Dynamics of neural stem cell-mediated neuroprotection in the presence of oxidative stress: studies in vitro and in vivo

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Dynamics of neural stem cell – mediated neuroprotection in the presence of oxidative stress: Studies in vitro and in vivo

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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For the Major Program
To my family for their unconditional support, love and patience
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CHAPTER II. INCREASED "VIGILANCE" OF ANTIOXIDANT MECHANISMS IN NEURAL STEM CELLS POTENTIATES THEIR CAPABILITY TO RESIST OXIDATIVE STRESS 37

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LIST OF ABBREVIATIONS

AD: Alzheimer's disease

ALS: Amytrophic lateral sclerosis

BBB: Blood brain barrier

BDNF: Brain derived neurotrophic factor

CAT: Catalase

CM-H$_2$DCFDA: 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

CNP: 2', 3'-cyclic nucleotide 3'-phosphodi-esterase

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

DETC: dethylthiocarbamate

ECM: Extracellular matrix

EGF: Epidermal growth factor

FGF: Fibroblast growth factor

GDNF: Glial derived neurotrophic factor

GDP: guanosine 5' diphosphate

GFAP: Glial fibrillary acidic protein

GFP: Green fluorescent protein

GPx: Glutathione peroxidase

HD: Huntington's disease

HNE: 4-hydroxy-2,3-nonenal

IL-6: Interleukin-6

LIF: Leukemia inhibitory factor
MAP2: Microtubule associated protein 2
MPTP: 1-methyl-4-phenyl-1,2,3,6, tetrahydropyridine
MS: Mercaptosuccinic acid
MTT: 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NGF: Nerve growth factor
NeuN: Neuronal nuclei
3-NP: 3 nitropropionic acid
NSC: Neural stem cell
NT: Neurotrophin
PD: Parkinson’s disease
PFA: Paraformaldehyde
PNP: Post-mitotic neural progenitors
ROS: Reactive oxygen species
SOD: Superoxide dismutase
SVZ: Subventricular zone
Tuj-1: class III beta-tubulin
UCP2: Uncoupling protein 2
VEGF: Vascular endothelial growth factor
This dissertation is based on the hypothesis that neural grafts may rescue/protect the host central nervous system (CNS) from induced and hereditary neurodegeneration. This hypothesis becomes especially intriguing in the context of neural stem cells (NSCs) that contribute to CNS plasticity and show therapeutic promise in neurodegenerative environments where oxidative stress plays a pathophysiological role. Unfortunately, little is known about the NSC’s intrinsic sensitivity to oxidative stress and how it relates to their potential to protect and repair the CNS. The present study therefore investigates the cellular and molecular bases of neural stem cell (NSC)-mediated neuroprotection, in a model of 3 nitropropionic acid (3-NP)-induced oxidative stress and neurodegeneration. Specifically, we studied in vitro and in vivo, the 1) alertness of antioxidant defenses and their contribution to the NSC’s sensitivity and response to 3-NP, and 2) interactions of NSCs with their local cellular microenvironment, which helped maintain homeostasis and cell survival.

The first part of our work analyzed in vitro cell survival and free radical levels in NSCs and their more differentiated counterparts (post-mitotic neural progenitors; PNPs) both at steady state and after exposure to 3-NP. We found that NSCs exhibited greater basal mitochondrial activity, lower intracellular reactive oxygen species (ROS) levels, and higher expression of key antioxidant enzymes, such as uncoupling protein 2 (UCP2) and glutathione peroxidase (GPx) than PNPs. On 3-NP exposure, PNPs showed rapidly increasing ROS levels and cell death. NSCs on the other hand were able control the ROS up rise and survive due to stronger antioxidant up-regulation. This heightened “vigilance” of antioxidant defense mechanisms might represent a basic NSC characteristic, which not only influences their fate, but also contributes to their ability to be therapeutically useful in the diseased CNS.
As the next step, we examined interactions of NSCs with their microenvironment that lead to neuroprotection from oxidative stress. For this purpose, we applied 3-NP *in vitro* (co-cultures of NSCs and primary striatal cells) and *in vivo* (NSC-transplanted mice) and studied NSC interactions with their cellular vicinity which may lead to the activation of antioxidant defenses and finally cell survival. In both models, the presence of NSCs significantly improved cell viability by interfering with the production of free radicals and increasing the expression of neuroprotective factors. *In vivo*, animals grafted with NSCs before treatment with 3-NP exhibited reduced behavioral symptoms and neuronal cell loss than vehicle-injected controls. Importantly, during the neuroprotective process, NSCs were found to solicit the assistance of neighbouring primary or host glial and endothelial cells. The interaction between NSCs, astrocytes and endothelial cells reciprocally increased expression of ciliary neurotrophic factor (CNTF) and vascular endothelial growth factor (VEGF). Also, both *in vitro* and *in vivo*, the increase in both factors was accompanied by a robust up-regulation of the antioxidant enzyme superoxide dismutase 2 (SOD2) in neurons, promoting their survival. This study therefore provides information on how NSCs, via cellular networking in the “niche”, may protect imperiled cells from the deleterious effects of oxidative stress.

In summary, this dissertation identifies two important properties of NSCs not explored before, namely their innate alertness to oxidative stress, and their ability to interact with and induce plastic responses from within their microenvironment. These features allow NSCs to survive in a hostile CNS environment and empower them to protect the host from the arriving impact of oxidative stress.
CHAPTER I. GENERAL INTRODUCTION

Dissertation Organization

This dissertation investigates the cellular and molecular dynamics underlying NSC-mediated neuroprotection. It contains the experimental results obtained by the author during her graduate study under the guidance and supervision of her major professors, Drs Jitka and Václav Ourednik. It is written in an *alternative thesis format*, and contains a general introduction, two research papers, a general conclusion and an acknowledgement.

The general introduction (Chapter I) includes a research objective, and background and literature review of NSCs, oxidative stress, CNS regeneration, graft-induced host plasticity, neuroprotection and neuroprotective factors, and NSC-host interactions.

