dopaminergic depletion or MPTP dosage (Rousselet et al., 2003).

There is now recent epidemiological evidence indicating a link between the use of pesticides and the development of PD (Ascherio et al., 2006; Dick et al., 2007a,b). Systemic administration of the pesticide rotenone has gained popularity as a potential new model of PD. Rotenone is a complex I inhibitor that is administered chronically for up to five weeks (Betarbet et al., 2000; Sherer et al., 2003a,b). The rotenone model of
PD is initially able to reproduce the loss of striatal dopamine followed by a progressive loss of the nigral dopamine cells (Betarbet et al., 2000; Sherer et al., 2003a,b). Rotenone-treated animals also have extra-nigral pathology, motor deficits and inclusions (Fleming et al., 2004). The drawback to this model is that it is highly variable and can affect cells other than dopaminergic neurons limiting its utility as a PD model (Beterbet et al., 2000; Sherer et al., 2003; Fleming et al., 2004).

There are a couple of strategies for developing genetic mouse models of PD. The first is deletion of genes that are important for the development or maintenance of dopaminergic neurons. The first of these are the Pitx3 (a homeobox transcription factor) KO mice. These mice lose dopaminergic cells of the SN early in postnatal development and show behavioral deficits that are responsive to levadopa (Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004a,b; Hwang et al., 2005). Unfortunately this model lacks the progressive nature and extra-nigral pathology normally associated with PD. Another mutant model with potential relevance to PD is the engrailed KO mouse. These mice display progressive postnatal dopaminergic cell loss with PD-associated behavioral deficits (Sonnier et al., 2007). Similar to the Pitx3 KO mice, engrailed KO mice do not present with any extra-nigral pathology. In both of these models the dopaminergic degeneration occurs earlier than the normal progression of PD. Thus the true utility of these KO mouse models has not been fully elucidated.

Another strategy for developing genetic mouse models of PD is to introduce mutations associated with familial forms of PD. One such model is overexpression of alpha-synuclein. The filamentous inclusions called Lewy bodies (one of the pathological hallmarks of PD), are made up of alpha-synuclein. Although normally present in the
adult CNS, the precise function of alpha-synuclein remains unknown (reviewed in Dawson et al., 2010). Several mouse lines overexpressing alpha-synuclein using different promoters to drive the expression have been generated (reviewed in Chesselet 2007; Meredith et al., 2008). While these mice present with alpha-synuclein inclusions and can show a wide range of behavioral pathologies, they are not necessarily associated with dysfunction of the nigrostriatal dopaminergic system (Meredith et al., 2008). Furthermore, few alpha-synuclein overexpressing mice have shown degeneration of dopaminergic cells in the SN (Masliah et al., 2000; Tofaris et al., 2006; Meredith et al., 2008). Another familial form of PD is due to loss of function in the parkin gene, which is an ubiquitin ligase (Hardy et al., 2006; Meredith et al., 2008). In a mouse model of the parkin mutation, age-related dopaminergic degeneration and motor deficits occur (Lu et al., 2009). Mutations in both the PINK1 and DJ1 genes have been utilized in mouse models of PD. For Pink1, deficits in dopamine release into the striatum have been described (Kitada et al., 2007). Although DJ1 KO mice do not have dopaminergic cell loss, they do exhibit decreased resistance to oxidative stress (Dodson et al., 2007; Yamaguchi et al., 2007). Overall, both the genetic and neurotoxin models described here have their advantages and disadvantages; unfortunately, none truly mimic all of the components of PD. Thus, it is important to choose a model that appropriately models the aspect of PD that is being addressed in a particular study. Due to the slower progression and clinically relevent changes in motor function, the current studies employ the rat striatal 6-OHDA model of PD.
Neurotrophic factors for dopaminergic cells

Although the exact pathogenesis of the development of PD is unknown, it is generally thought that a combination of genetic and environmental factors play a role in the development of this disease. 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 PD (Hefti and Weiner, 1986; Hymen et al., 1991; Unsicker 1994; Temlett et al., 1996). For example in comparisons of PD patients to healthy controls, decreases in glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF) are found in the nigrostriatal system, whereas there are little or no changes in neurotrophin-3 (NT-3), neurotrophin-4 (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., 1999,2001). Several classes of neurotrophic factors have shown to be neuroprotective both in vitro and in vivo for dopaminergic cells and thus may be potential therapeutic treatments for the progression of PD. Rather than discuss every known neurotrophic factor for dopaminergic cells in this introduction, I will focus on those most extensively studied – the neurotrophin and GDNF families - and one of the most recent families of neurotrophic factors for the dopaminergic nigrostriatal system – the NRGs.

Neurotrophins

The neurotrophin family of neurotrophic factors consist of NGF, BDNF, NT-3, neurotrophin 4/5 (NT-4/5), neurotrophin 6 (NT-6), and neurotrophin 7 (NT-7). All of the neurotrophins bind to a common low affinity receptor, p75 (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992; Chao 1994; Friedman and Greene 1999). The neurotrophic
activity of the neurotrophins is mediated through their binding to a member of the high affinity receptor tyrosine kinases, the trk receptors. Signaling by NGF occurs via binding to trkA, BDNF and NT-4/5 preferentially bind to trkB, and NT-3 binds to trkC with high affinity and to trkB with low affinity (Berkmeier et al., 1991; Kaplan et al., 1991a,b; Soppet et al., 1991; Klein et al., 1992).

Neurotrophins and their receptors are highly expressed in the nigrostriatal system during development as well as into adulthood. This suggests that neurotrophic factor support by neurotrophins may not only be important in the survival and growth of the developing nigrostriatal connections but also in the maintenance of these connections in the mature CNS. Dopaminergic cells in the ventral midbrain express both BDNF and NT-3 mRNA and protein, peaking during early postnatal development (approximately 2 weeks after birth), and declining into adulthood (Friedman et al., 1991a,b; Gall et al., 1992; Seroogy and Gall 1993; Seroogy et al., 1994; Venero et al., 1994; Altar et al., 1997; Conner et al., 1997; Yan et al., 1997; Yurek and Fletcher-Turner 2001; Numan et al., 2005). Both trkB and trkC mRNA are expressed in dopaminergic cells in the SNpc at similar levels in development and adulthood (Numan and Seroogy 1999; Numan et al., 2005). In the striatum, BDNF and NT-3 mRNA are not normally detected, although BDNF protein is present, presumably transported from other areas of the brain, such as the motor cortex and SNpc (Altar et al 1997; Conner et al., 1997; Katoh-Semba et al., 1999; Yurek and Fletcher-Turner, 2001). Similar to the SNpc, trkB and trkC are expressed within the striatum during development and into adulthood (Ringstedt et al., 1993; Fryer et al., 1996; Canals et al., 1999; Yurek and Seroogy 2000).
Neurotrophins administered to mesencephalic dopaminergic cell cultures are known to be neurotrophic and/or neuroprotective. NGF appears to have little effect on dopaminergic cells in vitro (Knusel et al., 1990). The addition of BDNF to dopaminergic cell cultures can increase the survival of neurons, dopamine uptake, dopamine content, cell body size, and neurite length (Hyman et al., 1991; Knusel et al., 1991; Hyman et al., 1994; Zhou et al., 1994; Spenger et al., 1995; Studer et al., 1995). BDNF administration also significantly protects against 6-OHDA-induced dopaminergic cell death (Skaper et al., 1993). NT-3 is able to increase the dopamine content, uptake and cell survival of mesencephalic dopamine cell cultures (Hyman et al., 1994). In PC-12 cells, NT-3 is able to significantly protect against 6-OHDA-induced apoptosis (Li et al., 2008). Lastly, NT-4/5, which appears to be the most potent survival factor of the neurotrophins, significantly increases dopaminergic cell body size, neurite length and number, and cell number (Hyman et al., 1994; Hynes et al., 1994). Similar to BDNF, NT-4/5 is able to protect mesencephalic dopaminergic neurons in vitro from neurotoxin-induced cell death (Hynes et al., 1994). Both NT-6 and NT-7, structurally most closely related to NGF, are only found in teleosts (Dethleffsen et al., 2003), and have not been examined for effects on dopaminergic neurons.

Neurotrophins are effectively used to modulate the nigrostriatal dopaminergic system and afford protection in in vivo animal models of PD. The most extensively studied neurotrophin in vivo is BDNF. In the intact nigrostriatal system BDNF is able to alter dopaminergic activity by increasing dopamine release, increasing dopamine turnover, increasing spontaneous electrical activity and induce contralateral rotations in amphetamine challenged animals (Altar et al., 1992; Martin-Iversen et al., 1994; Shen et
al., 1994; Shults et al., 1994). Implanting fibroblasts modified to produce BDNF prior to a striatal 6-OHDA lesion or MPP+ injection significantly protects nigral dopaminergic cells against neurotoxicity (Frim et al., 1994; Levivier et al., 1995). More recent studies have investigated increasing endogenous expression of BDNF (Chiocco et al., 2007). For example, animals treated with the anti-parkinsonian drug selegiline show increased expression of BDNF in the cortex and striatum (Gyarfas et al., 2009). For NT-3, supranigral infusions induce a decrease in dopamine turnover as well as an increase in amphetamine-induced contralateral rotations (Martin-Iversen et al., 1994). Infusing NT-3 into the SNpc prior to and during 6-OHDA injection does not protect against the dopaminergic cell loss but is able to improve against the functional deficits of this partial lesion model of PD (Altar et al., 1994). Infusing NT-4/5 into the intact SN is able to increase striatal dopamine turnover (Altar et al., 1994). In animals with a transection of the nigrostriatal pathway, NT-4/5 infusion promotes the survival of dopaminergic neurons in the nigra to a greater extent than BDNF or NT-3 (Hagg 1998). This, in vivo and in vitro data indicate that neurotrophins still hold promise as a potential treatment for PD.

**GDNF family**

The GDNF family of neurotrophic factors consists of GDNF, neurturin (NTN), persephin (PSP), and artemin (Art). While there has been extensive examination of GDNF, the other members of this neurotrophic factor family have not been comprehensively investigated. Signaling by these neurotrophic factors occurs through binding to the GDNF family of receptors (GDNFα1-4) and the activation of the receptor tyrosine kinase ret (Durbec et al., 1996; Jing et al., 1996; Saarma 2000). Specifically the
preferred binding partners are GDNF to GDNFα1, NTN to GDNFα2, Art to GDNFα3, and PSP to GDNFα4 (Saarma 2000).

Expression of GDNF family members and receptors is found within the nigrostriatal system. Protein and mRNA for GDNF are present within the striatum and SN of the adult rat brain (Schaar et al., 1993; Springer et al., 1994; Yurek and Fletcher-Turner 2001). The expression of NTN, Art, and PSP are also found within the rat striatum (Zhou et al., 2000). The GDNF family receptors are also highly expressed within the adult SN (Nosrat et al., 1997; Widenfalk et al., 1997; Glazner et al., 1998; Horger et al., 1998). This localization of GDNF family ligands and receptors suggests that midbrain dopaminergic cells would be responsive to treatment with these neurotrophic factors.

Dopaminergic neurons are responsive to administration of GDNF family ligands both in vitro and in vivo. Addition of GDNF to midbrain dopaminergic cell cultures increased dopamine uptake and promoted the survival and differentiation of the dopaminergic neurons (Lin et al 1993; Kaddis et al., 1996). GDNF treatment of 6-OHDA challenged dopaminergic cultures protect neurons from cell death and promote recovery of the damaged dopaminergic neurons as shown by increased dopamine uptake (Eggert et al., 1999; Kramer et al., 1999; Ding et al., 2004). Neurturin has also been shown to be a potent survival factor for mesencephalic dopamine cell cultures at doses comparable to GDNF (Horger et al., 1998). Data on the effects of Art and PSP on dopaminergic cell survival in vitro are sparse, although both appear to support midbrain cell cultures (Seigel and Chauhan 2000; Akerud et al., 2002).
There are many studies demonstrating the benefits of GDNF family ligands for midbrain dopaminergic neurons \textit{in vivo}. Although GDNF and NTN are the most thoroughly investigated, the administration of PSP (via overexpressing stem cells) significantly protects against 6-OHDA-induced dopaminergic cell death and behavioral deficits (Akerud et al., 2002). In the intact nigrostriatal system, GDNF infusion affects dopaminergic function. Intranigral infusions of GDNF have been shown to increase locomotor activity, increase striatal and nigral dopamine turnover, increase basal dopamine levels in the striatum and induce sprouting of nigral dopaminergic neurons (Hudson et al., 1995; Bowenkamp et al., 1996; Hebert et al., 1997; Lapchak et al., 1997). Furthermore, injecting GDNF into either the SN or the striatum is able to significantly protect the nigrostriatal dopaminergic system of rodents against neurotoxin injections (Kearns and Gash 1995; Tomac et al., 1995; Bowenkamp et al., 1996; Kearns et al., 1997; Sullivan et al., 1998; Kirik et al., 2000a,b; Kirik et al., 2004). In a study by Sun et al. (2005), the neuroprotective effects of GDNF and BDNF were directly compared, showing that GDNF is a more effective neuroprotective agent for the nigrostriatal dopaminergic system. The use of GDNF has been extended from rodent models of PD to primate models. Experiments using the primate MPTP model of PD indicate that GDNF infusion improved the behavioral outcomes in these hemiparkinsonian animals (Gash et al., 1996; Zhang et al., 1997; Grondin et al., 2002). Intraparenchymal infusions of GDNF into the putamen has been taken to phase I clinical trials in which the primary end point was met (patients exhibited motor function benefits without adverse side effects) (Gill et al., 2003; Love et al., 2005; Slevin et al., 2005). Unfortunately, in the multi-center, blinded phase II clinical trial that followed, the results did not confirm the preliminary
results of the phase I trial (Lang et al., 2006). Similar to GDNF, NTN has been shown to protect against the behavioral and morphological changes associated with neurotoxin injection in rodents (Horger et al., 1998; Rosenblad et al., 1999; Oiwa et al., 2002). Also, viral vector-mediated expression of NTN (AAV-NTN) in MPTP-treated monkeys demonstrated significant protection of dopaminergic neurons and motor function (Kordower et al., 2006; Gasmi et al., 2007; Herzog et al., 2007; Herzog et al., 2008). This use of AAV-NTN is currently in a phase II clinical trial for use in PD patients after promising data showed functional improvements in the phase I trial (Marks et al., 2008). Thus, the potential use of neurotrophic factors for the treatment of PD has already begun, although dose, delivery method, and even the optimal area of the brain to target remain to be fully elucidated.

Neuregulins

In our lab we have begun to study the role of NRGs as potential neurotrophic, neuroprotective and neurorestorative agents for the nigrostriatal system. As stated previously, the functional NRG receptor ErbB4 is highly expressed within the dopaminergic ventral midbrain indicating a potential functional role for NRGs in the nigrostriatal dopaminergic system (Steiner et al 1999; Gerecke et al 2001; Yurek and Seroogy 2001; unpublished results). Initial in vitro studies using fetal mesencephalic dopamine cell cultures demonstrate neurotrophic and neuroprotective properties of NRGs for dopamine cells. Administration of either a NRG1 isoform (GGF2) or the NRG2 isoform (NRG-2β) significantly increases neuronal survival, neurite outgrowth, neurite number and branches, and dopamine uptake under serum-free conditions (Zhang et al., 2004; K.B. Seroogy and D.M. Yurek, personal communication). In 6-OHDA-challenged
cultures, both NRGs significantly improved cell survival when compared to control cultures (Zhang et al., 2004; K.B. Seroogy and D.M. Yurek, unpublished results). Similar to BDNF and GDNF, NRG infusions show effects in the intact nigrostriatal system. When either GGF2 or heregulin-1β was infused supranigrally, a significant increase in striatal dopamine overflow was observed when compared to PBS controls indicating a potential functional role for NRGs in vivo (Yurek et al., 2004; K.B. Seroogy and D.M. Yurek, unpublished results). Initial studies in our lab indicate the NRGs have neuroprotective effects for the nigrostriatal system in vivo. Specifically the infusion of GGF2 supranigrally using osmotic minipumps is neuroprotective against the behavioral and morphological deficits found in both partial and full striatal 6-OHDA lesioned rats (Zhang, 2003; K.B. Seroogy, unpublished results). These initial studies indicate a role for NRGs as potential therapeutic agents for the treatment of PD.

Other neurotrophic factors

Although less popular that the neurotrophins or GDNF family ligands, there are several other classes of neurotrophic factors that could be potential neuroprotective/neurorestorative agents for the treatment of PD. One of these is the fibroblast growth factor (FGF) family. Both FGFs and FGF receptors are expressed within the nigrostriatal system (Wanaka et al., 1990; Bean et al., 1991; Cintra et al 1991; Bean et al., 1992; Tooyama et al., 1992) and in mesencephalic dopamine cell cultures, bFGF promotes the survival and growth of dopaminergic neurons through indirect (glial-mediated) mechanisms (Ferrari et al., 1989; Knusel et al., 1990; Engele et al., 1991; Mayer et al., 1993; Fawcett et al., 1995). Administration of FGF in MPTP-treated mice significantly protected against neurotoxicity (Chadi et al., 1993; Date et al., 1993). Other
neurotrophic factors that have shown promise include CNTF and the mesencephalic astrocyte-derived neurotrophic factor (MANF) family of neurotrophic factors. CNTF has been shown to prevent the loss of dopaminergic neurons in nigral-transected rats (Hagg and Varon 1993). More recently, members of the MANF family have been shown to be neuroprotective and potentially neurorestorative in a rat 6-OHDA model of PD (Lindholm et al., 2007; Voutilainen et al., 2009).