The second chapter of the dissertation examines the alertness of antioxidant defenses, and their contribution to NSC sensitivity and response to oxidative stress. The third determines interactions of NSCs with their cellular microenvironment under oxidative stress and their role in neuroprotection. Both chapters are arranged in a *paper format* with the titles “Increased vigilance of antioxidant mechanisms in neural stem cells potentiates their capability to resist oxidative stress” and, “Neuroprotection from oxidative stress in the stem cell niche: cellular networks and regulation of antioxidant expression” by Madhavan L, Ourednik V, Ourednik J, and have been accepted by *Stem Cells* and accepted with revisions by *The Journal of Neuroscience*, respectively.

Chapter IV consists of the general conclusions summarizing the current work on mechanisms underlying NSC-mediated neuroprotection. It then goes on to propose some possible directions for future research.
Research Objectives

It has been shown that the interaction of transplanted NSCs with the CNS environment can stimulate protection/regeneration of host cells exposed to oxidative stress (Ourednik et al., 2002). Once the donor NSCs engraft into the recipient parenchyma, they integrate with their surroundings and get involved in a constant dialogue with host elements. During these events, and in the case of a pathological condition such as increased oxidative stress, NSCs are exposed to the hostility of the diseased CNS. In other words, the local host environment begins to affect NSC behavior and fate. In a similar manner, the transplanted NSCs also act to influence the local environment and change host cells in their vicinity. Thus, there is a bidirectional interaction between the grafted cell and its local three dimensional milieu which determines the outcome of NSC-transplantation.

However, in this context of graft-host communication, not much is known about the NSC’s own intrinsic sensitivity to oxidative stress and the NSC interactions with the surrounding host cells which lead to neuroprotection. This dissertation investigates NSC behaviors and interactions with its microenvironment under oxidative stress, in vitro and in vivo, and in relation to the neuroprotection. The major objectives of the conducted research were to:

1. examine the intrinsic reactive oxygen species sensitivity and antioxidant capacity of NSCs and their effects on NSC behavior under oxidative stress,
2. characterize the neuroprotective ability of NSCs under an environment of oxidative stress, and
3. determine some of the cellular interactions with the local microenvironment underlying the neuroprotective potential of NSCs.
Background and Literature Review

This section provides background information and literature review related to the studies presented in this dissertation. The information has been divided into the following subdivisions:

1. Overview of neural stem cells (NSCs)
2. Oxidative stress
3. CNS regeneration and NSCs
4. The concept of “graft-induced host plasticity”
5. Neuroprotection and neuroprotective factors
6. NSC interactions with its local environment

Overview of neural stem cells (NSCs)

Recent work in neuroscience has challenged the credo that new neurons are not generated in the adult mammalian CNS: In 1992, it was demonstrated that stem cells isolated from the forebrain can differentiate into neurons in vitro (Reynolds and Weiss, 1992). These results along with technical advances in the development of immunocytochemical reagents led to a burgeoning of subsequent stem cell research. Now, normally occurring neurogenesis in the subventricular zone (SVZ)/olfactory bulb and dentate gyrus of the hippocampus has been well characterized in the adult mammalian brain (Alvarez-Buylla and Lim, 2004).

Stem cells in an organism can exist in various capacities (Gage, 2000). A totipotent stem cell can give rise to a full organism, including the nervous system. The pluripotent cell is the same as an embryonic stem cell (ES cell) and is somewhat more restricted than
totipotent cells. It can give rise to every cell of the organism, except the trophoblasts of the placenta. Most stem cells fall into the category of multipotent stem cells which are dividing cells with only limited self-renewal capacity and which can differentiate into at least two different cell lineages (Weiss et al., 1996; McKay, 1997; Gage, 2000). They are usually defined by the organ from which they are derived or by from where they are observed in vivo. Lineage-specific precursors/progenitors are cells which are restricted to one distinct lineage (e.g., neuronal, astroglial, glial, oligodendroglial). Together, CNS stem cells and all precursor/progenitor cell-types are broadly referred to as “precursors”. In a more rigorous sense, adult CNS “stem cells” exhibit three cardinal features: (1) they are “self-renewing”, with theoretically boundless capacity to give rise to progeny exactly like themselves; (2) they are proliferative, and continue to undergo mitosis; and (3) they are multipotent for all the three different neuroectodermal lineages of the CNS, including the numerous neuronal and glial subtypes.

Studying neurogenesis and neural precursor/stem cell biology in the adult CNS has two general aims: (1) to understand the fundamental and basic biology of the nervous system, and (2) to develop future cell therapy strategies for CNS disorders. From the more fundamental perspective, it is important to learn about the normal role and function of precursor cells and neurogenesis in the adult CNS, as this information is critical for our understanding of brain function during development, adulthood, and in pathology. With regard to NSC therapy, it is now known that endogenous stem cells can be stimulated and directed towards target areas to initiate “self-repair” in the brain (Lowenstein and Parent, 1999; Emsley et al., 2005; Goldman, 2005). Also, the possibility to isolate stem cells from the brain, and to expand and manipulate them in culture provides a rich source of grafting
material for neural transplantation. From what we know already, NSCs isolated from rodent and human brains survive and integrate well in the developing and adult, intact and damaged, brain and can migrate considerably to reach perturbed/changing brain areas (Emsley et al., 2005; Goldman, 2005). Whether these stem cells embrace the exact function of the cells they replace remains to be determined.

With respect to CNS therapy, NSCs offer more than being simply a source for cell replacement. Firstly, they can produce a multitude of growth and plasticity promoting factors which aid cell survival and create a plastic milieu similar to the one present during early development. Secondly, they can interact with host microenvironment, re-establish homeostasis and rescue/protect host elements in a pathological situation (Ourednik et al., 2002; Park et al., 2002; Ryu et al., 2004; Pluchino et al., 2005). A better comprehension of the above two NSC characteristics might help achieve the goal of disease prevention beyond the already evident goals of repairing what is already degenerating or damaged. Indeed, maintaining cellular plasticity might be one of the straightforward concepts in neuroprotection. The work presented in this dissertation addresses NSC-mediated neuroprotection from oxidative stress, and investigates some of the mechanisms underlying this phenomenon.