**Aging and the dopaminergic nigrostriatal system**

The nigrostriatal system is particularly susceptible to age-related degeneration as indicated by functional and histological/neurochemical changes. In humans and non-human primates, decreased striatal dopamine, reduced cell number in the SN, and decreased volume of the SN are all associated with chronological aging (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, effects on the nigrostriatal dopaminergic system are less conclusive. Aged Brown Norway/Fisher 344 (BN/F344) hybrid rats exhibit a significant decrease in spontaneous and amphetamine-induced locomotion that correlates with a decrease in dopamine content in the striatum (Yurek et al., 1998). Aged Sprague-Dawley rats also exhibited deficits in several tests of motor function that are associated with decreased neurochemical function rather than morphological changes of the SN (Emerich et al., 1993). Using unbiased stereology, Sanchez et al. (2008) observed a significant loss of dopaminergic cells in the SNpc but not the adjacent VTA in senile female Sprague Dawley rats that correlated with a loss of motor function. Conversely other studies are not able to verify these neurochemical or histological changes in aged
rats (Stanford et al., 2002, 2003; Tamas et al., 2005). In aged C57/B6 mice, Tatton et al. (1991) found a significant reduction of dopaminergic cells in the SN whereas McNeill and Koek (1990) showed no significant loss in TH-positive cells in the SNpc.

In animal models of PD, aged animals appear to be more susceptible to neurotoxic lesions than young animals. Older mice have increased degeneration of SNpc dopaminergic cells and increased depletion of dopamine after administration of MPTP (Ricaurte et al., 1987a,b; Date et al., 1990). This effect is increased in monkeys, in which middle-aged rhesus monkeys showed more pronounced behavioral deficits with a smaller dose of MPTP, when compared to younger animals (Ovadia et al., 1995). Aging also affects 6-OHDA lesions in rats, leading to more severe behavioral deficits (Tamas et al., 2005). Regardless, it is apparent that the nigrostriatal dopaminergic system is particularly susceptible not only to neurodegenerative diseases affecting the basal ganglia, but also to decline of function due to the normal aging process, although the underlying mechanisms remain to be determined.

There have been few studies indicating a decrease in neurotrophic support in the aged nigrostriatal system. In Sprague Dawley rats, a decrease in BDNF and trkB protein was detected in several areas of the aged brain, including a decrease of 18% for BDNF in the SN (Croll et al., 1998). Another study reported a significant reduction of BDNF protein in the striatum of aged Sprague Dawley rats (Katoh-Semba et al., 1998). Aged F344/BN rats exhibited a significant reduction of GDNF protein in the striatum and ventral midbrain (Yurek and Fletcher-Turner, 2001). Although knockout mice for many of these neurotrophic factors and/or their receptors (i.e. BDNF, NT-3, trkB, trkC) are non-viable, haploinsufficient animals have yielded insight as to the importance of trophic
factors in nigrostriatal function and survival during aging. Heterozygous mice for BDNF show age-related decreases in nigrostriatal function without mention of loss of cells (Dluzen et al., 2001, 2004). Also, mice with haploinsufficiencies of trkB and/or trkC exhibit a reduction in numbers of dopaminergic cells in the SNpc (von Bohlen und Halbach et al., 2005).

Changes in neurotrophic factor support in the nigrostriatal dopaminergic system have also been observed in aged animals following injury or other manipulations. Dopamine-depleted young animals exhibit 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). However, this compensatory striatal increase of BDNF and GDNF is attenuated in aged rats (Yurek and Fletcher-Turner, 2000, 2001). In addition, infusions of GDNF are less effective in combating 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 implanted dopaminergic cell grafts when compared to younger animals, possibly due to a diminished trophic factor environment (Collier et al., 1999; Sortwell et al., 2001). However, infusions of BDNF, GDNF, or Schwann cell cografts, are able to improve graft survival and outgrowth (Hudson et al., 1995; Sinclair et al., 1996; Yurek et al., 1996; Yurek 1998; Collier et al., 1999). Since PD is most prevalent in the aging population (in which there are many changes that have occurred in the brain, including neurotrophic factor alterations), it is important to investigate the mechanisms of neurodegeneration and develop potential therapies in aging animal models of PD.
Hypothesis/Specific Aims

This dissertation is designed to examine the role of NRGs in the injured and aged dopaminergic nigrostriatal system. The overall hypothesis is that NRG/ErbB signaling is essential for the maintenance of dopaminergic cells in the injured dopaminergic nigrostriatal system and that NRGs are neurotrophic/neurorestorative agents for the nigrostriatal system in young and aged models of experimental parkinsonism.

Specific Aim 1: Compare the neuroprotective/neurorestorative activities of two different NRGs in a striatal 6-OHDA model of PD.

The experiments in Chapter 2 tested the hypothesis that the NRG2 isoform NRG-2β will be more neuroprotective than GGF2 (a NRG1 isoform) for the dopaminergic nigrostriatal system in a progressive striatal 6-OHDA model of PD. The lesion paradigm used in this study not only better mimics the timing of therapeutic intervention in PD patients, but also allows for the assessment of the neurorestorative effects of NRGs for the dopaminergic nigrostriatal system. Moreover, this is the first description of NRG-2β as a neurotrophic factor for dopaminergic neurons in vivo.

Specific Aim 2a: Compare the expression of ErbB receptor and NRG1 mRNA and protein during chronological aging in the nigrostriatal system.

Specific Aim 2b: Determine whether GGF2 infusions are neuroprotective/neurorestorative in an aged model of PD.

In Chapter 3, two lines of experimentation were investigated. First, the hypothesis that a potential loss of NRG signaling (via loss of NRG ligands or receptors or
both) occurs in the chronologically aging animal was examined. Using two separate strains of rats, altered protein and mRNA levels were assessed in aged animals. The second set of experiments in this aim tested the hypothesis that GGF2 infusion will be a viable neuroprotective/neurorestorative agent for the dopaminergic nigrostriatal pathway in aged animals.

**Specific Aim 3: Determine the importance of maintaining ErbB4 receptor expression for the integrity of the injured nigrostriatal dopaminergic system.**

The experiments in Chapter 4 were designed to test the hypothesis that knockdown of ErbB4 expression in the SNpc will increase the susceptibility of dopaminergic cells in the ventral midbrain to neurotoxic lesions. To test this hypothesis lentiviral vector-driven siRNA targeted against ErbB4 was utilized. After establishing ErbB4 knockdown, a striatal 6-OHDA lesion was induced. Animals administered the siRNA vector were assessed for increased motor dysfunction and dopaminergic cell death in the SNpc compared to scrambled vector-injected animals.
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Chapter 2

Neuroprotective and neurorestorative effects of the neuregulins GGF2 and NRG-2β in a 6-OHDA model of Parkinson’s disease
Abstract

Neuregulins (NRGs) are pleiotrophic growth factors that serve many important functions in the developing nervous system. Previously, our lab has shown that the type II NRG1 protein, glial growth factor-2 (GGF2), is neurotrophic and neuroprotective for fetal mesencephalic dopamine cells in culture and neuroprotective for nigral dopaminergic cells in vivo in a pretreatment paradigm. In this study, we compare administration of two neuregulin ligands (GGF2 and NRG-2β) on improvement of behavioral functional recovery and survival of dopaminergic neurons following injury to the nigrostriatal system. Adult male Sprague Dawley rats received injections of 6-hydroxydopamine (6-OHDA) or vehicle into the right striatum to create a progressive, unilateral lesion of the nigrostriatal pathway. Two weeks later, the animals were continuously infused with GGF2, the NRG2 isoform NRG-2β, or appropriate vehicles for one week above the substantia nigra pars compacta (SNpc) via osmotic minipumps. The animals were evaluated behaviorally using the forelimb-use asymmetry test (cylinder test) prior to NRG infusion and every two weeks thereafter, and were assessed for tyrosine hydroxylase (TH) and neuronal nuclei (NeuN) immunohistocytochemistry eight weeks following infusion. The results indicate that the lesioned rats receiving infusions of GGF2 or NRG-2β demonstrated enhanced performance in the cylinder test compared to their vehicle-treated controls at 2, 4, and 8 weeks post-infusion. The improved behavioral effects of GGF2 and NRG-2β infusion were not significantly different from each other at any time point. Stereological cell counts showed that post-lesion infusion of both GGF2 and NRG-2β significantly protected nigral dopamine neurons from 6-OHDA-induced degeneration. As with the behavioral data, the dopamine cell survival effects of GGF2
and NRG-2β were not different from each other. These data implicate NRGs as functional trophic factors for the dopaminergic nigrostriatal system with neuroprotective/neurorestorative activity \textit{in vivo}, and raise the possibility of a therapeutic role for these growth factors in the treatment of Parkinson’s disease.
Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder that affects approximately 1% of the population over 60 years of age (de Lau et al., 2006; Olanow et al., 2009; Chen 2010). The hallmark motor symptoms associated with PD are due to a disruption of the normal dopaminergic signaling in the nigrostriatal pathway (Kapp 1992). Current treatment strategies for PD begin with dopaminergic replacement therapy (levadopa) (Obeso et al., 2010). Unfortunately the utility of this therapy fades necessitating increased doses of levadopa, potentially leading to the development of debilitating dyskinesias (Obeso et al., 2010). Furthermore, dopamine replacement therapies do not prevent the progression of the neurodegeneration.

Although the exact pathogenesis for the development of idiopathic PD is unknown it is generally thought to be a combination of genetic and environmental factors. Many have theorized that loss of neurotrophic factor support for the nigrostriatal pathway may increase susceptibility for degeneration in this system (Hefti and Weiner, 1986; Hyman et al., 1991; Unsicker 1994; Temlett et al., 1996). Neurotrophic factor treatment in rodent models of PD are neuroprotective for the dopaminergic nigrostriatal system (e.g. Altar et al., 1994; Frim et al., 1994; Martin-Iversen et al., 1994; Kearns and Gash 1995; Levivier et al., 1995; Tomac et al., 1995; Bowenkamp et al., 1996; Kearns et al., 1997; Sullivan et al., 1998; Kirik et al., 2000a,b; Kirik et al., 2004; Sun et al., 2005). These studies have led to clinical trials for the use of glial cell line-derived neurotrophic factor (GDNF) and neurturin in PD patients, which have had promising results in phase I, but failed to meet the primary end points in the phase II blinded trials (Gill et al., 2003; Love et al., 2005; Slevin et al., 2005; Lang et al., 2006; Marks et al., 2008). Using
neurotrophic factors may still be a viable therapy for the treatment of PD; however, the dose, delivery method, delivery area, and choice of neurotrophic factor or factors need to be further addressed.

Neuregulins (NRGs) are a family of structurally related growth and differentiation factors for tissues throughout the body (Burden and Yarden, 1997; Gassmann and Lemke, 1997; Buonanno and Fischbach, 2001; Yarden and Sliwkowski, 2001; Falls 2003; Seroogy and Zhang, 2006). Neuregulins and their receptors, the ErbB receptors, are widely distributed throughout the adult central nervous system (CNS) (Chen et al., 1994; Corfas et al., 1995; Steiner et al., 1999; Gerecke et al., 2001; Yurek and Seroogy, 2001; Law et al., 2004; Bernstein et al., 2006). In the dopaminergic ventral midbrain, ErbB4 and ErbB1 are highly expressed in both the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA), and are colocalized to dopaminergic cells as evidenced by reduced expression in dopamine-depleted animals (Seroogy et al., 1994; Steiner et al., 1999; Gerecke et al., 2001; Yurek and Seroogy, 2001). Although NRGs and ErbB receptors are present in the adult nigrostriatal system, the role of NRG support and signaling in dopaminergic function and dysfunction remains to be fully elucidated.

Not only are the ErbB receptors present in the nigrostriatal dopaminergic system, but they also appear to be functional both in vitro and in vivo. In fetal mesencephalic dopamine cell cultures the NRG-1 product, glial growth factor-2 (GGF2), is neuroprotective to dopamine cells; significantly increasing neuronal survival, neurite outgrowth, and increasing dopamine uptake in 6-OHDA-challenged cultures (Zhang et al., 2004). When injected directly into the SNpc, NRG1-β1 induces striatal dopamine overflow indicating functional effects of NRGs in the nigrostriatal system (Yurek et al.,
Furthermore, initial evidence in our lab indicates a neuroprotective effect of GGF2 pretreatment in 6-OHDA-treated animals (K.B. Seroogy and D.M. Yurek, unpublished results). Thus, in the adult animal NRGs may have a functional and/or neuroprotective role in the maintenance of the dopaminergic cells of the ventral midbrain.

There is now emerging evidence of differing spatial, temporal, and cellular distributions of NRG2 versus NRG1 proteins. Specifically, the expression of NRG1 is higher during development and decreases in postnatal animals, whereas NRG2 expression increases after development (Longart et al., 2004; Talmage and Role, 2004). They also appear to be expressed in complimentary areas of the brain (e.g. NRG1 in the hippocampal regions CA1 and CA3, while NRG2 in the dentate gyrus) (Longart et al., 2004; Talmage and Role, 2004). At the cellular level, NRG2 proteins are expressed almost exclusively within dendrites, whereas NRG1 proteins can be found within axons, dendrites, and somata (Longart et al., 2004; Talmage and Role, 2004). Furthermore, NRG2 and NRG1 proteins differ in the preferred ErbB receptor combinations they activate (Carraway et al., 1997; Nakano et al., 2000; Sweeney et al., 2001). Interestingly it appears that NRG2 proteins may be more effective than NRG1 at activating ErbB4/ErbB1 receptor combinations (Carraway et al., 1997). This may be particularly important for the ventral midbrain where ErbB4 and ErbB1 are the most highly expressed of the ErbB receptors. Thus, NRG1 and NRG2 proteins may differ in their putative neuroprotective activity for the nigrostriatal system.

In the present study we determine the availability of ErbB receptor mRNA in a striatal 6-OHDA model of PD in which there is a two week delay between the start of the lesion and subsequent NRG infusion. Thus, dopaminergic cell death has begun prior to
trophic factor therapy with NRGs. In using this delayed-infusion model of PD, we not only better mimic the timing of treatment in PD patients but also potentially assess neurorestoration of function. We examine the effects of GGF2 (a NRG1 isoform) and NRG-2β (a NRG2 isoform) infusion on functional and morphological changes of the nigrostriatal system after 6-OHDA lesion. We also determine if there is a difference in the efficacy of GGF2 versus NRG-2β to protect against 6-OHDA-induced dopamine cell death and associated behavioral motor dysfunction. This could indicate a role for NRG/ErbB signaling in the support of mesencephalic dopamine neurons in vivo against neurotoxic degeneration.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (n=72) were used in this study (Harlan Sprague Dawley, Frederick, MD) weighing 250-350 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.

Surgical procedures

Animals were anesthetized (87 mg/kg ketamine, 13 mg/kg xylazine; i.p.) and placed in a stereotaxic apparatus. Intrastriatal injection of the catecholamine-specific neurotoxin 6-OHDA was used to induce a progressive deterioration of the nigrostriatal pathway. Two unilateral injections of 6-OHDA (20μg in 2μl; Sigma Aldrich, St. Louis, MO) or vehicle (equivalent volume of 0.2% ascorbic acid physiological saline solution)
were administered to the striatum [AP: +0.5 ML: -2.5 DV: -5.0 and AP: -0.5 ML: -4.2 DV: -5.0 (Lee et al 1996; Kirik et al 1998; Deumens et al 2002; Paxinos and Watson, 2007)] via a 2-µl Hamilton syringe (Hamilton Company, Reno, NV). The syringe was lowered into the brain parenchyma and allowed to equilibrate for 5-min, the 6-OHDA or vehicle was then injected over a 10-minute period (0.2 µl/min) with a 5 min wait time post-injection before slowly withdrawing the syringe.

To better mimic the timing of therapeutic intervention (compared to neuroprotection studies) for the treatment of PD in this model, two weeks after initiation of the lesion (in which nigral dopaminergic cell degeneration has begun), the animals receiving NRG infusions were again anesthetized and placed in a stereotaxic apparatus. A cannula was lowered just above the SNpc [AP: -5.2 ML: -1.8 DV: -7.2 (Paxinos and Watson, 2007)] and attached to an osmotic minipump (model 1007D, Alzet, Cupertino, CA) that administered GGF2 (0.4 mg/ml; Acorda Therapeutics, Hawthorne, NY), NRG-2β (0.4 mg/ml; Acorda Therapeutics), phosphate-buffered saline (PBS), or the GGF2 vehicle (20 mM NaAcetate, pH 6.5, 100 mM Na₂SO₄, 100 mM arginine, and 1% mannitol) at a rate of 0.5 µl/hr for seven days for a total of 34 µg of NRGs infused. The pump was surgically placed between the shoulder blades and the cannula was held in place by 3 screws (Small Parts Inc., Logansport, IN) and dental cement (Dentsply International Inc., York, PA). The pumps continuously infused the reservoir contents for one week after which they were removed. Eight weeks following surgery, the rats were deeply anesthetized using an interperitoneal injection of sodium pentobarbital (60 mg/kg) followed by intracardial perfusion with 250 ml of cold 0.1M PBS and then 4% paraformaldehyde in PBS (pH 7.4). The brains were removed and post-fixed for 90
minutes in the fixative, then placed in a cryoprotectant solution of 30% sucrose in PBS at 4°C until being sectioned on a microtome. A timeline depicting the experimental paradigm and outcome measures is shown in Figure 1.

**In situ hybridization**

Rats processed for *in situ* hybridization in the initial 6-OHDA lesion studies were anesthetized with an overdose of sodium pentobarbital and rapidly decapitated. The brains were removed, mounted onto TBS tissue freezing medium (Ted Pella Inc., Redding CA) and frozen in powdered dry ice. The brains were subsequently sectioned (at 10-µm thickness) on a Microm Cryostat (Walldorf, Germany) throughout the ventral mesencephalon and thaw-mounted onto VWR micro slides (VWR, West Chester PA). The sections were then stored at -20°C until hybridization. Semi-adjacent sections throughout the rostrocaudal extent of the ventral mesencephalon were hybridized with 35S-labeled cRNA probes for detection and localization of ErbB1-4 and tyrosine hydroxylase (TH) mRNAs. The probes were prepared by *in vitro* transcription from linearized cDNA plasmids using the proper RNA polymerase and labeled with 35S-UTP (PerkinElmer, Waltham MA) as previously described (Seroogy and Herman, 1997). The TH cDNA plasmid (provided by J. Herman, University of Cincinnati) was contained in a pCR-TOPO vector and consisted of 366 bp. The ErbB2-4 plasmids (provided by H. Kornblum, UCLA) were contained in a pCR 2.1 vector and consisted of 1.8 kb (Kornblum et al., 2000). The ErbB1 (EGFr) plasmid consisted of 2.9 kb (Seroogy et al., 1994). The slides were pretreated as previously described (Seroogy and Herman, 1997; Numan and Seroogy, 1999; Numan et al., 2005). Briefly, slide-mounted sections were
Figure 1: Schematic depiction of experimental paradigm. The animals were injected with 6-OHDA at the start of the experiment (Day 0). Two weeks after initiation of the lesion, a cannula attached to an osmotic minipump is implanted to infuse NRGs supranigrally for the period of one week. The animals were assessed behaviorally with the forelimb-use asymmetry test (cylinder test) prior to NRG infusion and every two weeks thereafter. Ten weeks after 6-OHDA injection the animals were sacrificed for determination of lesion-induced morphological changes.
brought to room temperature and fixed in 4% paraformaldehyde. The sections were then taken through a sequential series of washes made with 0.1% diethylpyrocarbonate (DEPC)-treated water consisting of 0.1 M PBS, 0.1 M PBS/0.2% glycine, 0.1 M PBS, and triethanolamine (TEA) containing 0.25% acetic anhydride. The sections were dehydrated in a sequence of ethanol washes of increasing concentration, placed in chloroform for delipidation, and air-dried. Sections were hybridized overnight at 60°C in hybridization solution consisting of 50% deionized formamide, 10% dextran sulfate, 20 mM Tris-HCl, 1 mM EDTA, 1X Denhardt’s solution, 0.33 mg/ml denatured salmon sperm DNA, 0.15 mg/ml tRNA, 40 mM dithiothreitol, DEPC H2O and the 35S-labeled probe at a concentration of 1x10^6 cpm/50 µl. After hybridization, the coverslips were removed and the sections were washed in 4X standard saline citrate (SSC; 1X SSC = 0.015 M sodium citrate, 0.15 M sodium chloride at pH 7.0) with 10 mM sodium thiosulfate at 37°C. The sections were then washed in RNase buffer containing ribonuclease A (10 mg per 200 ml of buffer) at 45°C for 30 min and placed in an additional series of SSC washes of decreasing concentrations (2X, 0.5X, 0.1X) at 37°C. The final wash did not contain sodium thiosulfate. The sections were immersed briefly in dH2O and 95% ethanol and then air-dried. The slides were then exposed to BioMax MR film (Kodak, Rochester NY) for 2 days (TH cRNA) or 13 days (ErbB1-4 cRNA) for generation of film autoradiographs. The films were developed with Kodak GPX developer and fixer (Kodak). For the semi-quantitative in situ hybridization analysis, the mean corrected gray levels were obtained for each group to compare mRNA levels of hybridization using Scion Image software (National Institutes of Health). Measurements of the 6-OHDA-lesioned side were compared to the intact side. Values for the
hybridization data are expressed as mean corrected grey levels (as percent intact side) ±
the standard error of the mean (SEM). Measurements were taken from 6 sections per
animal for each probe. The data were analyzed using one-way ANOVA, followed by the
Tukey’s multiple comparison post-hoc test.

**Forelimb-use asymmetry**

The forelimb use asymmetry test (cylinder test) was used to assess motor
impairment of 6-OHDA-lesioned, NRG-treated animals and was performed as previously
described (Schallert et al 2000; Tillerson et al 2001; Woodlee et al 2005; Schallert 2006;
Anstrom et al 2007). The use of this behavior test has several advantages as a measure of
functional recovery for the dopaminergic nigrostriatal system. It is less stressful on the
rats, using their natural propensity to explore their environment to assess lesion severity.
Compared to amphetamine-induced rotational behavior, it is more sensitive to determine
recovery of motor function and does not have the disadvantage of repeated amphetamine
injections. Briefly, rats were placed in a clear plexiglass open cylinder, tall enough that
the rat cannot reach the top with its forepaws, and allowed to investigate the environment.
A mirror was placed behind the cylinder so that all movements the animals made could
be clearly seen with a video camera. All measurements were taken during the dark cycle
in low light conditions. The animals were allowed to naturally explore the cylinder; after
a total of 20 to 25 forelimb wall contacts were observed, the animal was removed from
the cylinder and placed back in the home cage. The number of contacts of each forelimb
or both forelimbs was later scored from the videos. An asymmetry score was calculated
by dividing usage of the impaired limb (impaired plus ½ both) by total limb usage
(impaired + unimpaired + both). The cylinder test was performed every two weeks after
6-OHDA injection. Statistical comparisons included a one-way ANOVA (with repeated measures for neurorestoration data) followed by the Tukey’s multiple comparisons post hoc test.

**Immunohistochemistry**

Six sections (one out of every twelve sections) of the ventral midbrain and striatum were stained with an antibody for TH or neuronal nuclei (NeuN) using the ABC method. Representative free-floating sections were rinsed using 0.1 M phosphate buffer (PB). Non-specific staining was blocked using 10% normal horse serum (NHS; Vector Laboratories, Burlingame, CA) in PB for TH and 10% normal goat serum (NGS; Vector Laboratories) in PB for NeuN for an hour. The sections were then incubated in TH (1:8000; Chemicon International, Temecula, CA) or NeuN (1:1000; Chemicon International) primary antibody with 1% normal serum and 0.1% Triton X-100 overnight at 4°C with constant agitation. The next day the sections were washed in PB and then rinsed in 2% NHS (TH) or NGS (NeuN) in PB. Sections were incubated with the anti-mouse IgG biotin-conjugated secondary antibody (1:200 made in horse for TH and 1:400 made in goat for NeuN; Vector Laboratories) for an hour. The secondary antibody was rinsed off with 3-5 min PB washes. The sections were then incubated for 30 min in ABC-peroxidase reagent (Vector Laboratories) and rinsed with 50 mM Tris buffer (pH 7.5). Antisera labeling was visualized using the diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories) containing 0.3 % H2O2, 7.5 pH Tris buffer, and DAB reagent followed by 50mM Tris buffer. The sections were then mounted onto Superfrost Plus glass slides, (VWR International, West Chester, PA) dehydrated through a series of alcohols of increasing concentration, a clearing agent, and coverslipped.
**Stereological cell counting**

To investigate neuronal cell survival after 6-OHDA lesion and subsequent NRG treatment, the number of TH+ and NeuN+ cells in the SNpc was estimated using unbiased stereologic principles and techniques (West 1993). Immunostained sections were visualized with an Olympus BX-60 microscope (Melville, NY) using a CCD video camera (HV-C20, Hitachi, San Jose, CA). The SNpc was outlined under 2X magnification whereas counting was performed under 60X magnification using the optical fractionator technique via the Stereo Investigator software (Microbrightfield, Williston, VT). For each section analyzed, a guard zone of 2-4 μm was established. The stereology software randomly selected sampling areas to count using a 170X100 grid size for TH and 220X220 grid size for NeuN. Cells were counted if they fell within the 50X50 μm counting frame by standard inclusion criteria. The coefficient of error (CE) was calculated for each animal using the Gundersen correction and was lower than 0.15. Statistical comparisons included a one-way ANOVA followed by the Tukey’s multiple comparison post-hoc test.

**Striatal TH fiber density**

Striatal TH immunoreactive fiber density (as represented by mean grey level) was determined using Scion image software (National Institutes of Health). The mean grey level of immunostained sections was obtained in 1 out of every 12 sections throughout the striatum for a total of 6 sections analyzed per animal. The intact side striatum was used as a control for the DAB staining, with the lesioned side being represented as a percent of the intact side. Statistical comparisons included a one-way ANOVA followed by the Tukey’s multiple comparison post-hoc test.
Results

ErbB receptor availability

Previous experiments have shown that 6-OHDA lesions decrease ErbB1 and ErbB4 mRNA expression in the dopaminergic ventral midbrain (Seroogy et al., 1994; Steiner et al., 1999). To determine the availability of ErbB receptors in the 6-OHDA-lesioned SNpc at the time of intervention for the current experiments, in situ hybridization for ErbB1-4 and TH was performed. The animals were injected with 6-OHDA into the striatum and sacrificed one day, one week or two weeks later. In the ventral midbrain, robust expression of ErbB4 and ErbB1 mRNA was observed in the SN and the VTA as previously described, whereas only low levels of ErbB3 and ErbB2 were present (Figure 2; see also Seroogy et al., 1994; Steiner et al., 1999; Gerecke et al., 2001). The injection of 6-OHDA into the striatum significantly altered the expression of TH, ErbB4 and ErbB1 in the SNpc. For TH mRNA, a significant reduction in expression occurred by 1 week post-lesion to a value of 34.8±1.8 percent of the intact side (p<0.01 versus vehicle; see Figure 3) and further decreased two weeks after the lesion to 12.8±3.0 percent intact side (p<0.01 versus vehicle and lesion 1 week; see Figure 3). The expression of ErbB4 decreased in a similar pattern but to a lesser degree than TH mRNA. At 1 week post-lesion ErbB4 mRNA measured 64.0±3.6 percent of the intact side, and at two weeks ErbB4 expression declined to 55.0±3.3 percent of the intact side (Figure 2 and 3; p<0.01 versus vehicle-treated animals). ErbB1 mRNA expression was also significantly decreased at one week post-lesion to 75.8±7.2 percent intact side and at two weeks following the 6-OHDA lesion the expression decreased to 57.9±2.1 percent intact side (p<0.01 versus vehicle). Thus by two weeks after neurotoxin injection both ErbB4 and
ErbB1 expression declined by approximately 45% while TH expression was about 87% less than the intact side (see Figure 3). Neither ErbB2 nor ErbB3 expression changed in the ventral midbrain due to 6-OHDA injection (data not shown). These data indicate that in this model of PD, ErbB4 and ErbB1 receptors are present within the SN at the time of intended intervention (two weeks after striatal 6-OHDA injection).

**Neurorestoration: GGF2 and NRG-2β**

The cylinder test was used to determine changes in motor function due to 6-OHDA lesions and NRG treatment in animals infused with either GGF2 or NRG-2β compared to their vehicle controls (“PBS” for NRG-2β and “vehicle” for GGF2 as described in the Methods). There were no changes in motor function for any of the intact (unlesioned) animals treated with supranigral infusions of either NRG protein or their appropriate vehicles for any of the time-points assessed. All lesioned animals exhibited significant forelimb-use asymmetry versus the intact control animals at all time-points. As early as 4 weeks after 6-OHDA lesion (2 weeks after beginning supranigral infusion), treatment with either GGF2 or NRG-2β significantly improved limb usage impairments relative to the appropriate vehicle control. The 6-OHDA/veh animals displayed an asymmetry score of 1.9±1.2 percent impaired limb usage versus 15.3±3.6 percent for the 6-OHDA/GGF2-treated animals at 4 weeks (p<0.01), while 6-OHDA/PBS animals exhibited an asymmetry score of 7.0±2.8 impaired limb usage versus 6-OHDA/NRG-2β treated animals at 17.8±2.8 percent (p<0.01; see Figure 4). At 6 weeks post-lesion
Figure 2: Representative reverse image film autoradiograms depicting TH and ErbB receptor mRNA expression after striatal 6-OHDA lesion. For vehicle-treated animals, representative examples from an animal 2 weeks after vehicle injection are shown (no reduction in expression of any mRNA is observed). The expression of TH mRNA in the SNpc declines by 1 week after striatal 6-OHDA injection and appears to be almost completely diminished after 2 weeks of lesion progression (left column; arrow indicates side of lesion). Expression of ErbB4 and ErbB1 mRNA also declines after striatal 6-OHDA lesion (right two columns), although the decrease proceeds at a slower rate than that of TH mRNA. Scale bar=1000 μm. n=4 animals in each group.
Figure 3: Quantification of in situ hybridization signal in the SN after striatal 6-OHDA lesion. 

**A:** Vehicle injection did not alter the mRNA expression levels of the injected side compared to the contralateral side. The expression of TH mRNA was significantly decreased 1 week after striatal 6-OHDA injection. Two weeks after lesion, TH mRNA continued to decline when compared to 1 week post-lesion. **B and C:** Similarly, there was no change in ErbB4 and ErbB1 mRNA expression after vehicle injection. In 6-
OHDA-lesioned animals the expression of ErbB4 and ErbB1 mRNA declined 1 and 2 weeks post-lesion. Note that the degree of ErbB4 and ErbB1 loss is less than that of TH mRNA and further progressed from the 1-week to the 2-week time point. *p<0.01 versus saline control, ^p<0.01 versus lesion 1-week group. n=4 per group.
GGF2-infused animals continued to show improvement of impaired limb usage with an asymmetry score of 22.1±4.2 compared to vehicle-infused animals at 9.3±2.2 (p<0.01); similarly NRG-2β-treated animals exhibited a percent impaired limb usage of 24.9±4.0 versus PBS-infused animals at 11.0±3.9 percent impaired limb usage (p<0.01; see Figure 4). At 8 weeks post-lesion, administration of GGF2 or NRG-2β appeared to improve motor function although it did not reach statistical significance (p=0.07 for 6-OHDA/GGF2- versus 6-OHDA/veh-treated animals; p=0.10 for 6-OHDA/NRG-2β versus 6-OHDA/PBS animals; Figure 4). By 10 weeks post-lesion, NRG infused animals again displayed significant improvement of impaired limb usage, 6-OHDA/veh- and 6-OHDA/GGF2-treated animals had asymmetry scores of 4.9±3.4 and 26.4±2.7 percent impaired limb usage respectively (p<0.01), and 6-OHDA/PBS and 6-OHDA/NRG-2β animals displayed asymmetry scores of 11.7±4.3 and 25.5±2.8 percent impaired limb usage, respectively (p<0.01; Figure 4). Infusions of NRG-2β did not improve motor function when compared to GGF2 infusions at any of the time-points. The 6-OHDA/GGF2- and 6-OHDA/NRG-2β-treated animals were compared at 4 and 10 week post-lesion to determine if there was any potential neurorestoration of motor function. Lesioned animals receiving either GGF2 or NRG-2β both significantly improved motor function at 10 weeks post-lesion compared to the 4 week time point while vehicle-or PBS-infused animals did not (Figure 5; p<0.05).

**Stereology of TH+ cells and striatal fiber density**

To determine whether NRG-2β or GGF2 were neuroprotective for the dopaminergic nigrostriatal system against neurotoxin-induced morphological changes,
Figure 4: Quantification of the forelimb-use asymmetry test. All lesioned animals displayed significantly impaired forelimb use at all of the time-points assessed when compared to unlesioned controls. By 4 weeks post-lesion (two weeks after NRG infusion) and at 6 weeks post-lesion, lesioned animals infused with either GGF2 or NRG-2β showed significant improvement of motor function (increased use of impaired limb).
when compared to control lesioned animals. At 8 weeks post-lesion, GGF2 and NRG-2β infused lesioned animals use their impaired limb more often than their appropriate control lesioned animals, although it did not reach statistical significance. At 10 weeks post-lesion, the significant motor function improvement afforded by NRG infusion is present and also appears to increase when compared to 4 weeks post-lesion potentially indicating neurorestoration. *p<0.01 versus appropriate lesioned control group. ^p=0.07 $p=0.10. n=6-8 per group.
Figure 5: Comparison of forelimb-use asymmetry test results at 4 and 10 weeks post-lesion. Treatment of lesioned animals with GGF2 or NRG-2β resulted in significant enhanced recovery of motor function at 10 weeks compared to the 4-week time-point indicating a neurorestorative effect on impaired limb usage in this model of PD.