**Oxidative Stress**

Oxygen is necessary for life but is also potentially hazardous. Therefore, an intricate system of pro-oxidant and anti-oxidant processes exists in cells to use oxygen efficiently. Oxidative stress occurs when an imbalance in the pro-/antioxidant homeostasis leads to unregulated generation of toxic reactive oxygen species (ROS) such as hydrogen peroxide,
superoxide, nitric oxide and the highly reactive hydroxyl radicals (Mates and Sanchez-Jimenez, 1999). In particular, cells in the brain are quite susceptible to ROS toxicity because of their high oxygen consumption, relatively low antioxidant levels and low regenerative capacity; oxidative stress is now known as an important event which leads to neurodegeneration (Andersen, 2004).

As their name suggests, ROS are very reactive, interacting with a multitude of molecules such as lipids, proteins and DNA to initiate neuronal cell death and neurodegeneration through an array of pathways. For example, ROS can oxidatively modify lipids and result in the formation of compounds such as acrolein and 4-hydroxy-2,3-nonenal (HNE) causing cellular toxicity (Barnham et al., 2004). More specifically, acrolein decreases glutamate and glucose uptake, whereas ROS modified HNE causes protein modification resulting in inhibition of the neuronal glucose transporter type-3, the glutamate transporter GLT-1 as well as Na\(^{+}\)-K\(^{+}\) ATPases (Barnham et al., 2004). ROS modified HNE also activates c-Jun aminoterminal kinases and mitogen-activated protein kinase (p38) leading to apoptosis (Barnham et al., 2004). In terms of proteins, ROS interactions can lead to the impairment of enzymes (like glutamine synthase and superoxide dismutase); in terms of DNA, they can cause mutations. Furthermore, excessive levels of ROS lead to dysregulation of intracellular calcium signaling (as observed in many neurodegenerative conditions) inducing the apoptotic cascade (Ermak and Davies, 2002). Also, ROS-induced calcium influx causes excitotoxicity which has been implicated in several neurodegenerative conditions such as Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington’s disease (HD) (Ermak and Davies, 2002).
Agents which induce oxidative stress such as 1-methyl-4-phenyl-1,2,3,6, tetrahydropyridine (MPTP), rotenone, and 3-nitropropionic acid (3-NP) are able to cause neurodegeneration (Beal et al., 1993; Betarbet et al., 2002). In particular, 3-NP is a mitochondrial toxin which produces oxidative stress in vitro and in vivo and evokes progressive degeneration of striatal neurons leading to behavioral changes similar to those accompanying Huntington's disease in humans (Beal et al., 1993). Based on post-mortem analysis of human brains which reveal the presence of elevated levels of oxidative damage products such as malondialdehyde, 8-hydroxy-deoxyguanosine, 3-nitrotyrosine and HNE in areas of degeneration, and the occurrence of increased free radical production in animal models, oxidative stress is considered either as a causative event, or a secondary component of the cell death cascade in HD and other neurodegenerative diseases (Bowling and Beal, 1995; Andersen, 2004). For these reasons, in our studies, we used in vitro and in vivo models of 3-NP-induced oxidative stress to analyze the behavior of NSCs and their potential to guard the host environment from impending neurodegeneration.

Cells possess an array of endogenous antioxidant defense mechanisms against oxidative stress. Among these mechanisms is the presence of various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as nonenzymic compounds such as α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C) which determine the redox potential and ROS concentration within cells (Mates and Sanchez-Jimenez, 1999). SOD destroys the free radical superoxide by converting it into hydrogen peroxide which then can be detoxified by CAT or GPx. Four SOD subtypes have been identified containing either dinuclear copper (Cu)/zinc (Zn), or mononuclear iron (Fe), nickel (Ni) or manganese (Mn) co-factors. Recent reports involving SOD knockouts have
revealed that Mn-SOD (manganese-superoxide dismutase/SOD2) is essential for life whereas the other forms of SOD are not (Macmillan-Crow and Cruthirds, 2001). CAT, on the other hand, reacts with hydrogen peroxide to form molecular oxygen and water and thereby protects cells from oxidative damage. Similarly, GPx also detoxifies a variety of hydroperoxides (including hydrogen peroxide) by utilizing glutathione and protects cells against oxidative injury. Furthermore, proteins such as the uncoupling proteins play an important role in mildly “uncoupling” the mitochondria to reduce their free radical load and consequently protect the cell against oxidant injury (Arsenijevic et al., 2000; Jezek, 2002; Mattiasson et al., 2003).

In many neurodegenerative diseases, the activities of the antioxidant molecules which would normally counteract the injurious effects of ROS are reduced or changed making them useful indicators of oxidative stress in these conditions. SOD, CAT, and GPx, for example, display reduced activities in affected brain regions in AD (Andersen, 2004). PD is characterized by reduced levels of the thiol-reducing agent, glutathione, in the substantia nigra, including in dopaminergic neurons (Andersen, 2004). SOD activity is reduced in HD brains (Borlongan et al., 1996; Browne et al., 1997). Also, mice with SOD2 and GPx deficiency have been shown to be more vulnerable to HD-like effects of 3-NP treatment (Andreassen et al., 2001; Kim and Chan, 2002). Mutations in Cu, Zn-SOD have been demonstrated to be involved in 20% of all familial cases of ALS (Andersen, 2006). Though circumstantial, all this evidence taken comprehensively indicates that reduced antioxidant potential might contribute to increased oxidative stress present in these diseases.

Stem cells show therapeutic potential in many neurodegenerative diseases where oxidative stress is a prominent entity (Ourednik et al., 2002; Goldman, 2005). Hence, it is
important to understand the behavior and potential of NSCs under oxidant stress and their cellular and molecular underpinnings before cell-based therapy can be used as a viable option. In this context, this dissertation investigates NSC sensitivity and reaction to 3-NP-induced free radicals and how they relate to the NSC potential to protect the CNS from oxidant injury.