*p<0.05. N=6-8
stereological cell counts of TH+ and NeuN+ cells were performed in the SNpc and striatal TH+ fiber density was quantified. Neither GGF2 or NRG-2β administration significantly altered TH+ or NeuN+ cell number in the SNpc of vehicle-injected animals (unlesioned animals; data not shown). All of the 6-OHDA-lesioned animals displayed significantly less numbers of TH+ and NeuN+ cells when compared to unlesioned control animals (p<0.01). Treatment of lesioned animals with either GGF2 or NRG-2β significantly protected against dopaminergic cell death when compared to the appropriate lesioned control groups (p<0.01 versus appropriate control group; Figure 6). Counts of NeuN+ cells were assessed in order to distinguish between potential loss of TH phenotype rather than cell death of dopaminergic neurons. There were no significant differences between TH+ cell loss and NeuN+ cell loss indicating that the TH+ cell loss in these animals is due to cell death rather than phenotypic change (data not shown). The density of TH+ fibers in the striatum was also assessed as a measure of morphological integrity of the nigrostriatal pathway due to 6-OHDA lesion and subsequent NRG treatment. Infusions of either of the NRGs investigated resulted in a nonsignificant increase in TH+ fiber density in the striatum compared to lesioned control animals. In 6-OHDA/veh animals fiber density was 53.4±3.9 percent of the intact side compared to 67.9±5.8 for the 6-OHDA/GGF2 group (p=0.07). The 6-OHDA/PBS group exhibited 50.9±3.3 percent fiber density versus 61.8±5.0 for the 6-OHDA/NRG-2β-treated animals (p=0.10; Figure 7).
Figure 6: TH immunohistochemistry and stereological cell counts in the SNpc. 

**A:**
Representative photomicrographs of the SNpc of lesioned animals infused with GGF2, NRG-2β, or appropriate vehicle. Note the increase in TH+ cell survival in the SNpc of NRG-treated animals when compared to lesioned control (vehicle-treated) animals.

**B:**
Quantification of TH+ cells in the SNpc revealed a significant increase in TH+ cell survival in the NRG-treated groups when compared to the appropriate lesioned control animals. *p<0.05, **p<0.01. compared to appropriate control-lesioned group. Scale bar=100 μm. n=6-8 per group.
Figure 7: TH+ fiber density in the striatum. 

A: Representative photomicrographs of the striatum from lesioned animals infused in the SNpc with PBS or NRG-2β. There appears to be increased fiber density in the striatum of NRG-2β treated animals (GGF2 infusion displayed similar results).

B: Quantification of fiber density (mean grey levels) indicated a nonsignificant increase of TH+ fiber density in the striatum of NRG-treated animals compared to the lesioned control groups. ^p=0.07, $p=0.10. Compared to appropriate control-lesioned group. Scale bar=500 μm. n=6-8 per group.
Discussion

These data demonstrate the neuroprotective and potential neurorestorative effects of NRGs for the dopaminergic nigrostriatal system in 6-OHDA-lesioned animals. In this paradigm, the infusions of NRGs occurred 2 weeks after the onset of the 6-OHDA lesion to better mimic the timing of therapeutic intervention for PD patients, in which significant neurodegeneration has occurred prior to diagnosis and treatment initiation. At this time, expression of the NRG receptor ErbB4, and one of its heterodimerization partners ErbB1, mRNAs were still present at a higher level than the remaining TH mRNA. In lesioned animals infused with NRGs, both of the NRGs tested (GGF2 and NRG-2β) significantly improved dopaminergic cell survival in the SNpc of lesioned animals. This protection of the nigrostriatal system translated to protection of motor function in these animals. Thus, there was neurorestoration of behavioral motor function when the animals were compared at the beginning versus the end of this study.

There are previous reports of decreased ErbB4 and ErbB1 mRNA expression due to 6-OHDA lesions. By injecting 6-OHDA directly into the SNpc, Steiner et al. (1999) showed that virtually all of the ErbB4 mRNA expressing cells were lost 20 days after the neurotoxin injection (a time at which there is severe dopaminergic cell death). Those animals were also assessed for changes in ErbB3 mRNA, in which no difference in ErbB3 expression in dopamine-depleted animals was found (Steiner et al., 1999). In animals with a medial forebrain bundle lesion, ErbB1 expression is also almost completely extinguished two weeks after the lesion (Seroogy et al., 1994). In the striatal 6-OHDA model of PD that is employed in this study, progression of the lesion occurs over a course of 4-6 weeks (Przedborski et al., 1995; Meredith et al., 2008). Thus, it was
unknown how this progressive neurotoxic lesion would affect expression of ErbB receptors at a time earlier than complete dopaminergic denervation. This study is in agreement with the previous data, showing that a striatal 6-OHDA lesion significantly decreases ErbB4 and ErbB1 mRNA and does not effect ErbB3 mRNA expression. The loss of ErbB1 and ErbB4 expression begins within one week of the neurotoxin injection and continues to decrease two weeks after injection. Interestingly, the progression of the decrease in the ErbB receptor mRNA occurred at a slower pace than that of TH mRNA (about 45% lost for ErbB1 and ErbB4 and 87% lost for TH at two weeks post-lesion). This indicates that in this model, two weeks after 6-OHDA injection, there are ErbB receptors available in the injured nigrostriatal system that could be activated by NRG ligand treatment. Although we report a significant 87% loss of TH mRNA expression here, it is unlikely that there is as large a loss of cells within the SNpc. Recent data on the time course of TH cell survival compared to NeuN cell survival in a striatal lesion indicate a phenotypic change of the dopaminergic cells in the SNpc two weeks after initiation of the lesion (Spieles-Engemann et al., 2010). The loss of NeuN+ cells then matches the TH+ cell loss at 4 and 6 weeks after the lesion (Spieles-Engemann et al., 2010). Thus there is a large population of cells within the SNpc that have lost their phenotype and that may be potentially restored by activation of ErbB receptors through NRG treatment.

There are several lines of evidence showing that neurotrophic factors are neuroprotective for the nigrostriatal dopaminergic system in animal models of PD. The most notable of these are brain-derived neurotrophic factor (BDNF) and GDNF (e.g. reviewed in Chiocco et al., 2007). In animals with neurotoxic lesions, treatment with
either intrastriatal injections of astrocytes or fibroblasts genetically engineered to express BDNF significantly protect against dopaminergic cell degeneration and rotational behavior (Frim et al., 1994; Levivier et al., 1995; Yoshimoto et al., 1995; Galpern et al., 1996). Using gene transfer strategies to induce BDNF expression in neurons of the SNpc significantly protected against lesion-induced rotational behavior, but had no protective effect against dopaminergic cell death (Klein et al., 1999). The use of GDNF is neuroprotective in both rodent and primate models of PD. For rodents, infusions of GDNF into the SNpc or the striatum significantly protects against neurotoxin lesions of the nigrostriatal dopaminergic pathway, improving behavioral function and dopamine cell survival (Hoffer et al., 1994; Tomac et al., 1995; Bowenkamp et al., 1996; Kearns et al., 1997; Sullivan et al., 1998; Kirik et al., 2000). Intrastratal or intracerebroventricular infusion of GDNF in primate MPTP models of PD improve against parkinsonian behaviors, protect dopaminergic cells of the SNpc and increase dopamine levels in the striatum (Gash et al., 1996; Gerhardt et al., 1999; Costa et al., 2001; Grondin et al., 2002). In a more recent study, gene transfer in the striatum of BDNF, GDNF or both were compared in a rodent 6-OHDA model of PD (Sun et al., 2005). These data showed that GDNF was more effective than BDNF at protecting against lesion-induced rotational behavior and dopaminergic cell death (Sun et al., 2005). Simultaneous expression of both BDNF and GDNF in this model did not afford increased protection from neurotoxic lesioning than with GDNF alone (Sun et al., 2005).

In our lab, we have begun characterizing the use of NRGs as novel survival factors for dopaminergic cells in vitro and neurotrophic factors for the SNpc in vivo. Treatment of fetal mesencephalic dopaminergic cell cultures with either NRG-2β or
GGF2 significantly increase cell number, neurite number, and neurite outgrowth in serum free and 6-OHDA challenged cultures (Zhang et al., 2004; K.B. Seroogy and D.M. Yurek, unpublished results). Infusions of GGF2 supranigrally significantly increases dopaminergic release in the striatum in intact animals and protects against the behavioral deficits and dopaminergic cell loss with pretreatment in a striatal 6-OHDA lesion (Yurek and Seroogy, 2001; Yurek et al., 2004; K.B. Seroogy and D.M. Yurek, unpublished results). The current results indicate that infusions of GGF2 or NRG-2β significantly protect dopaminergic cells of the SNpc after striatal 6-OHDA lesions when compared to lesioned control animals. Based on previous data indicating differences of NRG2 versus NRG1 activation of ErbB receptors, we hypothesized that NRG-2β would be more neuroprotective of dopaminergic cells than GGF2 (Carraway et al., 1997; Longart et al., 2004). Contrary to our prediction, NRG-2β was equal to, but not more beneficial than, GGF2 for improvement of dopaminergic cell survival and associated behavioral function.

While there is an abundance of studies indicating the neuroprotective effects of neurotrophic factors for models of PD, data showing neurorestoration of the nigrostriatal system are more difficult to demonstrate. Lentiviral vector-mediated expression of GDNF in the striatum and SNpc of MPTP-treated monkeys induced neurorestoration of motor function as evidenced by improvement in clinical rating scores (Kordower et al., 2000). Similarly, adeno-associated virus neurturin-treated MPTP-lesioned monkeys showed improvements in motor function consistant with neurorestoration (Kordower et al., 2006). Recently, Voutilainen et al. (2009) showed that both GDNF and mesencephalic astrocyte-derived neurotrophic factor (MANF) infused 4 weeks after lesion restored behavioral function in 6-OHDA-lesioned rats. Similarly, infusions of
conserved dopamine neurotrophic factor (CDNF) 4 weeks after 6-OHDA lesion also restored dopaminergic function (Lindholm et al., 2007). In the current study, the start of the 6-OHDA lesion and infusion of NRGs are separated by two weeks in order to determine if there are any neurorestorative effects of NRGs for the nigrostriatal dopaminergic system. Both GGF2 and NRG-2β significantly improved motor function in 6-OHDA-lesioned animals compared to control lesioned animals. Furthermore, comparing the forelimb-use asymmetry scores of the NRG-treated animals at 4 and 10 weeks post-lesion indicated significant neurorestoration over the course of the study. Similar to the immunohistochemical data, there were no differences in enhancement of behavioral function between GGF2 and NRG-2β. Taken together, these data indicate NRGs may slow the neurodegeneration of midbrain dopamine cells and act as potential neurorestorative agents after neurotoxin-induced injury.

In conclusion, both NRG-2β and GGF2 significantly protected against 6-OHDA-induced cell death and were neuroprotective/neurorestorative for the behavioral deficits found in this model. These data indicate that NRGs may be novel neurotrophic factors for the injured dopaminergic nigrostriatal system in vivo. Although further studies are needed to determine the full potential of NRGs as protective/restorative compounds in models of PD, the present findings raise the possibility that NRGs could be potential therapeutic agents for the treatment of PD.
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Chapter 3

Age-related changes in NRG/ErbB receptor expression in the ventral midbrain and neurorestoration by GGF2 in an aging model of experimental parkinsonism

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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 survival factors for dopaminergic cells of the ventral midbrain. To determine if levels of NRG and their ErbB receptors are maintained during aging in the dopaminergic ventral mesencephalon, expression of NRG1, ErbB and tyrosine hydroxylase (TH) mRNAs and protein was examined in young (3-4 months), middle-aged (16-18 months), and old (22-24/25 months) Brown Norway/Fischer 344 F1 (BN/F344) and Sprague Dawley rats. ErbB4 and TH mRNA and protein levels in the ventral midbrain were significantly reduced in the old animals when compared to young rats in both strains. In the Sprague Dawley rats, ventral midbrain ErbB1 protein expression was reduced in the aged animals whereas striatal NRG1 protein increased. These ErbB4 and ErbB1 alterations were not due to any naturally occurring age-related loss of dopaminergic cells in the ventral midbrain as assessed by unbiased stereology of TH+ cells in the SNpc. In light of these age-related decreases in nigral ErbB receptors, the effectiveness of GGF2 treatment was tested in aging (16-month-old) Sprague Dawley rats in the partial lesion 6-OHDA model of experimental parkinsonism. In these aged animals, post-lesion supranigral GGF2 infusion significantly protected against 6-OHDA-induced forelimb asymmetry and increased dopaminergic cell survival in the SNpc and TH+ fiber density in the striatum. These results indicate that GGF2 significantly protects dopaminergic cells of the ventral midbrain and improves behavioral motor function in aged animals in spite of the age-related decrease in ErbB receptor availability within the substantia nigra. These findings suggest that although mesostriatal dopaminergic neurons
may experience diminished NRG/ErbB4 trophic support during aging, neuroprotection and neurorestoration of the neurotoxin-damaged nigrostriatal system can still be obtained with sufficient GGF2 administration.
Introduction

Neuregulins (NRGs) are a family of structurally related growth and differentiation factors. During development, NRGs play a central 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 participate 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). Furthermore, 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; K.B. Seroogy, unpublished results). In fetal mesencephalic dopamine cell cultures 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). When injected directly above the SNpc, GGF2 and another NRG isoform, NRG1-β1, induce striatal dopamine overflow indicating functional effects of NRGs in the nigrostriatal system in vivo (Yurek et al., 2004; Seroogy and Zhang, 2006). As shown in Chapter 2, NRG infusion is neuroprotective and neurorestorative for mesencephalic dopamine cells in a rat 6-OHDA model of PD. Although NRGs and ErbB receptors are present in the adult CNS, their functional roles through the lifespan remain to be fully elucidated, including within the midbrain dopaminergic cells.

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 include reduced nigral volume and cell number, and a decrease in striatal dopamine (reviewed in Stark and Pakkenberg, 2004). Similarly, aged non-human primates exhibit decreased numbers of ventral midbrain tyrosine hydroxylase (TH)-positive cells, reduced volume of the SNpc, and decreased 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 nigrostriatal 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). Aging animals have been reported to have increased
susceptibility to neurotoxic lesions in both mouse and rat models of PD (Ricaurte et al., 1987a,b; Date et al., 1990; Tamas et al., 2005). There is also evidence of a compromised neurotrophic factor environment and impaired responsiveness to therapies (e.g. dopaminergic cell grafts, neurotrophic factor infusion) in aged animals (Collier et al., 1999; Yurek and Fletcher-Turner, 2000, 2001; Fox et al., 2001; Sortwell et al., 2001). This is of particular importance given that PD is most prevalent in the aged population. 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 diminution of dopaminergic function during aging remain unknown.

The objectives of the present study are twofold. First, we examine the effects of aging on the expression of NRG1, TH, and ErbB1-4 mRNA and protein in the aging brain of both Brown Norway/Fischer 344 rats (BN/F344) and Sprague Dawley rats. The purpose is to determine if there is an age-related decline in NRG/ErbB receptors that would suggest a decrease in neurotrophic factor support to mesencephalic dopamine neurons. The BN/F344 strain is recommended by the National Institute on Aging (NIA) for use in aging studies because of their low incidence of age-related pathologies (Lipman et al., 1996). Sprague Dawley rats were examined because most studies of experimental parkinsonism (including our lab) use this strain. Secondly, given that GGF2 is protective and restorative for young animals in a model of PD, we now determine the efficacy of GGF2 treatment in an aged/lesioned model of PD.
Materials and Methods

Animals

Male Brown Norway/Fischer 344 F1 hybrid and Sprague Dawley rats (NIA/Harlan Sprague Dawley) were used in this study at ages 3-4 months, 16-18 months, and 22-24/25 months (n=30 for BN/F344 F1 and n=90 total for Sprague Dawley). Adult rats weighed 300-850 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

For in situ hybridization the animals were processed as described in the Methods from Chapter 2. Briefly, semi-adjacent sections through the levels of the prefrontal cortex (PFC), striatum, hippocampus, and ventral mesencephalon were hybridized with $^{35}$S-labeled cRNA probes for detection and localization of pan-NRG1, ErbB1-4 and TH mRNAs. The probes were prepared by in vitro transcription from linearized cDNA plasmids using the proper RNA polymerase and labeled with $^{35}$S-UTP (PerkinElmer, Waltham MA) as previously described (Seroogy and Herman, 1997). The TH cDNA plasmid (provided by J. Herman, University of Cincinnati) was contained in a pCR-TOPO vector and consisted of 366 bp. The ErbB2-4 plasmids (provided by H. Kornblum, UCLA) were contained in a pCR 2.1 vector and consisted of ~1.8 kb each (Kornblum et al., 2000). The ErbB1 plasmid was containted in a pCR 2 vector and consisted of 2.9 kb (Seroogy et al., 1994). The NRG1 plasmid was contained in a pCR-TOPO vector and consisted of 500 bp. The in situ hybridization procedure was
performed as detailed previously (Seroogy and Herman, 1997; Numan and Seroogy, 1999; Numan et al., 2005; see also Methods of Chapter 2). After drying, the slides were exposed to BioMax MR film (Kodak, Rochester NY) for 2 days (TH cRNA) or 13 days (NRG1 and ErbB1-4 cRNA) for generation of film autoradiographs.

**Western Blots**

Rats were euthanized by CO₂ asphyxiation for the protein analysis. The ventral midbrain, PFC, striatum, hippocampus, and retrosplenial cortex (RSC) were 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 μg/ml aprotinin, and 10 μg/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 that was loaded onto a 7.5% acrylamide gel was 25 ul. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) analysis was included as a loading control. For the list of antibodies used and concentrations see Table 1. 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 appropriate primary antibody. Following primary antibody removal, the membranes were washed in 2% milk PBS/tween (1 hour) and then incubated in the appropriate secondary antibody diluted in 2% milk PBS/tween for an hour. The membranes were then washed in
**Table 1:** Antibodies and concentrations for Western Blots

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<th>Antigen</th>
<th>Manufacturer</th>
<th>Isotype</th>
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<th>Secondary AB concentration</th>
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PBS/tween alone 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. The level of mRNA expression of NRG1, ErbB1-4 or TH in the PFC, striatum, motor cortex, anterior cingulate cortex, corpus colossum (for ErbB3), hippocampus, SNpc, SNl, VTA, or RSC was determined using Scion Image software (National Institutes of Health). Figure 1 depicts these areas as they were delineated for analysis in this study. Control measurements of the background were taken within each section (from an area of non-expression), and then subtracted from the grey level 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 ± the standard error of the mean (SEM). 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 Tukey’s multiple comparison post-hoc test.

Western blot films were analyzed using Scion Image software. The mean corrected grey levels were determined by measuring the grey level within 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) ± SEM. The Western blot data were analyzed using one-way ANOVA followed by the Tukey’s multiple comparison post-hoc test.
Figure 1
**Figure 1:** Schematic depiction of delineated areas analyzed for *in situ* hybridization. **A:** In the levels containing the striatum, the areas analyzed included the striatum, corpus callosum (CC; for ErbB3), anterior cingulate cortex (ACCtx), and motor cortex (MCtx).

**B:** In sections containing the hippocampus, regions CA1 and CA3, and the dentate gyrus (DG) were measured. **C:** Sections containing the ventral midbrain were analyzed for the ventral tegmental area (VTA), substantia nigra pars compacta (SNpc), substantia nigra pars lateralis (SNl; in BN/F344 rats), and retrosplenial cortex (RSC). Sections of the prefrontal cortex were also analyzed (not shown on schematic). Modified from Paxinos and Watson (2007).
**Surgical procedures**

Middle-aged (16-month-old) Sprague Dawley rats were anesthetized using isoflurane and placed in a stereotaxic apparatus. Two unilateral injections of 6-OHDA (10 μg in 2 μl per injection; Sigma Aldrich, St. Louis, MO) or vehicle (equivalent volume of 0.2% ascorbic acid physiological saline solution) were administered to the striatum ([AP: +0.5 ML: -2.5 DV: -5.0 and AP: -0.5 ML: -4.2 DV: -5.0 (Lee et al., 1996; Kirik et al., 1998; Deumens et al., 2002; Paxinos and Watson, 2007)] via 2-μl Hamilton syringe (Hamilton Company, Reno, NV). The syringe was lowered into the brain parenchyma and allowed to equilibrate for 5 min. The 6-OHDA or vehicle was then injected over a 10-minute period (0.2 μl/min). The needle was left in place for an additional 5 min before being slowly withdrawn to allow dispersion and avoid reflux along the needle track.

Two weeks after initiation of the lesion the rats were again anesthetized and placed in a stereotaxic apparatus. A cannula was lowered just above the SNpc [AP: -5.2 ML: -1.8 DV: -7.2 (Paxinos and Watson 2007)] and attached to an osmotic minipump (model 1007D, Alzet, Cupertino, CA) that delivered GGF2 (0.6 mg/ml; Acorda Therapeutics, Hawthorne, NY) or a vehicle (20 mM NaAcetate, pH 6.5, 100 mM Na₂SO₄, 100 mM arginine, and 1% mannitol) at a rate of 0.5 μl/hr for two weeks for a total amount of 100 μg of GGF2 infused. The pump was surgically placed between the shoulder blades and the cannula was held in place with Loctite 454 glue (Alzet, Cupertino, CA). The pumps continuously infused the contents of its reservoir for two weeks after which they were removed. Eight weeks following surgery, the rats were deeply anesthetized via an interperitoneal injection of sodium pentobarbital (60 mg/kg).
followed by intracardial perfusion with 250 ml of cold 0.1M PBS and then 4% paraformaldehyde in PBS (pH 7.4). The brains were post-fixed for 90 minutes and placed in a cryoprotectant solution of 30% sucrose in PBS at 4°C until they were sectioned on a microtome.

**Forelimb-use asymmetry.**

The forelimb-use asymmetry test was used to assess motor impairment and recovery of aged 6-OHDA-lesioned animals and was performed as previously described (Schallert et al., 2000; Tillerson et al., 2001; Woodlee et al., 2005; Schallert 2006; Anstrom et al., 2007; see Methods in Chapter 2).

**Immunohistochemistry**

Six sections (from a series of one out of every twelve sections) of the ventral midbrain and striatum for chronologically aging and aged lesioned animals were stained with an antibody for TH or neuronal nuclei (NeuN) using the ABC method as described in Methods of Chapter 2.

**Stereological cell counting**

To investigate numbers of dopaminergic cells in the SNpc during normal aging and after 6-OHDA lesion and GGF2 treatment, TH+ and NeuN+ cells were estimated using unbiased stereologic principles (West 1993). The parameters for stereology were the same as described in the Methods section of Chapter 2.

**Striatal TH fiber density**

Striatal TH immunoreactive fiber density was determined using Scion image software (National Institutes of Health) as described in the Methods of Chapter 2.
Results

Age-related changes in BN/F344 rats

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, SNl, 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 (Figure 3A; p<0.01). Young animals had a mean corrected grey level of 55.80 ± 0.87, compared to 42.83 ± 1.37 and 41.87 ± 1.99 for middle-aged and old animals, respectively. 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. In the adjacent VTA or SNl, expression of ErbB4 mRNA did not differ significantly with aging (Figure 3A; see Figure 2 for representative autoradiographs). In the VTA, ErbB4 mRNA mean corrected grey levels were 51.52 ± 1.82 for young animals versus 46.06 ± 1.13 for middle-aged and 46.77 ± 1.79 for old animals (p>0.05). In the SNl, ErbB4 mRNA levels were 34.51 ± 2.94 for young animals, 32.96 ± 1.77 for middle-aged animals and, 32.23 ± 1.68 for old animals respectively (p>0.05). Thus, age-related reduction in ErbB4 mRNA is not evident in all dopaminergic cell populations and appears to be specific for the SNpc among the regions analyzed in this study.

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. The mean corrected grey levels were 28.5 ± 5 for the young animals, 30.2 ± 1.9 for the middle aged animals, and 31 ± 3.5 for the old animals. There were no significant differences among any of the age groups in the retrosplenial cortex (Figure 3A). Taken together with the VTA and SNl data, these results suggest that the reduction in ErbB4 mRNA in the aged BN/F344 rat 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, SNl, and VTA. The mean corrected grey levels in the SNpc were 112.4 ± 2.00 for the young animals, 104.0 ± 2.23 for the middle-aged animals, and 99.78 ± 2.37 for the old animals (Figure 3B). Expression levels for TH mRNA were significantly reduced between the young and the old animals (p<0.01). In the VTA, there were no significant changes in TH mRNA during aging with young animals having a mean corrected grey level of 102.0 ± 2.0, 95.39 ± 3.15 for the middle-aged animals, and 96.83 ± 2.33 for the old animals (Figure 3B). There was a decrease in TH mRNA levels in the VTA that did not reach statistical significance (p=0.12). In the SNl, young animals had a mean corrected grey level of 55.37 ± 1.61, versus 54.03 ± 2.36, and 51.42 ± 1.71 for the middle-aged and old animals, respectively, there were no changes in any of the age groups (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 examined in the ventral midbrain of young, middle-aged, and old BN/F344 rats (Figure 4B). The young animals had a corrected mean grey level of $100 \pm 5.8$, middle-aged animals had a level of $83 \pm 5.1$, and old animals had a level of $65.7 \pm 9.5$. There was a significant decrease between the young and the old rats ($p<0.05$), and a decrease in the middle-aged animals when compared to the young animals that did not reach statistical significance ($p=0.054$).

Expression of TH protein (migrating at 60 kDa) in the ventral midbrain during aging was also examined. The young animals exhibited a mean corrected grey level of $100 \pm 4.6$, middle-aged animals had a level of $91 \pm 6.3$, and old animals had a level of $81.8 \pm 6.5$ (Figure 4B). There was a significant decrease in TH protein between the young and old rats ($p<0.05$). No differences in the protein levels of GAPDH were observed in any of the animals regardless of age (Figure 4B).

Age-related changes in Sprague Dawley rats

Expression of ErbB, NRG1 and TH mRNAs

To determine if loss of ErbB4 expression in the ventral midbrain was a consequence of aging rather than a strain-specific (for BN/F344 rat) observation, ErbB4 mRNA and protein was examined throughout brains of adult and aging Sprague Dawley
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 when compared to the young animals in the SNpc but not in the SNl or VTA (D-F). Scale bar = 1000 μm.
Figure 3. Densitometric analysis of ErbB4 and TH cRNA hybridization during aging in BN/F344 rats. **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 had no significant changes due to aging. **B:** Expression of TH mRNA was significantly decreased in the SNpc during aging, but not in the VTA or SNI. Data are expressed as mean corrected grey level ± SEM. *p<0.01 compared to the 3-month (young) age group. N=4 per age group.
Figure 4

**A**

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**B**

![Graph showing protein expression levels across different age groups](image)

**Figure 4.** **A:** Representative autoradiograms from Western blots of the ventral midbrain of BN/F344 rats 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. **B:** Quantification of blots revealed a significant decrease in protein expression between young and old animals for ErbB4 in the ventral midbrain. There was also decreased expression of ErbB4 protein in the middle-aged animals when compared to the young
animals that did not reach statistical significance. 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) ± SEM. *p<0.05, ^p=0.054 compared to the 3-month (young) age group. N=6 per age group.
rats. The results in the BN/F344 rats were also extended in the Sprague Dawley rats by investigating additional ErbB receptors other brain regions (beside the ventral midbrain). Using a pan-NRG1 probe, age-related changes in NRG ligands were also determined. Sprague Dawley rats were also examined because past and present neuroprotective studies with NRG in our lab use this strain. Results of general expression levels and changes in mRNA expression are summarized in Table 2. Similar to the changes observed in BN/F344 rats, ErbB4 mRNA expression in Sprague Dawley rats was decreased in both the middle-aged and old animals when compared to young animals in the SNpc (mean corrected grey levels: young=58.3±1.7; middle-aged=49.6±1.0; old=49.3±1.8; p<0.01 for middle-aged and old versus young animals; see Figures 5 and 6). Within the same sections there was no change in either the VTA or the RSC due to chronological aging, indicating that the decrease in ErbB4 is again specific for the SNpc. There were no alterations for any of the other ErbB receptors (ErbB1-3) in any of the areas assessed in the ventral midbrain sections. The expression level of TH mRNA in the SNpc was also decreased in the old animals compared to young animals whereas TH expression in the VTA was unchanged (mean corrected grey levels: young=124.0±3.2; middle-aged=116.5±1.8; old=113.8±3.4; p<0.05 in old versus young animals; see Figures 5 and 6). The expression of NRG1 mRNA remained unaltered due to chronological aging (Table 2). Similarly ErbB receptor expression remained unchanged during aging in the other forebrain areas assessed (Table 2).
Table 2: Summary of ErbB, NRG1 and TH mRNA expression in the normal and aging brain of Sprague Dawley rats. + = low expression, ++ = moderate expression, +++ = high level expression, nc = no change, nd = not determined. Regions of significant change are highlighted in red.

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- 151 -
**Figure 5:** Representative reverse image autoradiograms of young middle-aged and old Sprague Dawley rat brains hybridized with ErbB4 or TH cRNA probes. Note that while there is a reduction in ErbB4 mRNA due to chronological aging in the SNpc, no changes were observed in the VTA or the RSCtx. A slight reduction in the expression of TH mRNA is apparent in the old animals when compared to the young animals. Scale bar=1000 μm.
Figure 6: Quantification of *in situ* hybridization signal in ventral midbrain sections from Sprague Dawley rats. **A:** Quantification of ErbB4 mRNA revealed a significant decrease in expression in the SNpc starting at middle-age and continuing in the old animals when compared to the young animals (*p<0.01). This decrease was specific to the SNpc, with no changes in expression observed in the VTA or the RSCtx. **B:** In the SNpc there was also a significant decrease in expression of TH mRNA that occurred in the old animals compared to the young animals that was not apparent in the VTA of those animals (*p<0.05). N=10 per age group.
Expression of ErbB, NRG1 and TH protein

Age-related modulation of ErbB, NRG, and TH protein was investigated in the PFC, striatum, hippocampus, ventral midbrain, and RSC of Sprague Dawley rats. Chronological aging led to several changes in the aging brain when compared to young animals. For ErbB4 in the ventral midbrain, protein expression was decreased in both the middle-aged and old animals when compared to young animals (mean corrected grey levels: young=100.0±8.7; middle-aged=60.5±5.4; old=61.8±4.9; p<0.01 versus young animals; see Figures 7 and 8). Also in the ventral midbrain, old animals exhibited a significant decrease in ErbB1 protein when compared to young animals (mean corrected grey levels: young=100.0±8.0; middle-aged=81.1±7.9; old=66.3±8.0; p<0.05 in old versus young animals; see Figures 7 and 8). Aging led to decreased expression of TH protein in the old animals compared to young animals in the ventral midbrain (mean corrected grey levels: young=100.0±6.4; middle-aged; 99.7±6.5; old=83.1±5.8; p<0.05 in old versus young animals; Figures 7 and 8). There were no changes in ErbB2 or ErbB3 in the ventral midbrain due to chronological aging. The expression of ErbB protein was unaltered in any of the other areas assessed (hippocampus, striatum, PFC, and RSC).

Protein expression of NRG1 using a pan-NRG1 antibody was used to determine if changes in NRG1 ligands occurred during aging. There were no changes in NRG1 protein in the ventral midbrain, hippocampus, or RSC during chronological aging. However, in the striatum the expression of NRG1 protein was increased in the middle-aged and old animals when compared to the young animals (mean corrected grey levels: young=100.0±5.4; middle-aged=138.6±9.6; old=151.3±12.0; p<0.01 for middle-aged and old animals versus young animals; Figures 7 and 8). There was also a non-significant
**Figure 7**: Representative bands from Western blots in aging Sprague Dawley rats.  

**A**: In the ventral midbrain, the expression of ErbB4 protein decreases starting at 16 months of age. This decreased ErbB4 protein is still apparent at 22 months of age. The expression of ErbB1 and TH protein are decreased in the old (22 months) animals. There is no change in ventral midbrain GAPDH expression.  

**B**: In the striatum of aged animals there is no change in TH or ErbB4 protein due to chronological aging. However, aged animals (22 months) exhibited an increase in NRG1 protein. There is no change in striatal GAPDH expression.
**Figure 8**: Quantification of Western blot data in aging Sprague Dawley rat brains.  

**A**: In the ventral midbrain, quantification of Western blots revealed a significant decrease in ErbB4 protein in the middle-aged and old animals compared to the young rats. The expression of ErbB1 and TH protein was also significantly decreased in the old animals compared to young rats. There was no change in the housekeeping protein GAPDH.  

**B**: In the striatum, there were no changes in ErbB receptor (only ErbB4 shown), TH, or GAPDH protein due to aging. There was, however, a significant increase in NRG1 protein expression in the middle-aged and old animals.  

**C**: In the PFC, there were no significant changes in any of the ErbB receptor proteins (only ErbB4 shown) or GAPDH...
protein due to chronological aging. There was an increase in NRG1 protein that was apparent in the PFC of the old animals when compared to the young animals that did not reach statistical significance. Data are expressed as mean corrected grey level (represented as percent control) ± SEM. *p<0.05, **p<0.01, ^p=0.08 compared to young animals. N=10 per age group.
**Figure 9:** TH+ cell number and fiber density in aging Sprague Dawley rats. **A:** Dopaminergic cells in the SNpc were quantified using unbiased stereology. There were no age-related changes in numbers of TH+ cells in the SNpc throughout aging. **B:** Quantification of striatal TH+ fiber density revealed no differences due to aging. N=10 per age group.
increase in NRG1 protein in the PFC of old animals compared to young animals (mean corrected grey levels: young=100.0±10.5; middle-aged=122.9±11.4; old=132.9±12.9; p=0.08 in old versus young animals; see Figures 7 and 8). The expression of GAPDH was used as a loading control for all areas of the brain and remained unaltered in all regions during aging.

*Stereological analysis of TH+ cells and striatal fiber density*

To determine if the decreases in ErbB receptor mRNA and protein in the ventral midbrain were due to changes in nigrostriatal dopaminergic neuron survival and morphology, the number of nigral TH+ cells and striatal fiber density were measured across the aging spectrum. Using unbiased stereology, nigral TH+ cells remained unchanged in young, middle-aged, and old animals (mean cell counts: young=23338±1401; middle-aged=24146±979; old=23369±1407; Figure 9). There were also no changes in TH+ fiber density in the striatum due to chronological aging in the Sprague Dawley rats (Figure 9).