**CNS regeneration and NSCs**

It is a common assumption that neurons of the mature mammalian CNS have minimal ability to regenerate following injury. In contrast, however, it is known that peripheral nerves can regenerate after various types of trauma. The difficulty of CNS neurons to regenerate following damage has been attributed principally to the presence of extracellular signaling factors which inhibit axonal growth and/or an inability of mature central neurons to respond adaptively to damage.

Associated with the pessimism regarding CNS regeneration, a prevailing dogma is that central neuron maturation leads to decreased structural plasticity. Obviously, CNS injuries such as stroke, trauma and neurodegenerative processes do not fully reverse themselves. However, in recent years there have been several fundamental discoveries which indicate that the mature CNS has a greater potential for producing new neurons and retains more significant capacity for structural plasticity than previously thought (Alvarez-Buylla and Lim, 2004). Examples include the elaboration of neurons from stem cells which subsequently integrate into existing neuronal circuitry (Hermann et al., 2005; Muotri et al., 2005), the sprouting of axons and resulting cortical re-organization (Darian-Smith and Brown, 2000), and the activity-dependent remodeling of dendritic spines and synaptic
plasticity (Xie et al., 2005). Also, it has been demonstrated that axonal sprouting and cellular changes occur in the brain following structural injury (Dancause et al., 2005), and that there is a general and systematic cellular response to injury which involves growth factors (Connor and Dragunow, 1998). In addition, the mature CNS continues to express a variety of molecules required for the formation of neuronal networks during embryonic development. These include growth factors, axon guidance molecules, cell-adhesion molecules and proteins which all determine cell fate. Although the functional importance of most of these molecules in the adult is unknown, their expression pattern goes beyond the regions of known neurogenesis, suggesting that the degree of potential network remodeling in the mature CNS may be more extensive than generally believed.

In terms of NSCs and their connection to CNS regeneration and plasticity, acute injury can cause attraction of proliferating precursor cells and the differentiation of local, dormant progenitor cells into neurons and glia. Hence, NSCs may be one of the components of fundamental 'programs' persisting within the CNS throughout life which not only ensure normal development, but also maintain homeostasis when perturbations occur. When neural progenitors are actually transplanted into the adult CNS, they are able to survive, differentiate, and become incorporated. Thus, NSCs not only show therapeutic potential, but also can answer basic biology questions regarding cell proliferation and differentiation, target-oriented migration in the CNS, neuronal synapse formation, and reaction to injury in the brain. In summary, exogenous NSC transplantation or endogenous NSC stimulation can be used as a tool to interrogate and explore the abnormal and normal host CNS and revive the brain’s capacity for self-repair, regeneration and neuroprotection.
The concept of “graft-induced host plasticity”

NSC transplantation has been proposed to help structural and functional recovery of the CNS in the following ways:

(1) replacing dead or dying cells;

(2) correction of a biochemical deficit by delivering necessary enzymes, neurotransmitters or hormones;

(3) local innervation and reconstruction of neural circuitries resulting in the restoration of synaptic transmitter release, and the re-establishment of functional nerve cell connections;

(4) creation of highly growth permissive tissue bridges for axonal regeneration and target-oriented guidance of growing axons;

(5) and as shown recently, by promoting CNS regeneration and protection through growth factor secretion and inflammation-induced stimulation of plastic responses which improve survival and function of host neurons.

Already, early reports by Ourednik et al. (1992 and 1995) suggested that fetal neural grafts containing germinative cells (stem cells) could elicit a significant regenerative response within the host CNS. The idea that NSCs could have been the important carriers and mediators of this process was later investigated and confirmed by the same authors (Ourednik et al., 2002) and others (Park, Teng et al. 2002; Teng, Lavik et al. 2002; Lu, Jones et al. 2003; Ryu, Kim et al. 2004; Yan, Welsh et al. 2004; Pluchino, Zanotti et al. 2005), and supports statement (5) above. The report by Ourednik et al (2002) showed that the damaged CNS can benefit from transplanted NSCs not only because of cell replacement but also due to rescue of dead or dying cells. In particular, the focus of their report was on aging and
MPTP affected dopaminergic neurons in the substantia nigra. After transplantation, although some NSCs did differentiate into dopaminergic neurons, most remained undifferentiated and expressed glial derived neurotrophic factor (GDNF), resulting in the rescue of the non-apoptotic but dysfunctional tyrosine hydroxylase (TH)-positive cells and finally reconstituted the mesostriatal system. Their results thus indicated that the host can benefit from NSC-derived cell replacement and also importantly the ability of NSCs to alter the microenvironment leading to host cell survival.

These above results have been corroborated by several other studies: NSCs seeded on a synthetic biodegradable scaffold and transplanted into the traumatized spinal cord significantly reduced the necrosis and initiated functional recovery (Weiss et al., 1996; Teng et al., 2002). Also, NSCs constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury (Lu et al., 2003). In addition, neural stem cells can protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors (Llado et al., 2004). NSCs furthermore, have been shown to exert a protective effect on the host brain when proactively transplanted into a rodent model of HD (Ryu et al., 2004). And finally, a quite recent report showed NSC-mediated neuroprotection through interactions with the immune system (Pluchino et al., 2005). Together, these observations lend support to the concept that increased host regeneration and resistance, or "graft-induced host plasticity," in addition to differentiation and integration of new neurons, can be an important contributor to the observed improvements after NSC engraftment.

However, the mechanisms underlying the observed protective and regenerative effects of NSCs remain elusive. They are likely be a replay of basic developmental processes.
It is now known that many of the molecules active in development also contribute to recovery after CNS injury (Connor and Dragunow, 1998; Imitola et al., 2004). Therefore, a guiding idea is that the presence of NSCs and their interaction with the surrounding CNS environment, post-transplantation, may lead to expression of developmentally important molecules, and finally neuroprotection and/or regeneration.

One group of such developmental molecules are neurotrophic factors like, neurotrophins 3, 4 & 5 (NT-3, 4 & 5), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), nerve growth factor (NGF), vacular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF) and others spontaneously expressed by neural stem cells (Ourednik et al., 2002; Emsley and Hagg, 2003; Lu et al., 2003; Maurer et al., 2003; Imitola et al., 2004; Llado et al., 2004). These factors, found to be playing key roles in the observed neuroprotection in our past and present studies (Ourednik et al., 2002), will be extensively discussed in the next section.

Another group of development-related molecules, possibly involved in graft-induced host plasticity, is the group of molecules involved in cellular growth and guidance, such as components of the extracellular matrix (ECM), cell adhesion molecules, diffusible factors such as sonic hedge hog, and bone morphogenic proteins. These molecules may promote axonal outgrowth/sprouting and correct targeting towards their post-synaptic counterparts. Additionally, they may be involved in signaling leading to cell survival. In terms of the cell adhesion molecule LI, by using its over-expression in NSCs in a MPTP-based lesioning paradigm, beneficial effects on stem cell migration and survival as well as the recovery of dying host dopamine neurons have been found (Ourednik et al., submitted). Thus, molecular interactions between stem cells and the adult CNS via adhesion/recognition molecules may
play a significant role in helping stem cells recreate/maintain a homeostatic milieu which ultimately can lead to cell survival.

Finally, a third group of molecules, likely to play a role in NSC-mediated neuroprotection, are injury-induced cytokines (Lucas et al., 2006). These cytokines are an important part of the host immunological reaction after NSC transplantation. Evidence exists that these cytokines can enhance host plasticity (Tonelli et al., 2005) and that the suppression of these molecules can lead to dampening of the host regenerative response (Bresjanac et al., 1997). Also, it was recently shown, when neuroinflammation predominates, transplanted NSCs can survive recurrent inflammatory episodes by retaining an undifferentiated phenotype and the ability to proliferate (Pluchino et al., 2005). The same study also showed that the transplanted NSCs, together with resident CNS cells, could persist and exert neuroprotective effects in this highly inflamed environment due to a continuous communication with inflammatory cells within perivascular niche-like areas.

Thus, it is reasonable to conjecture that NSC-host interactions, via the aforementioned groups of molecules under pathological stress and the resulting graft-induced host plasticity, may be a route by which dormant self-repair and neuroprotective cascades of the CNS can re-surface. The details of such interactions in the context of the dissertation work are discussed in the last section of this chapter.

**Neuroprotection and neuroprotective factors**

'Neuroprotection' is a term used to describe the putative effect of interventions protecting the brain from pathological damage. One obvious group of molecules likely to
play a key role in NSC-induced host plasticity and neuroprotection (as mentioned in the previous section) is the group of trophic and neuroprotective factors.

Various neuroprotective factors such as BDNF, NGF, NT-3,4,5, GDNF, CNTF and VEGF influence a wide range of cell functions in the developing, mature and injured nervous system. It has become increasingly clear that these factors do not have a single function but in fact exert many regulatory and protective effects on various type of central and peripheral neurons (Barde, 1989; Gomazkov, 2005; Levy et al., 2005). It has been proposed that growing axons compete for limited amounts of neurotrophic factors produced by target tissues. Neurons, failing to obtain a sufficient quantity of the necessary neurotrophic factor, die by a process of programmed cell death thus regulating the number of neurons and neuronal connections within the developing CNS (Johnson et al., 1989). The neurotrophic factor family has been also been proposed to play a role in the protection of specific types of neurons by suppressing the expression of “suicide genes” which, when activated, induce apoptosis (Talapatra and Thompson, 2001; Biswas and Greene, 2002). In other words, when the level of neurotrophic factors falls below the level required to suppress activation of “suicide genes”, transcriptional processes may initiate apoptosis.

The implications of trophic support deficits in the adult mature CNS are still unclear. If mature neurons still depend on the presence of neurotrophic factors for survival, then the reduction in neurotrophic factors, causing loss of transcriptional suppression of apoptosis, may result in the neuronal atrophy observed during aging or neurodegeneration. A theme of ongoing research is to determine whether members of the neurotrophic factor family have roles in preventing/and or reducing neuronal death/atrophy occurring in neurodegenerative disorders such as PD, AD, HD or ALS. Indeed, it has been shown that neurotrophic factors
have the potential to protect diseased and injured neurons from dying, and to induce neuronal sprouting and increase neuronal metabolism and function (Connor and Dragunow, 1998). One of the important areas of current research then is to identify potential ways to deliver neurotrophic factors to selectively vulnerable regions of the CNS so as to restore and maintain neuronal function. In this vein, in terms of stem cell therapy, it has been demonstrated that NSCs can express neuroprotective substances, spontaneously or after genetic modification, such as VEGF, CNTF, GDNF, NT-3, BDNF or stem cell derived neural stem/progenitor supporting factor (SDNSF) (Maurer et al., 2003; Toda et al., 2003; Imitola et al., 2004) and promote host cell survival, making them even more attractive as candidates to treat CNS disorders. The following sections provide information on CNTF and VEGF, the major neurotrophic factors which were found to be contributing to NSC-mediated neuroprotection in the present dissertation.