**Infusion of GGF2 in aged lesioned animals: neuroprotection/neurorestoration**

To determine if GGF2 infusion is effective in aged animals despite the decreased availability of ErbB receptors, GGF2 was infused supranigrally two weeks after striatal 6-OHDA injection. Vehicle-infused and lesioned animals were compared to GGF2-infused lesioned animals for changes in motor function and dopaminergic cell survival. In the forelimb-use asymmetry test, all lesioned animals were significantly impaired when compared to vehicle-injected controls (p<0.01). Infusion of GGF2 in 6-OHDA-injected animals significantly improved motor function (i.e. improved use of the impaired
Figure 10: Forelimb-use asymmetry in 16-month-old lesioned animals. **A and B:** At 2 and 4 weeks after GGF2 infusion in lesioned animals there is no difference in motor function when compared to control lesioned animals. **C and D:** By 6 weeks and continuing out to 8 weeks post-infusion of GGF2 there is a significant enhancement of impaired limb use in GGF2-treated lesioned animals when compared to control lesioned animals. **E:** Comparing the forelimb-use asymmetry scores of the GGF2-infused lesioned animals at 2 weeks versus 8 weeks post-infusion revealed a non-significant improvement of motor function over the course of the study. *p<0.01 compared to 6-OHDA/veh group. N=8 per group.
Figure 11: TH and NeuN+ cell counts for aged lesioned animals. **A:** Representative photomicrographs of lesioned control and GGF2-infused lesioned animals. Notice that GGF2 infusion protected against dopaminergic cell death in the SNpc. **B:** Quantification of TH+ cells in the SNpc revealed a significant increase in TH+ cells in the GGF2-infused lesioned animals compared to the control lesioned animals. **C:** The loss of NeuN+ cells was statistically similar to the loss of TH+ cells providing evidence that frank cell death occurred rather than phenotypic downregulation. *p<0.05 compared to 6-OHDA/veh-treated animals. N=8 per group. Scale bar=500 μm
Figure 12: Striatal TH fiber density in aged lesioned animals. Supranigral infusion of GGF2 after 6-OHDA injection significantly protected against TH+ fiber density loss.

*p<0.05. N=8 per group. Scale bar=500 μm
limb) at 6 and 8 weeks post-infusion (8 and 10 weeks post-lesion). At 6 weeks post-infusion, 6-OHDA/veh rats used the impaired limb 3.7±1.4 percent of the time compared to 14.3±1.8 percent of the time for 6-OHDA/GGF2-treated rats; p<0.01; at 8 weeks post-lesion, use of the impaired limb for 6-OHDA/veh rats was 2.8±0.8 percent compared to the 6-OHDA/GGF2 group at 17.1±1.4 percent; p<0.01; see Figure 10). When GGF2-infused lesioned animals were compared over the course of the study there was a non-significant improvement of motor function from 2 weeks post-lesion to 8 weeks post-lesion that was not evident in vehicle-lesioned animals, indicating potential neurorestoration in GGF2-treated animals (6-OHDA/GGF2 at 2 weeks=10.5±3.0 versus 6-OHDA/GGF2=17.1±1.4; p=0.07; Figure 10). All lesioned animals demonstrated significant loss of TH+ dopaminergic cells in the SNpc when compared to vehicle-injected controls (p<0.01). The infusion of GGF2 afforded significant protection of dopaminergic cells in the SNpc when compared to lesioned control animals (6-OHDA/veh=14.0±1.3 percent of intact side versus 6-OHDA/GGF2=26.0±3.5; p<0.01; Figure 11). To distinguish possible phenotypic changes from cell death, NeuN+ cells were quantified in the lesioned animals. Both the control and GGF2-lesioned animals exhibited a similar loss of NeuN+ and TH+ cells indicating dopaminergic cell death rather than phenotypic alterations (cell counts: 6-OHDA/veh for TH=8761±101; 6-OHDA/veh for NeuN=10619±1639; 6-OHDA/GGF2 for TH=6930±766; 6-OHDA/GGF2 for NeuN=7837±2690; see Figure 11). Fiber density in the striatum was also significantly protected by GGF2 infusion in lesioned animals when compared to lesioned control animals (6-OHDA/veh=56.5±1.3 percent intact side versus 6-OHDA/GGF2=66.3±3.3; p<0.05; Figure 12).
Discussion

In the present study we demonstrate an age-related reduction of both mRNA and protein for the ErbB4 receptor in the SNpc of BN/F344 and Sprague Dawley rats. Tyrosine hydroxylase was analyzed and exhibited decreases in mRNA and protein in the old animals when compared to young animals. The expression of ErbB1 protein in the ventral midbrain of aged rats was also decreased, whereas NRG1 protein in the striatum increased with age. We also demonstrate that treatment of 16-month-old rats in the 6-OHDA model of PD with GGF2 significantly increased dopaminergic neuron survival and improved behavioral functional recovery. Thus, the loss of ErbB receptor availability did not prevent the neuroprotective effects of GGF2 infusion in aging 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 is emphasized in studies of mutant mice. Both NRG1 and ErbB receptor knockout animals result in embryonic lethality with cardiac and hindbrain defects (Gassmann et al., 1995; Meyer and Birchmeier 1995; Lee et al., 1995; Rietmacher et al., 1997). Reduced expression of NRG1 leads to abnormalities in the function of N-methyl-D-aspartic acid receptors and increased behaviors associated with schizophrenia are noted (Mohn et al., 1999; Gerlai et al., 2000; Stefansson et al., 2002; Chen et al., 2008). Brain-specific ErbB4 knockout mice have abnormal neural crest cell migration, an increased number of large interneurons in the cerebellum, deficits in differentiation of olfactory interneurons, and behavioral disturbances including lower spontaneous motor activity and reduced grip
strength (Tidcombe et al., 2003; Anton et al., 2004; Golding et al., 2004; Golub 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 to be due to a strong compensatory response (Thuret et al., 2004). 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 and ErbB1 are highly expressed in the SN and VTA where they are present in almost all dopaminergic cells (Seroogy et al., 1994; Steiner et al., 1999; Yurek and Seroogy, 2001; 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. The expression of ErbB1 also decreased in aged animals, adding to the loss of potential NRG signaling pathways. There were no changes in the other NRG receptor, ErbB3, or in the heterodimerization partner ErbB2. The loss of ErbB4 mRNA and protein in both the BN/F344 and Sprague Dawley rats occurs before the changes in TH mRNA and protein. Furthermore, in aged Sprague Dawley rats there was no loss of TH+ cells in the SNpc or of TH+ fiber density in the striatum indicating that the mRNA and protein changes are not due to frank cell loss. This progressive loss of NRG/ErbB4 activation may have important functional implications for the mesostriatal dopaminergic system.

It is 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., 1994; Tomac et al., 1995; Kearns and Gash 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 nigrostratal 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 BN/F344 rats exhibited a significant reduction of GDNF protein in the striatum and ventral midbrain (Yurek and Fletcher-Turner, 2001). The current study demonstrates a decrease in nigral ErbB4 message and protein, both significant reductions, by old age in BN/F344 hybrid and Sprague Dawley rats. We also show that there is a significant decrease in ventral midbrain ErbB1 protein in aged Sprague Dawley rats. Interestingly, and in contrast, a significant increase in NRG1 protein was found in the striatum of middle-aged and old animals when compared to the young animals. Whether this is a compensatory mechanism for the aging nigrostriatal system remains to be determined. Regardless, these data indicate a deficit in availability of ErbB receptors in the SNpc of aged animals, which may play a role in the selective vulnerability of the nigrostriatal pathway to neurodegeneration.

Changes in neurotrophic factor support in the nigrostriatal dopaminergic system are observed in aged animals following injury or other manipulations. Young animals that receive a neurotoxic lesion of the SNpc increase 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 animals (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 cografts, 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. Thus, it is important to investigate the ability of neurotrophic factors to protect the nigrostriatal system in aged models of PD. Our experimental data indicate that GGF2 infusion after 6-OHDA injection in 16-month-old Sprague Dawley rats significantly protects against the behavioral and morphological changes associated with the lesion.

In comparisons of Parkinson’s disease patients to healthy controls, decreases of GDNF, BDNF, and ciliary neurotrophic factor (CNTF) are 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 initial 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. To ensure that the reduced ErbB4 expression in the ventral midbrain was not a BN/F344 strain specific result, aged Sprague Dawley rats were examined. The age-related decline in ErbB4 expression for the ventral midbrain was confirmed. Also in the Sprague Dawley rats, the investigation was extended to determine if changes in other ErbB receptors and NRG1 occurred in the aging brain. This revealed that ErbB1 protein was decreased in the ventral midbrain of aged rats, whereas NRG1 protein was increased in the striatum. Furthermore, neurotoxin models of PD (including those used in our lab) most commonly use Sprague Dawley rats, thus necessitating examination of age-related alterations in that strain.

In conclusion, we have found decreases in ErbB4 mRNA and protein and ErbB1 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. These findings raise the possibility that a progressive loss of NRG receptor 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. Importantly, however, we also show that GGF2 infusion is neuroprotective/neurorestorative for the injured nigrostriatal system in aged animals. Thus, these findings suggest that any potential untoward effects due to the loss of the ErbB4 receptor during chronological aging could be overcome by relatively large doses of the ErbB4 ligand GGF2. Moreover, these results may indicate that administration of neurotrophic factors (in this case GGF2) to older animals could be a viable and effective treatment for experimental parkinsonism in the aging nigrostriatal system.
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Chapter 4

ErbB4 knockdown and susceptibility of the dopaminergic nigrostriatal system to neurotoxin-induced degeneration
Abstract

The neuregulin (NRG) receptor ErbB4 is highly expressed by dopaminergic cells in the ventral midbrain. During aging, the expression of ErbB4 mRNA and protein decreases in middle-aged and old rats, raising 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. In the present study, we hypothesize that reduction of ErbB4 receptor availability in the substantia nigra will exacerbate the detrimental effects of neurotoxin-induced damage upon the nigrostriatal system. To address this issue, we employed a gene therapeutic approach to specifically inhibit RNA synthesis of the ErbB4 receptor protein. Adult male Sprague Dawley rats were injected with a siRNA construct targeted against ErbB4, mediated by a lentiviral vector, or a scrambled control into the substantia nigra pars compacta (SNpc). Two weeks later, the animals received a striatal 6-OHDA or vehicle injection. Changes in motor function were assessed with the forelimb-use asymmetry test every two weeks, whereas the movement initiation test and sensory motor preference tests were performed once each. At the end of the study, dopaminergic cell numbers were determined using unbiased stereology to count nigral tyrosine hydroxylase (TH) positive cells. The results indicate that dopaminergic cells in the SNpc take up the lentiviral vector (as indicated by a GFP reporter), and express the ErbB4 siRNA. Animals injected with both the interfering RNA and 6-OHDA did not worsen motor dysfunction in any of the functional tests assessed. Similarly, ErbB4 knockdown combined with 6-OHDA lesion did not exacerbate dopaminergic cell death in the SNpc when compared to scrambled control injected animals. While this may be due
to insufficient dispersal of the injected lentiviral vector, at present these data indicate that
decreased ErbB4 expression is not sufficient to increase the susceptibility of the
nigrostriatal dopaminergic pathway to neurotoxic lesions.
Introduction

Neuregulins (NRGs) are pleiotrophic growth factors that bind to receptor tyrosine kinases of the ErbB family and mediate many cellular processes including survival, proliferation, 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). Although present in the adult central nervous system (CNS), functional roles for NRGs and their receptors remain to be fully elucidated but are implicated in several disease states including stroke, multiple sclerosis, peripheral neuropathy and most recently schizophrenia (Chen et al., 1994; Corfas et al., 1995; Cannella et al., 1999; Steiner et al., 1999; Gerecke et al., 2001; Yurek and Seroogy, 2001; Bruce et al., 2002; Parker et al., 2002; Law et al., 2004; Talmage and Role, 2004; Fox and Kornblum, 2005; Bernstein et al., 2006; Esper et al., 2006; Norton et al., 2006; Thompson et al., 2007). We have begun to characterize a role for NRGs as neurotrophic factors for the dopaminergic nigrostriatal system.

Dopaminergic neurons in the substantia nigra pars compacta (SNpc) are selectively vulnerable to age and neurotoxin-induced degeneration. Although the exact mechanism by which this occurs is unknown, it is generally thought to be a combination of genetic and environmental factors. The functional NRG receptor, ErbB4, is highly expressed in the substantia nigra of both rodents and primates (including humans), where it is present within almost all of the dopaminergic cells (Steiner et al., 1999; Yurek and Seroogy, 2001; Abe et al., 2009; Zheng et al., 2009). Previous data from our lab indicate a functional and neuroprotective role for NRGs in the dopaminergic ventral midbrain.
unpublished results; Chapter 2). In Chapter 3, we found age-related decreased expression of the functional ErbB4 receptor specific to the dopaminergic ventral midbrain in both Brown Norway/F344 and Sprague Dawley rats. The ramifications of this potential loss of NRG/ErbB4 signaling remain to be determined but raise the possibility that a progressive loss of NRG/ErbB4 signaling may contribute to the natural age-related decline of nigrostriatal dopaminergic function and perhaps to the pathogenesis of age-related neurodegenerative disorders such as Parkinson’s disease.

RNA interference is the process of posttranscriptional gene silencing originally discovered in plants as a defense from viral invasions (Hamilton and Baulcombe 1999). Mammalian cells employ the use of endogenous RNA interfering machinery and small inhibitory RNA (siRNA), called micro RNAs, to silence abundant mRNA or to silence developmentally programmed RNAs (Zamore 2002; Plasterk 2006). Although designing siRNA constructs can be difficult due to the specifications that need to be followed to be effective in mammalian cells, the machinery is endogenously expressed in mammalian cells and, thus, this could be a powerful technique to knockdown RNA in various animal models. Moreover, long-term and stable expression of siRNA can be achieved through the use of viral vectors (Miest et al., 2009).

In the present study, using lentiviral vector-driven siRNA, we propose to reduce nigral ErbB4 receptor expression in young animals, before other age-related changes have occurred. The results will indicate whether decreased NRG signaling is sufficient to increase susceptibility of nigral dopamine cells to subsequent neurotoxicity. If so, this
could imply a role for NRG/ErbB4 signaling in the maintenance and performance of the
dopaminergic nigrostriatal system and in the pathogenesis of neurodegeneration in PD.

Materials and Methods

Animals

Adult male Sprague Dawley rats (n=40) were used in this study (Harlan Sprague
Dawley) weighing 250-350 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.

Vector constructs and cell culture

Three lentiviral vector constructs were obtained from America Pharma Source
(Gaithersburg, MD), and verified in cortical cell cultures for ErbB4 (NM_021687)
knockdown. The lentiviral vector consists of a three plasmid packaging system (see
Figure 1A) with green fluorescent protein (GFP) used as a reporter. Expression of the
vector was driven by the U6 promoter. The three sequences for the siRNA constructs, as
determined by the America Pharma Source algorithm, are shown in Figure 1B.

Primary rat cortical neurons were purchased from Invitrogen (Carlsbad, CA). The
cells were grown in standard conditions on poly-D-lysine coated plates in Neurobasal
media supplemented with B27 and 200 mM glutamine. Cells were plated at a
concentration of 1x10^5 per well and the media was replaced after the first 24 hours and
every three days thereafter. On day in vitro (DIV) four, half of the media was removed
and replaced with one of the siRNA lentiviral vectors or the scrambled vector at a
**Figure 1**

**A**  
**Packaging construct**

![Diagram of packaging construct](image)

**Vector**

![Diagram of vector](image)

**Env**

![Diagram of env](image)

**Position 301**

Top: 5'- GATCCAGCCCTCAACCAGTTTCGTTACTCAAGAGGTTACGAAACTGGTTGAGGGCTTTTTGG -3'  
Bottom: 5'- AATTCCAAAAAGCCCTCAACCAGTTTCGTTACCTCTTGAGTAACGAAACTGGTTGAGGGCTG -3'

**Position 522**

Top: 5'- GATCCAGCAAGATATTGTTCGGAATCCTCAAGAGGGATTCCGAACAATATCTTGCTTTTTGG -3'  
Bottom: 5'- AATTCCAAAAAGCAAGATATTGTTCGGAATCCCTCTTGAGGATTCCGAACAATATCTTGCTG -3'

**Position 592**

Top: 5'- GATCCAGGAAGATGCCATAAGTCTTGCTCAAGAGGCAAGACTTATGGCATCTTCCTTTTTGG -3'  
Bottom: 5'- AATTCCAAAAAGGAAGATGCCATAAGTCTTGCCTCTTGAGCAAGACTTATGGCATCTTCTG

**Figure 1:** Lentiviral vector system. **A:** The lentiviral vector is divided into three plasmids in order to prevent spontaneous recombination. In the vector plasmid, siRNA and GFP reporter expression are driven by the U6 promoter. **B:** Maps of the three siRNA clone sequences as determined by the commercial siRNA generating algorithm. The resulting lentiviral vectors were then tested for inhibitory effects on Erbb4 expression *in vitro.*
concentration of $1 \times 10^6$ plaque forming units (pfu) per ml. One week after lentiviral vector administration, wells were visualized with a fluorescent microscope to confirm infection. Wells in the same treatment groups were scraped, combined, and centrifuged at 14,000 x g for 30 min and processed for Western blotting.

**Surgical procedures**

Animals were anesthesized (ketamine-87 mg/kg; xylazine-13 mg/kg i.p.) and placed in a stereotaxic apparatus. A Hamilton syringe (Hamilton Company, Reno, NV) was lowered just above the SNpc (AP: -5.2 ML: -1.8 DV: -7.2 (Paxinos and Watson, 2007)), and the lentiviral vector with siRNA directed against ErbB4 or a scrambled vector was injected over a period of 10 min (2 μl total volume with lentiviral vector of concentration $1 \times 10^9$ pfu/ml) with 5 min wait times pre and post-injection. Two weeks after lentiviral vector injections, intrastriatal injection 6-OHDA was used to induce a progressive deterioration of the nigrostriatal pathway. Two unilateral injections of 6-OHDA (5 μg in 2 μl; Sigma Aldrich, St. Louis, MO) or equivalent volume of 0.2% ascorbic acid physiological saline solution were administered to the striatum [AP: +0.5 ML: -2.5 DV: -5.0 and AP: -0.5 ML: -4.2 DV: -5.0 (Lee et al 1996; Kirik et al 1998; Deumens et al 2002; Paxinos and Watson, 2007)] via a 2-μl Hamilton syringe (Hamilton Company). The syringe was lowered into the brain parenchyma and allowed to equilibrate for 5 min, and the 6-OHDA or vehicle was then injected over a 10-minute period (0.2 μl/min) with a 5 min wait time post-injection to avoid reflux along the needle track.