**Ciliary neurotrophic factor (CNTF)**

Early studies identified CNTF as a trophic factor which supports chick embryonic ciliary ganglionic cells (Adler et al., 1979; Lin et al., 1989) from which it derives its name. The cloning and sequencing of CNTF after its discovery revealed that it is part of a cytokine family that includes more generally acting cytokines such as leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) (Lin et al., 1989). Although involved in neuronal differentiation processes in vitro, CNTF is primarily considered as a lesion factor because of the lack of a signal peptide in the CNTF gene and its immunohistochemically determined cytosolic location (Ip and Yancopoulos, 1996).
Expression-wise, CNTF is restricted to the schwann cells in the peripheral and astrocytes in the central nervous system and it is now well established that a wide variety of central and peripheral neurons respond to CNTF (Ip and Yancopoulos, 1996; Schuster et al., 2003). Studies show that CNTF is important for the survival of motor neurons (Holtmann et al., 2005) and in preventing retrograde cell death of neurons in thalamic nuclei (Clatterbuck et al., 1993). CNTF can also enhance the survival of sensory, hippocampal, and cerebellar neurons (Larkfors et al., 1994; Semkova et al., 1999; van Adel et al., 2005), and protect retinal neurons and endothelial cells from oxidative stress (Koh, 2002; Yu et al., 2004). In addition, CNTF is known to slow the progress of motor neuropathy (Sagot et al., 1995), and has been shown to have neuroprotective effects in different models of HD (Alberch et al., 2004). With respect to HD, CNTF can protect striatal neurons in vitro (Petersen and Brundin, 1999) and in vivo in rodent and monkey models (Emerich et al., 1997; Emerich, 2004; Emerich and Winn, 2004). Also, CNTF-treated human NSC transplants improved motor function in a rodent model of HD (McBride et al., 2004). Furthermore, a study using polymer-encapsulated cells engineered to secrete human CNTF in human HD patients is ongoing (Bloch et al., 2004). The ability of CNTF to resist oxidative stress by stimulating the JAK/STAT and MAPK pathways and promote cell survival (De-Fraja et al., 2000; Kuroda et al., 2001; Kaur et al., 2005) may be contributing to these HD effects mentioned above.

In addition to neurons, there are also non-neuronal cells that respond to CNTF. For example, CNTF is involved in the survival and maturation of oligodendrocytes (Linker et al., 2002). It is also known to be involved in the maturation, proliferation, differentiation of astrocytes and is regarded as one of the signals that leading to astrocyte activation following
a CNS lesion (Ip and Yancopoulos, 1996). Even NSCs can respond to CNTF. Recently, it was shown that endogenous and exogenous CNTF enhance forebrain neurogenesis in adult mice (Emsley and Hagg, 2003). Thus all these cell types have the ability to respond to CNTF which is made available as a part of a series of complex events occurring after injury, making it an important cell survival and neuroprotective factor.

Vascular endothelial growth factor (VEGF)

VEGF was originally discovered as an endothelial-specific growth factor. It was discovered as vascular permeability factor (VPF) by Dvorak and colleagues in 1983 (Senger et al., 1983) and then termed vascular endothelial growth factor (VEGF) after the cloning of its gene by Ferrara and coworkers in 1989 (Ferrara and Henzel, 1989). Since then insurmountable evidence has been presented that VEGF/VPF is a major player in angiogenesis during development and in pathology (Ruhrberg, 2003). Recent observations, though, indicate that VEGF also has direct effects on neurons and glial cells, and stimulates their growth, and survival (Rosenstein and Krum, 2004; Storkebaum et al., 2004). Furthermore, VEGF has also been found to be an autocrine survival factor for embryonic stem cells (Brusselmans et al., 2005) and also be involved in the regulation of neurogenesis and learning in the hippocampus (Cao et al., 2004). Because of these pleiotropic effects, VEGF has now been implicated in several neurological disorders such as leukomalacia, stroke, neurodegeneration, cerebral and spinal trauma, ischemic and diabetic neuropathy, and nerve regeneration.

The VEGF gene contains a hypoxia response element that is essential for binding hypoxia inducible factor (HIF-1) which is a transcription factor serving as an oxygen sensor
During development, transient expression of VEGF, possibly by the neuroepithelium and perinatal neurons (Ogunshola et al., 2000), may regulate hypoxia-driven angiogenesis so that vessel formation is matched to oxygen and metabolic demand. In the mature brain, angiogenesis occurs only after injury and it is now well known that ischemic or implantation injuries cause an up-regulation of VEGF and its receptors in neurons and astrocytes (Rosenstein and Krum, 2004; Storkebaum et al., 2004). Thus, it is possible that nervous system trauma may promote re-emergence of certain developmental cellular events strongly influenced by VEGF leading to a process of physiological repair by normalizing vascular supply and metabolic demand. During this process of normalization, VEGF, by increasing vascular bed and oxygen tension, may have other, perhaps indirect, effects on non-vascular cell types in the neuropil. In this context, both neuronal and glial cells have been shown to possess VEGF receptors (Sondell et al., 2000; Krum and Khambullina, 2003; Rosenstein et al., 2003) and it is hypothesized that VEGF may help orderly developmental patterning or reparation of vessels and neurites, under normal and pathological conditions, by communication with chemical determinants of neuritic and vascular growth and motility. It is also becoming increasingly apparent that VEGF may carry out multiple repair functions in the CNS and PNS which include angiogenic, blood brain barrier (BBB) permeabilizing, neurotrophic, gliotrophic, and anti-apoptotic actions (Rosenstein and Krum, 2004; Storkebaum et al., 2004). Other growth factors such as BDNF and GDNF may support neural growth and survival, but only VEGF has the added ability to affect the physiology of the extracellular space where neural cells function. Therefore, VEGF can no longer be characterized solely as an endothelial mitogen as its nervous system effects may be quite broad. Furthermore, in terms of resistance to oxidative stress, VEGF is known to be
regulated by cellular redox (Maulik, 2002; Schafer et al., 2003; Eyries et al., 2004) and can up-regulate or modulate the antioxidant enzyme SOD2 (Abid et al., 2001; Abid et al., 2004). Thus, by virtue of its strong expression, VEGF could contribute to brain repair, regeneration, and neuroprotection by initiating basic cellular cascades, and play a significant role extending beyond simply blood vessel growth.