Eight weeks following initial surgery, the rats were deeply anesthetized via interperitoneal injection of sodium pentobarbital (60 mg/kg) and intracardially perfused
with 250 ml of cold 0.1M PBS followed by 4% paraformaldehyde in PBS (pH 7.4). The
brain tissue was post-fixed for 90 minutes, then placed in a cryoprotectant solution of
30% sucrose in PBS at 4°C until they were sectioned on a microtome.

**In situ hybridization**

The brains were prepared for *in situ* hybridization as described in the Methods of
Chapter 2. Semi-adjacent sections throughout the rostrocaudal level of the ventral
mesencephalon were hybridized with $^{35}$S-labeled cRNA probes for detection and
localization of ErbB4 and tyrosine hydroxylase (TH) mRNAs as previously described in
the Methods of Chapter 2. 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) were contained in a pCR 2.1 vector and
consisted of 1.8 kb (Kornblum et al., 2000). 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.

**Behavioral assessments**

The forelimb-use asymmetry test, tactile stimulation test, and forelimb akinesia
test were used to assess sensorimotor impairment and recovery of 6-OHDA-lesioned
animals and were performed as previously described (Schallert et al., 2000; Tillerson et
al., 2001; Woodlee et al., 2005; Schallert 2006; Anstrom et al., 2007). Briefly, for the
forelimb-use asymmetry test, rats were placed in a clear plexiglass open cylinder, tall
enough that the rat cannot reach the top with its forepaws, and allowed to investigate the
environment. A mirror was placed behind the cylinder so that all movements the animals
made could be clearly seen with a video camera. All measurements were recorded during
the dark cycle in low light conditions. The animals were allowed to naturally explore the cylinder, and after a total of 20 to 25 forelimb wall contacts was observed, the animals were removed from the cylinder and placed back in their home cage. The number of contacts of each forelimb or both forelimbs were later scored from the videos. An asymmetry score was calculated by dividing usage of the impaired limb (impaired plus $\frac{1}{2}$ both) by total limb usage (impaired + unimpaired + both). For the tactile stimulation test, sticky pieces of paper were placed bilaterally on the forepaws. The animals were then placed in their home cage and forelimb preference for touching the paper with the mouth to remove it was recorded. A total of 5 trials were performed with paw preference calculated as number of trials where the impaired limb is touched first/total number of trials. For the forelimb akinesia test, the animals were held so that the hindquarters were raised and one of the forelimbs was immobilized. All of the animal’s weight was supported by the other forelimb. The animal was then allowed to initiate movement and the number of steps initiated was recorded for a period of 10 seconds. The percent use of the impaired limb was calculated by dividing the number of initiated steps by the impaired limb by total number of steps taken. Statistical comparisons included a one-way ANOVA followed by the Tukey’s multiple comparisons post hoc test.

**Immunohistochemistry**

Immunohistochemistry for TH was performed as previously described in the Methods of Chapter 2.

**Stereological cell counting**

The number of TH+ cells in the SNpc was estimated using unbiased stereology as previously described in Chapter 2 Methods.
Western Blots

Primary rat cortical cells treated with lentiviral vector driven siRNA against ErbB4 or a scrambled vector were centrifuged at 14000 x g for 30 minutes. The Western blotting procedure was performed as described previously (see Methods in Chapter 2; Wolf et al., 1999).

Results

In vitro knockdown of ErbB4

To determine which of the siRNA constructs (designated 465, 466, and 467) was most effective at knocking down ErbB4 expression, they were tested in primary cortical cell cultures. Primary cortical neurons express ErbB4 and are commercially available, thus, they were chosen to test the neuronal ErbB4 knockdown by the siRNA vectors. On DIV four, each of the siRNA constructs or the scrambled construct were added to a series of wells. All of the lentiviral vector constructs were taken up by the primary cortical cells as determined by GFP expression within three days of vector administration (Figure 2). The level of GFP expression appeared higher in the cultures treated with constructs 466 and 467 than 465, possibly indicating increased expression of the lentiviral vector in those constructs. One week after administration of the lentiviral vectors, GFP expression was still present and wells from each treatment were combined and processed for Western blot analysis of ErbB4 protein to determine possible alterations in ErbB4 protein expression. Constructs 465 and 466 demonstrated decreased ErbB4 protein levels, whereas construct 467 resulted in only slightly lowered ErbB4 protein expression when compared to scrambled vector-treated cultures (Figure 3). In scrambled construct-treated
Figure 2: Lentiviral vector-driven expression of GFP reporter. All of the lentiviral vectors (siRNA and scrambled) expressed GFP reporter. Note that construct 465 appears to have a lower level of GFP expression than either construct 466 or 467. In control wells (with no lentiviral vector added) cells were present (see phase contrast picture in lower right panel), but no GFP expression was observed. All photomicrographs were taken at 40X magnification.
wells, there was no decreased expression of ErbB4 when compared to control cultures (data not shown). Expression of GAPDH was used as a loading control, with no differences observed for GAPDH levels in any of the treatment groups (Figure 3). Based on the substantial knockdown of ErbB4 and the high expression level of GFP in vitro, construct 466 was chosen for the in vivo experiments.

**In vivo expression of siRNA**

For in vivo expression of siRNA, the concentration of the lentiviral vector was increased to $1 \times 10^9$ pfu/ml. Animals received injections of the siRNA construct 466 or scrambled control into the SNpc. Two weeks later, expression of GFP was assessed in order to determine if neurons expressed the lentiviral vector construct in vivo (Figure 4). At two weeks post-injection, GFP expression was visible within cells in the area of the SNpc in both siRNA- and scrambled-injected animals. This expression was detected up to 8 weeks post-injection (data not shown). In situ hybridization for ErbB4 or TH mRNA was performed to determine if there was specific knockdown of ErbB4 mRNA due to the siRNA vector (Figure 4). Injection with the ErbB4 siRNA vector substantially decreased ErbB4 mRNA expression in the SNpc when compared to the uninjected side or to the scrambled construct-injected control (Figure 4). Importantly, in adjacent sections, there were no changes in TH mRNA expression due to the siRNA injection (Figure 4).

**siRNA in 6-OHDA-injected animals**

To determine if decreased ErbB4 expression is sufficient to increase the susceptibility of the nigrostriatal dopaminergic system to neurotoxic lesions, animals previously injected with the ErbB4 siRNA vector or scrambled control were given an intrastratal 6-OHDA injection to create a partial lesion model of PD. The animals were
Figure 3: Representative Western blots of cortical cells treated with the three ErbB4 siRNA constructs or scrambled vector. The siRNA constructs 465 and 466 both show obvious reductions in ErbB4 expression (~185 kd band) versus scrambled construct, whereas the 467 vector does not appear to be as efficient at knocking down ErbB4. There were no changes in GAPDH expression (~36 kd band) due to any of the lentiviral vectors assessed.
Figure 4: Representative photomicrographs of siRNA vectors *in vivo*. **A:** Representative reverse image autoradiographs showing ErbB4 and TH mRNAs in an animal injected with siRNA vector against ErbB4. The top arrow indicates the decreased expression of ErbB4 mRNA compared to uninjected side. Note that there is no change in expression of TH mRNA in an adjacent section (bottom panel arrow). Scale Bar=1000 μm. **B:** Photomicrographs demonstrating GFP expression in lentiviral vector-injected animals in the SN. Both the siRNA vector- and scrambled vector-injected animals express the GFP indicator. Scale Bar=250 μm.
assessed for modulation of sensorimotor function and dopaminergic cell death due to the neurotoxin lesion. For the all of the behavioral tasks, the 6-OHDA lesion significantly increased sensorimotor dysfunction compared to non-lesioned control animals. The forelimb-use asymmetry test was performed 2, 4, and 6 weeks after 6-OHDA injection. In the siRNA vector-treated animals, there were no differences in forelimb asymmetry when compared to the scrambled vector-injected animals (Figure 5A). The forelimb akinesia and tactile stimulation tests were performed 5 weeks after 6-OHDA lesion. There were no changes in forelimb preference for the tactile stimulation test due to siRNA vector injection in lesioned animals (Figure 5B). Similarly, the forelimb akinesia test revealed no significant increase in motor dysfunction for the siRNA vector-injected lesioned animals compared to scrambled vector-injected lesioned animals (p=0.17; Figure 5C). Stereological cell counts of TH+ neurons in the SNpc were conducted to determine possible modulation of dopaminergic cell death due to siRNA vector injection in lesioned animals. Both siRNA vector-injected and scrambled construct-injected animals demonstrated significantly less TH+ cells when compared to unlesioned control animals (p<0.01). However, when siRNA vector-injected lesioned animals were compared to lesioned scrambled vector-injected animals, there were no significant differences in TH+ cell numbers (Figure 6).

Discussion

The present data demonstrate that lentiviral vector-mediated siRNA designed against ErbB4 is functional in vitro and in vivo, as verified by cognate protein knockdown with Western blotting and mRNA knockdown with in situ hybridization, respectively. However, animals receiving an injection of the siRNA vector were not
Figure 5: Behavioral assessment in siRNA vector-injected lesioned animals. A: In the forelimb-use asymmetry test, there were no differences among any of the groups prior to 6-OHDA injection. After 6-OHDA lesioning, all of the 6-OHDA-injected animals exhibited significantly decreased motor function compared to unlesioned animals (*p<0.01). Injections of the siRNA vector (466) did not significantly worsen the motor
deficits due to 6-OHDA lesion compared to scrambled-injected lesioned animals. **B:** Similarly in the tactile stimulation task, forelimb preference was not altered in siRNA vector-injected lesioned animals compared to scrambled vector-treated lesioned animals. **C:** In the forelimb akinesia test, there was a slight worsening of the motor symptoms in siRNA vector-injected animals compared to the scrambled vector-lesioned animals that did not reach statistical significance ($p=0.17$). N=8 for each group.
Figure 6: Stereological cell counts of TH+ cells in SNpc following vector injections and unilateral 6-OHDA lesions. **A:** Representative photomicrographs of lesioned animals with either the scrambled vector (top panel) or 466 vector (bottom panel) injected into the right SNpc. There appear to be no qualitative differences in TH immunostaining due to siRNA vector treatment. **B:** Stereological quantification of TH+cells revealed no significant differences due to siRNA vector treatment in lesioned animals compared to scrambled vector-injected lesioned animals. Scale bar=500 μm. N=8 per group.
significantly worse (in terms of either motor function or dopaminergic cell degeneration) than scrambled vector-injected animals due to striatal 6-OHDA lesioning. This indicates that reduced NRG signaling through the ErbB4 receptor may not be sufficient to increase vulnerability of the nigrostriatal dopaminergic pathway to neurotoxin-induced degeneration.

The dopaminergic nigrostriatal pathway is particularly vulnerable to age-related and pathogenic degeneration. For example aged animals are more sensitive to 6-OHDA-induced degeneration of dopamine cells in the SNpc (Tamas et al., 2005) and less responsive to growth factor treatment in animals models of PD (Fox et al., 2001; Chapter 3). The underlying mechanism that is responsible for this increased susceptibility to neurodegeneration and decreased responsiveness to trophic factor therapy is currently unknown. A reduced neurotrophic factor environment for the dopaminergic nigrostriatal pathway has been described, including not only direct loss of trophic factor expression, but also loss of compensatory mechanisms in the aged nigrostriatal pathway (Croll et al., 1998; Katoh-Semba et al., 1998; Yurek and Fletcher-Turner, 2000, 2001). As we have shown in Chapter 3, there is age-related decreased expression of ErbB4 mRNA and protein specific to the SNpc. With respect to NRGs and nigrostriatal function, conditional nervous system ErbB4 knockout mice exhibited no changes in motor behavior or mesencephalic dopamine neurons suggesting that ErbB4 is not needed for the development and maintenance of dopaminergic cells in the ventral midbrain (Thuret et al., 2004). However, the authors acknowledge the potential for compensatory mechanisms to account for these results (Thuret et al., 2004). Aged animals were not
assessed in that study to address the potential need for ErbB4 in the aging ventral mesencephalon.

In the present experiment using siRNA to knockdown ErbB4 expression specifically in the SNpc, it appears that NRG signaling through ErbB4 may not be essential to the injured dopaminergic nigrostriatal system. This knockdown of ErbB4 prior to a striatal 6-OHDA lesion did not increase dopaminergic cell death in the SNpc or the motor asymmetry normally associated with the lesion. One important caveat with this set of experiments is the degree of knockdown that was achieved in vivo. Unlike the age-related loss of ErbB4 expression (which was decreased throughout the entire extent of the SNpc; see Chapter 3), the injection of the siRNA vector did not spread throughout the rostrocaudal extent of the nigra but rather stayed localized to the site of injection. Accordingly, the knockdown of ErbB4 mRNA achieved was limited to only a few sections (at 50-μm thickness each) through the SN. Future experiments using multiple injections of the siRNA vector into the SN are needed to achieve a more substantial spatial reduction of ErbB4 throughout the entire nigra. Also, the timing between the injection of the siRNA and the neurotoxin may be a factor. Two weeks in-between the siRNA and 6-OHDA was sufficient for knockdown of ErbB4 but may not be sufficient to change the physiology of the cells enough to increase their vulnerability to neurotoxic lesioning. Lastly, modulation of ErbB4 may not result in cell death in the ventral midbrain, but rather may effect more subtle changes in dopaminergic signaling. Thus, assessing changes in dopamine release in the striatum or other indicators of activation of dopaminergic cells of the SNpc may be necessary.
In conclusion, limited ErbB4 knockdown in the SNpc was not sufficient to increase the susceptibility of mesencephalic dopamine cells to 6-OHDA-induced degeneration. Future studies are needed to determine if more extensive suppression of ErbB4 in the midbrain is required for functional dysregulation or if there are more subtle changes in dopamine cell physiology in the SNpc due to knockdown of ErbB4. These data may have implications for the potential role, or lack thereof, of NRG/ErbB4 signaling in the pathogenesis of neurodegenerative diseases like PD.
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Chapter 5

General discussion and conclusions
The overall objective of this dissertation was to investigate the neuroprotective/neurorestorative activity of neuregulins (NRGs) for the injured nigrostriatal dopaminergic system and to determine if NRG/ErbB signaling played a role in the increased susceptibility of the mesencephalic dopaminergic connections to age and toxin-induced degeneration. The neuroprotective/neurorestorative function of NRGs for dopaminergic cells of the ventral midbrain in young animals was first addressed in Chapter 2 using a full striatal 6-OHDA lesion. In Chapter 3, age-related changes in NRG1 and ErbB receptors were assessed as well as the impact of NRG infusion in the aged substantia nigra pars compacta (SNpc) in an aged 6-OHDA model of PD. In the final experiment in Chapter 4, RNA interference technology was employed to knockdown ErbB4 receptor expression in the SNpc to determine if loss of ErbB4 expression was sufficient to increase the susceptibility of the nigrostriatal dopaminergic system to 6-OHDA-induced toxicity.

**NRGs as neurotrophic factors for the nigrostriatal system**

There are many examples indicating the importance for neurotrophic factor support in the injured nigrostriatal dopaminergic system—both in Parkinson’s disease (PD) patients and in animal models of PD. In PD patients, reduced brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) have been described in the SNpc (Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000; Seigel and Chauhan, 2000; Chauhan et al., 1999, 2001). Importantly, this is not only due to loss of dopaminergic cells within the SNpc; expression of these growth factor within the remaining neurons is significantly less then
age-matched controls indicating a compromised neurotrophic factor environment in PD patients not entirely due to chronological aging (Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000; Seigel and Chauhan, 2000; Chauhan et al., 1999, 2001). In normal animals and models of PD, the relationship between neurotrophic factors and support of mesencephalic dopamine cells are further exemplified. In the intact nigrostriatal system, infusions of either BDNF or GDNF are known to alter dopaminergic function, including increasing dopamine turnover in the striatum and affecting motor function in these animals (Altar et al., 1992; Martin-Iversen et al., 1994; Shen et al., 1994; Shults et al., 1994; Hudson et al., 1995; Bowenkamp et al., 1996; Hebert et al., 1997; Lapchak et al., 1997). In rodent models of PD, both GDNF and BDNF have proven to be neuroprotective, using several strategies to get them into the brain (e.g. direct infusion in both the SNpc and striatum, viral vectors, cell-mediated) (Frim et al., 1994; Levivier et al., 1995; Kearns and Gash 1995; Tomac et al., 1995; Bowenkamp et al., 1996; Kearns et al., 1997; Sullivan et al., 1998; Kirik et al., 2000a,b; Kirik et al., 2004; Sun et al., 2005). Furthermore, in the primate MPTP model of PD, GDNF has been shown to be not only neuroprotective, but also neurorestorative of motor function (Gash et al., 1996; Gerhardt et al., 1999; Kordower et al., 2000; Costa et al., 2001; Grondin et al., 2002). These studies led to using GDNF in clinical trials for the treatment of PD. Initial trials using intracerebroventricular administration of GDNF not only had no therapeutic effects, it also had adverse side effects in patients receiving large doses (Nutt et al., 2003). Similarly, although intraparenchymal infusions of GDNF met the primary endpoints of safety and efficacy in phase I clinical trials, the phase II trial did not confirm the initial findings (Gill et al., 2003; Love et al., 2005; Slevin et al., 2005; Lang et al., 2006). The
authors acknowledge that the differences could be due to inconsistent delivery methods and patient choices (Lang et al., 2006). Regardless, these trials exemplify not only that there is a potential for neurotrophic factor treatment for PD, but also the importance of consistency between phase I and phase II trials. Furthermore, these studies indicate that future work identifying novel neurotrophic factors or even combining neurotrophic factors for the treatment of PD is necessary.