**NSC interactions with its local environment**

The potential of stem cells today relies upon removing them from their natural habitat, expanding them in culture, and placing them into a foreign tissue environment. Hence, it is essential to understand how stem cells interact with their extracellular microenvironment, the so called stem cell “niche”, to establish and maintain their properties and therapeutic effects. Niche is a word originally coined in ecology, and is a definition of where the cell lives, what it does and how it communicates with its environment. As mentioned before in the research objective of this dissertation, it is being realized that once the NSCs engraft into the host tissue they are engaged in constant crosstalk with the extracellular niche that they inhabit (Imitola et al., 2004; Ourednik and Ourednik, 2005). This constant and reciprocal communication between NSCs and their surrounding milieu *in vivo*, leads to proliferation, migration, differentiation, repair, protection and other effects. Thus the local microenvironment in which a stem cell resides regulates its behavior and fate. For example, it was shown that adult neural stem cells produced neurons when transplanted into the neurogenic zone of the hippocampus, but produced astrocytes in the environment of the spinal cord (Song et al., 2002a; Song et al., 2002b). In this study, further investigation showed that a specific component of the local environment, the regional astrocytes of the
hippocampus, were capable of instructing these stem cells to adopt a neuronal fate \textit{in vitro} (Song et al., 2002a). In addition to the regional differences within the nervous system, the microenvironment encountered by the stem cell may vary as a function of age of the host organism. Similarly, the alteration of the niche by injury, drugs or other circumstances is likely to affect the ability of transplanted stem cells to survive, integrate into the circuitry, protect, differentiate, and migrate. Just as the surrounding host microenvironment affects the fate of stem cells, transplanted cells similarly can also act to influence and alter neighbouring host cells. Stem cells, via soluble- or contact-mediated factors, affect the activity or resiliency of damaged host cells. Therefore, teasing apart and understanding these interactions between the stem cell and its environment poses a major challenge that must be faced in order to develop realistic cell therapies.

With regard to NSC-host cell cross-talk, stem cells \textit{in vivo} are likely to be influenced by a multitude of signals originating from the many neighboring cell types in their environment. For example in the subventricular zone, where neurogenesis exists throughout life, interactions between neural stem cells, ependymal cells, astrocytes, blood vessels and their lining endothelial cells are known to be important in its maintenance and functionality (Alvarez-Buylla and Lim, 2004). Similarly in NSC niches created after transplantation, cells such as astrocytes, endothelial cells, microglial cells and neurons present in the local microenvironment may all be important in NSC-host communication (Aarum et al., 2003; Wurmser et al., 2004). These cell-cell interactions may be mediated via direct contact, extracellular matrix (ECM), diffusible molecules such as trophic factors and sonic hedgehog, or immune response and inflammation (Alvarez-Buylla and Lim, 2004; Imitola et al., 2004;
Ourednik and Ourednik, 2005), and contribute to cell differentiation, regeneration and neuroprotection after NSC transplantation.

In the context of cellular interactions in the NSC niche, there are several recent reports which suggest that there is a reciprocal communication between the endothelial and NSC microenvironments (Louissaint et al., 2002; Cao et al., 2004; Schanzer et al., 2004; Shen et al., 2004). It has now been established that the brain distribution of NSCs is not random, but rather exists in close association with blood vessels in vascular niche’s (Palmer et al., 2000; Zhang et al., 2003; Wurmser et al., 2004). This location places NSCs in close proximity to endothelial cells which line blood vessels facilitating communication between these two cell types. Also, endothelial cells can regulate proliferation and the conversion of NSCs to neurons in vitro (Palmer et al., 2000; Shen et al., 2004). Additionally, recent evidence suggests that endothelial cell-derived VEGF, a hypoxia inducible molecule promoting angiogenesis, also has neurotrophic effects (Rosenstein and Krum, 2004; Storkebaum et al., 2004). VEGF also promotes neurogenic effects on neuronal progenitors (Jin et al., 2002; Cao et al., 2004; Schanzer et al., 2004; Shen et al., 2004) and has been found to be a chemo-attractant for NSCs (Zhang et al., 2003). Chapter III includes data and discussion of how endothelial cell-NSC interactions can be important in attaining neuroprotection from chemically induced oxidative stress.

Another important component of stem cell niches are astrocytes (Doetsch, 2003). Astrocytes are uniquely poised to act as sensors and regulators in the niche. Their meandering processes contact all niche cell types, their end feet terminate on the basal lamina of blood vessels and also, they are extensively coupled by gap junctions. Hence they can detect alterations, and also initiate as well as promote cellular communication in the niche.
In addition, they are vast repositories of several trophic and neuroprotective factors (Ridet et al., 1997; Svendsen, 2002; Horner and Palmer, 2003). Furthermore, astrocytes are known to promote the proliferative and neurogenic abilities of NSCs (Song et al., 2002a). In this thesis, we elucidate how NSC-astrocyte communication can contribute to host cell survival after transplantation.

Taken together, these findings imply that it is the combination of the intrinsic characteristics of stem cells and their microenvironment which shapes them and their potential. After transplantation, a consortium of such factors via NSC-host interactions may result in the deployment of appropriate defenses (such as neuroprotective factor production and anti-oxidant up-regulation as shown in this dissertation), and later in the modulation of gene transcription, thus determining the fate of both donor and host cells. Thus, in order to find realistic NSC-based therapies, biologists will need to understand how stem cells and their neighbours depend on each other for survival and then test how far a system’s equilibrium can be pushed before stem cells are wiped out or turn cancerous. In this dissertation, we focus on detailing the neuroprotective ability of NSCs and the molecular/cellular nature of graft-host interactions underlying this neuroprotection with regard to oxidative stress.

References


CHAPTER II. INCREASED “VIGILANCE” OF ANTIOXIDANT MECHANISMS IN NEURAL STEM CELLS POTENTIATES THEIR CAPABILITY TO RESIST OXIDATIVE STRESS

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ABSTRACT

Although the potential value of transplanted and endogenous neural stem cells (NSCs) for the treatment of the impaired central nervous system (CNS) has widely been accepted, almost nothing is known about their sensitivity to the hostile microenvironment in comparison to surrounding, more mature cell populations. Since many neuropathological insults are accompanied by oxidative stress, this report compared the alertness of antioxidant defense mechanisms and cell survival in NSCs and postmitotic neural progenitors (PNPs). Both, primary and immortalized cells were analyzed. At steady-state, NSCs distinguished themselves in their basal mitochondrial metabolism from PNPs by their lower reactive oxygen species (ROS) levels and higher expression of the key antioxidant enzymes UCP2 and GPx. Following exposure to the mitochondrial toxin 3-nitropropionic acid (3-NP), PNP cultures were marked by rapidly decreasing mitochondrial activity and increasing ROS content, both entailing complete cell loss. NSCs, in contrast, reacted by fast up-regulation of UCP2, GPx, and SOD2 and successfully recovered from an initial deterioration. This recovery could be abolished by specific antioxidant inhibition. Similar differences between NSCs and PNPs regarding redox control efficiency were detected in both primary and
immortalized cells. Our first in vivo data from the subventricular stem cell niche of the adult mouse forebrain corroborated the above observations and revealed strong baseline expression of UCP2 and GPx in the resident, proliferating NSCs. Thus, an increased “vigilance” of antioxidant mechanisms might represent an innate characteristic of NSCs, which not only defines their cell fate, but also helps them to encounter oxidative stress in diseased CNS.