This dissertation investigates the neurotrophic effect of a novel group of dopaminergic survival factors, the NRGs. Initial studies *in vitro* indicated that the NRG isoforms glial growth factor-2 (GGF2) and NRG-2β are trophic and protective for fetal mesencephalic dopamine cells (Zhang et al., 2004; K.B. Seroogy and D.M. Yurek, unpublished observations). Similar to BDNF and GDNF, the NRG1 isoforms NRG-1β and GGF2 are able to increase striatal dopamine overflow after supranigral infusion indicating functional significance for NRGs *in vivo* (Yurek and Seroogy 2001; Yurek et al., 2004). In neuroprotection studies using both full and partial striatal 6-OHDA lesions, GGF2 infusion supranigrally decreased dopaminergic cell death in the SNpc and improved motor function as measured by the cylinder test when compared to vehicle-infused lesioned animals. In Chapter 2, the neuroprotective effects of GGF2 and NRG-2β are compared in a more stringent striatal 6-OHDA model of PD. This model of PD involved injecting 6-OHDA into the striatum followed by a two-week delay before NRG infusion to allow the lesion to progress prior to therapeutic intervention and, thus, better mimic the point at which treatment for PD would begin in patients. This design also allowed for assessment of potential neurestoration of the behavioral deficits in these animals. At the two-week time point as measured by *in situ* hybridization, ErbB4 and
ErbB1 are still available, albeit diminished, in the injured nigrostriatal system in which TH mRNA is severely depleted. The reason for comparing a NRG2 and NRG1 isoform in these studies was due to evidence that indicated NRG2 isoforms may be more effective than NRG1 isoforms at activating the ErbB receptor isoforms most prevalent in the SNpc, ErbB4 and ErbB1 (Carraway et al., 1997; Longart et al., 2004). Moreover, preliminary in vitro studies indicated that NRG-2β protected fetal dopaminergic cell cultures from 6-OHDA-induced cell death at a 10-fold lower concentration than GGF2 (K.B. Seroogy and D.M. Yurek, unpublished observations). When GGF2 and NRG-2β supranigral infusions were compared in Chapter 2, both were equally neuroprotective for dopaminergic cells in the SNpc against 6-OHDA-induced cell death. Both NRGs were also equally neuroprotective against motor deficits normally associated with the 6-OHDA lesion. Interestingly, both NRGs appeared to have neurorestorative effects on motor function in this model, increasingly improving motor function from two weeks after infusion to eight weeks post-infusion. Thus, in this set of experiments, there was no statistical difference in enhancement of either motor function or dopaminergic cell survival between the GGF2- and NRG-2β-treated animals. Taken together, these data indicate that GGF2 and NRG-2β at the very least prevent the further progression of 6-OHDA-induced degeneration in this model. In PD patients, development of therapies that similarly halt the progression of the neurodegeneration would significantly improve quality of life, in part by increasing the therapeutic window for current dopaminergic replacement treatment.

There are several potential caveats that need to be addressed for these experiments. First, there was no dose response performed for either of these NRGs in
The potential importance of a dose-response study comparing GGF2 and NRG-2β is exemplified by the differences in neuroprotection displayed for these NRGs in vitro. Whereas GGF2 and NRG-2β exhibited similar neuroprotective effects for fetal mesencephalic dopamine cell cultures, NRG-2β protected against 6-OHDA challenge at a 10-fold lower concentration than GGF2 (K.B. Seroogy and D.M. Yurek, unpublished observations). The present dose was chosen based on the effective dose for the initial neuroprotection studies involving GGF2. Whereas protective and restorative effects were observed in the current study with the chosen dose it is unknown what the most effective dose for either of the NRGs is for the nigrostriatal system in vivo. Potentially the dose that was used could be lower than the most therapeutic dose for either GGF2 or NRG-2β. Thus these NRGs may not be reaching their full neuroprotective/neurorestorative activities. Alternatively, the dose used may be above the effective dose and thus could be masking not only the full neurotrophic activity of these NRGs, but also any of the potential differences between them. Future dose-response experiments to determine the most efficacious doses for GGF2 and NRG-2β will be important to identify which NRG is most therapeutic for the injured nigrostriatal system. Second, the amount of 6-OHDA used in these studies typically induced a large, full lesion; thus, differences may have been indistinguishable due to the extent of the lesion. Lastly, the duration of the infusion may have affected the outcome in this model; the duration of one week was again chosen due to the previous neuroprotection study. Nevertheless, these studies further define a role for NRG signaling as neuroprotective/neurorestorative in the dopaminergic ventral midbrain and its potential as a treatment for PD.
NRGs and ErbB receptors in the aging brain

Age-related changes are described throughout the central nervous system (CNS) although not all parts of the brain are affected equally. The dopaminergic ventral midbrain is particularly susceptible to age-related degeneration. For example, humans exhibit age-related decreases in striatal dopamine, cell number in the SNpc, and volume of the SNpc (Stark and Pakkenberg 2004). The underlying mechanisms for these age-related changes in the nigrostriatal system are unknown, though loss of neurotrophic factor support for the midbrain dopaminergic cells is proposed. Several lines of evidence indicate a compromised neurotrophic factor environment in the aged mesencephalic dopaminergic system in rodents. Decreased expression of BDNF in the striatum and of both BDNF and its receptor trkB in the ventral midbrain have been reported in chronologically aging rats (Croll et al., 1998; Katoh-Semba et al., 1998). Reduced GDNF expression also observed in the striatum and ventral midbrain of aged rats (Yurek and Fletcher-Turner 2001). The present data from Chapter 3 indicate a potential role for NRG signaling in age-related vulnerability of dopaminergic cells in the ventral midbrain. In the SNpc, decreased expression of mRNA and protein for the NRG receptor ErbB4 was found within the SNpc in two different strains of rats (Sprague Dawley and Brown Norway/F344 hybrid rats) and occurred prior to the decreases in tyrosine hydroxylase (TH) mRNA and protein (ErbB4 was decreased by middle-age and TH was not significantly reduced until old-age). There was also decreased expression of ErbB1 protein in the ventral midbrain of aged rats indicating that both the most ‘functional’ receptor for NRGs (ErbB4) and one of the heterodimerization partners (ErbB1) may be deficient in the aged nigrostriatal system. One concern with these findings was that these
decreases in ErbB4 and ErbB1 protein may be due to natural, age-related loss of dopaminergic cells in the SNpc rather than downregulation of those receptors within those cells. Thus, unbiased stereological cell counts of dopaminergic cells within the SNpc were performed and revealed no significant TH+ cell loss in the SNpc due to chronological aging, providing evidence phenotypic downregulation of the ErbB receptors is indeed occurring within aging dopaminergic neurons. Another potential concern was that the age-related decline in these receptors was due to globally reduced ErbB4 expression throughout the brain. As shown in Chapter 3, the decreased expression of ErbB4 and ErbB1 was specific to the ventral midbrain, with no other areas showing any significant age-related changes. There were, however, somewhat unexpected aging-associated alterations in NRG1 protein as assessed by a pan-NRG1 antibody using Western blot. Interestingly, aging increased the expression of NRG1 protein in the striatum of middle-aged and old animals compared to young animals. Moreover a trend toward an increase in NRG1 protein was noted in the prefrontal cortex in old animals, whereas no changes in NRG1 mRNA were detected in any of the areas assessed. This striatal NRG ligand increase could be a compensatory reaction to aging within the nigrostriatal dopaminergic system, perhaps in response to the downregulation of its receptors in the midbrain. Future experiments investigating specific isoforms of NRG1 and addressing increased NRG1 as a compensatory response to aging would need to be performed.

Besides direct decreases in neurotrophic factors/signaling, the aged nigrostriatal system is compromised in its ability to respond to environmental toxins and treatments for PD. Specifically in the 6-OHDA model of PD, Tamas et al. (2005) observed that
aged lesioned rats were more susceptible to the behavioral deficits associated with the lesion. As shown in Chapter 3, our data agree with the Tamas et al. (2005) study in that the amount of 6-OHDA that we injected (20 μg total) causes less than 70% dopaminergic cell death in young animals and less severe behavioral deficits. In the aging 16-month-old Sprague Dawley rats, that same amount of 6-OHDA caused more than 80% cell death with more severe behavioral deficits in the control lesioned animals. In addition, when aged animals are lesioned with 6-OHDA, there is an impaired compensatory trophic factor response when compared to young lesioned animals (Yurek and Fletcher-Turner 2000, 2001). Aging also alters the responsiveness of the dopaminergic nigrostriatal pathway to potential therapeutic treatments presumably due to a reduced neurotrophic factor environment. For example, dopaminergic cell grafts in aged lesioned animals have lower viability than in younger animals (Collier et al., 1999; Sortwell et al., 2001). This decreased viability can be corrected with neurotrophic factor infusion (Hudson et al., 1995; Sinclair et al., 1996; Yurek et al., 1996; Yurek 1998; Collier et al., 1999).

Furthermore, GDNF infusions are less effective at ameliorating the effects of 6-OHDA lesions in aged versus young rats (Fox et al., 2001).

Unfortunately, experiments involving aged animals are not only more expensive to perform, they also tend to be more difficult (e.g. higher fatality rate due to surgery, aged animals are less apt to do the behavioral tasks). As PD is a disease most prevalent in the aged population, these studies underscore the important differences between the young adult and aged brain especially with regard to the development of potential treatments for PD. Thus, while we have shown GGF2 to be neuroprotective/neurorestorative in young animals in both partial and full 6-OHDA lesion
models of PD, the reduction of ErbB4 expression in aged animals necessitated testing GGF2 infusions in aged animals (Chapter 3). For these studies using 16-month-old lesioned animals, the infusion of GGF2 was again delayed by two weeks from the start of the lesion. Furthermore in order to increase potential success of the infusions, the total amount of GGF2 administered was increased by extending the length of the infusion by 1 week. In these aging studies, lesioned animals receiving GGF2 infusions were significantly protected against dopaminergic cell death when compared to lesioned control animals. Similarly, GGF2 infusion in aging lesioned animals protected against the motor deficits caused by the 6-OHDA lesion. There also appeared to be significant improvement of motor function over the course of the study indicating potential neurorestorative effects in these aged animals.

Throughout this series of studies in this dissertation, it should be noted that aged and young animals were not directly compared. Thus, in the separate experiments, there were some notable differences between the studies in young versus aged rats. In the aged animals, again, a lower dose of 6-OHDA was used in order to induce a partial lesion and potentially distinguish more subtle differences in these animals. As stated earlier, the aged lesioned animals exhibited approximately 85% dopaminergic cell death indicating that the dose normally used to create a partial lesion in young animals creates a more severe lesion in the aged animals. The timing of the protective effects of the young and aged animals also differed. In young animals there was protection of motor function as early as two weeks after GGF2 infusion, whereas in the 16-month-old animals the protection of motor function was delayed to 6 weeks after the start of infusion. This occurred despite increasing the length of infusion and, thus, the total dose in the aged
animals. Therefore, whereas overall these studies indicate NRG treatment (specifically GGF2) as effective in an aged-lesion model of PD, it appears that it could be less potent in the aged animals than in young animals. This has important ramifications for developing any potential growth factor therapies for PD using NRGs. Specifically, GGF2 treatment in aging brains may require higher doses of GGF2.

Given the findings that the dopaminergic nigrostriatal system experiences age-related decreases in ErbB4 and that aged animals may be less responsive to GGF2 than young animals, Chapter 4 aimed to address whether loss of ErbB4 expression in the SNpc was sufficient to increase the susceptibility of dopaminergic neurons in the ventral midbrain to neurotoxin-induced degeneration. Using lentiviral vector-driven siRNA against ErbB4, the expression of ErbB4 mRNA was knocked down \textit{in vivo} in young animals. These ErbB4 siRNA animals were then injected with 6-OHDA into the striatum in order to induce a partial lesion of the nigrostriatal system. The ErbB4 siRNA animals were compared to scrambled vector-injected lesioned animals and assessed for possible exacerbation of motor dysfunction and dopaminergic cell death in the SNpc. Lesioned animals receiving ErbB4 siRNA did not have any increase in motor deficits compared to the scrambled vector-lesioned animals as determined by three separate behavioral tasks. Furthermore, ErbB4 siRNA did not increase dopaminergic cell death in the SNpc after 6-OHDA lesion. These data imply that loss of ErbB4 expression in the SNpc may not be sufficient to increase the vulnerability of dopaminergic neurons in the SNpc to neurotoxic degeneration. Thus, NRG signaling through ErbB4 in the ventral midbrain may not be essential to maintain nigrostriatal dopaminergic integrity after injury in the adult. This is in agreement with Thuret et al. (2004), in which adult conditional brain-specific ErbB4
knockout mice did not display motor deficits or dopaminergic cell loss. The authors concede that compensatory mechanisms may be account for the lack of phenotype that they reported (Thuret et al., 2004). Also, aged animals were not assessed but would be important to investigate in order to determine if ErbB4 signaling was essential in maintaining dopamine cell survival in the ventral midbrain of aged animals. In our study, several potential caveats may explain the lack of differences between the ErbB4 siRNA-lesioned animals and the scrambled-lesioned animals. The knockdown of ErbB4 was visible \textit{in vivo} by \textit{in situ} hybridization, however, using Western blots change in ErbB4 protein was indistinguishable. This is not surprising since the spread of the siRNA vector did not extend not very far from the injection site, thus remaining very localized. Unlike the differences that occur in the aging animals (decreased ErbB4 expression throughout the SNpc) this was, in effect, a high level of knockdown in a small number of neurons in the SNpc. It may take multiple injections throughout the SNpc in order to obtain a large enough knockdown of ErbB4 to elicit any changes in motor function or dopaminergic cell death. Also the timing between the injection of the siRNA vector and the lesion may be a factor. Although two weeks was sufficient to knockdown ErbB4 expression, it may not have been enough time to increase the susceptibility of infected cells to neurotoxicity.

\textit{Conclusions and Future Directions}

Overall this dissertation investigated the role of NRG/ErbB signaling in the injured and aging nigrostriatal system. In young animals there was no difference in the neuroprotective activity of GGF2 or NRG-2β for our 6-OHDA model of PD. These data indicate NRGs as potential neurotrophic factor therapies for the treatment of PD. Future
studies would need to be performed including a dose response for GGF2 and NRG-2β to confirm the similar trophic activities of these NRGs. Furthermore, increasing the length of the infusion in this model could increase the neuroprotection/neurorestoration. This could also be achieved through the use of a lentiviral vector system to deliver one or several NRG isoforms and may be even more beneficial for the treatment of animal models of PD. There is the possibility that an optimal NRG ligand for the dopaminergic nigrostriatal system remains to be identified and examined. Screening several other NRG1 and NRG2 ligands and even NRG3 in vitro may provide insight as to which NRG ligand would be the most effective dopaminergic trophic agent. To further determine the neurorestorative properties of these NRGs, future experiments should include increasing the delay between the 6-OHDA injection and NRG infusion.

There were several age-related alterations that occurred in the dopaminergic nigrostriatal pathway. Age-related decreased expression of ErbB4, ErbB1, and TH were observed in the ventral midbrain. In the aged striatum, NRG1 protein exhibited a significant increase. For the NRG ligands, future investigations are needed to address age-related alterations in specific NRG1 isoforms and in NRG2 or NRG3 and whether the increased expression of NRG1 protein in the aged striatum is a compensatory response to aging. In spite of the age-related decline in ErbB receptor availability, GGF2 was found to be neuroprotective/neurorestorative for the dopaminergic nigrostriatal system in an aged-lesion model of PD. Additional investigations will be needed to determine dose, length of infusion, and particular NRG ligand optimal for aged-lesioned animals.
Decreasing ErbB4 expression in young animals via siRNA in these studies did not increase the susceptibility of nigral dopaminergic neurons to neurotoxic lesioning. Future experiments are needed to further determine the role of ErbB4 in the maintenance of the nigrostriatal system in the adult and aging brain. Specifically, in the present experiments the spread of the ErbB4 siRNA was very restricted. Thus, using multiple injections to further decrease ErbB4 over the extent of the SNpc may elucidate a role for NRG/ErbB4 signaling in the injured nigrostriatal system. Alternatively, examining aged brain-specific ErbB4 knockout mice with and without a neurotoxic lesion, could identify increased vulnerability of the dopaminergic cells in the SNpc to age- and neurotoxin-related degeneration. Thus, these and additional future experiments will be needed to further address the role of NRG/ErbB4 signaling in the aging and injured nigrostriatal dopaminergic system.
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