INTRODUCTION

The restorative potential of NSCs is based on their abilities to provide cell replacement, serve as vehicles for gene therapy, but also, and importantly, on their capacity to stimulate reparative responses in the diseased host while promoting re-establishment of homeostasis [1-3]. During these events, NSCs and their progeny, together with all the other, more mature cell types, become exposed to the hostile environment of the diseased central nervous system (CNS). Although many investigations are addressing the deteriorating behavior of the mature neurons and glia in such conditions, interestingly, almost nothing is known about the vulnerability and the response of the NSCs which, frequently, appear to better resist environmental stress [4, 5].

Oxygen is necessary for life but, paradoxically, its metabolism produces reactive oxygen species (ROS) as by-products highly toxic to cells. Because of its elevated metabolic rate, high oxygen consumption, and relatively reduced capacity for cellular regeneration compared with other organs, the brain is believed to be particularly susceptible to the damaging effects of ROS. This becomes evident in diseases such as Parkinson’s disease or Huntington’s disease, where various indices of ROS damage have been reported within the specific brain regions that undergo selective neurodegeneration. To make matters worse,
although mammalian cells have evolved several resistance and repair mechanisms to deal with oxidative stress and the associated damage, the activities of various antioxidant defense molecules that would normally counteract the injurious effects of ROS are reduced in the brain [6 and cit. herein]. This is particularly true for the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

In regard to stem cells, the maintenance of a balance between self-renewal and differentiation is pivotal for their function in development, tissue repair, and homeostasis. In this context, a new role of the cellular redox state control has been recognized, affecting multiple processes related to cell proliferation and differentiation [7]. The redox potential and the concentrations of free radicals in cells are determined by a balance between their rates of production and clearance and are controlled by various antioxidant compounds like SOD, GPx, and CAT. Moreover, uncoupling of the mitochondrial respiratory chain by proton transporters like the uncoupling proteins (UCPs) reduces free radical production and consequently regulates the cellular redox state [8]. The latter’s control may also depend on the convergence of different signaling pathways, while its changes may, in turn, influence processes controlling self-renewal and differentiation. Thus, as demonstrated recently, the degree of cellular oxidation/reduction in progenitor cells can be altered by growth factor signaling. Depending on the nature of the signaling molecules, it can render the progenitors more reduced or oxidized [9]. In the present study, we therefore hypothesized that NSCs and their postmitotic progeny differ in their free radical household to an extent that also changes their ability to resist oxidative stress. This should be reflected, among others, in distinctive expression patterns of their antioxidant enzymes.
To explore this thought, we examined cultured NSCs and their 7-day-differentiated progeny (postmitotic neural progenitors or PNPs) and compared 1) their mitochondrial activities and ROS levels at steady-state and after exposure to the mitochondrial toxin 3-nitropropionic acid (3-NP), and 2) the corresponding expression levels of antioxidant molecules and their importance in the response to 3-NP intoxication. Two types of NSCs and PNPs often employed in current stem cell research were examined: primary NSCs isolated from the subventricular zone (SVZ) of newborn mice and the immortalized NSC clone C17.2 [10,11] successfully used in our previous studies of NSC-mediated rescue of neurons [5]. Assessed were their cellular characteristics relating to redox modulation and cell viability, such as mitochondrial activity, concentration of free radicals, ratios of surviving and apoptotic cells, and intensity of cell proliferation. Next, we analyzed the expression of antioxidative enzymes, focusing on the four key modulators UCP2, GPx, manganese-containing mitochondrial superoxide dismutase (Mn-SOD or SOD2), and CAT [8,12,13].

This report also includes our pilot immunohistochemical data pertaining to steady-state expression of antioxidant modulators in the subventricular stem cell niche in adult mouse brains. This data will serve as baseline in future studies addressing the vulnerability of endogenous NSCs to 3-NP-induced oxidative stress.

**MATERIALS AND METHODS**

**Cell Cultures**

*Primary cells*  NSCs isolated from the subventricular zone of newborn C57BL/6 mice were grown under standard conditions in uncoated dishes and serum-free Neurobasal medium supplemented with 2% B27 (NB27), 20ng/ml epidermal growth factor (EGF), 10ng/ml basic
fibroblast growth factor (bFGF), and 8μg/ml heparin. After 7 and 14 days, primary and secondary neurosphere cultures were split and plated at $10^4$ cells/ml in NB27 containing EGF, bFGF, and heparin to allow formation of tertiary neurospheres. The latter were used for all the experiments regarding proliferating NSCs, or differentiated in culture on poly-L-lysine for another 7 days without growth factors but in presence of 1% FBS for the derivation of PNPs.

*Immortalized cells* To match the growth conditions of the primary cells, immortalized cells (clone C17.2 [10]) were grown in serum-free DMEM/F12 medium supplemented with N2, 50 units/ml penicillin and 50 μg/ml streptomycin, heparin (8μg/ml), bFGF (20 ng/ml) and EGF (10 ng/ml) as described [14]. Only early (1-3) passage stocks of the originally immortalized cells were used for the preparation of tertiary neurospheres. To derive PNPs, the latter were grown on poly-L-lysine without growth factors and in presence of 1% FBS.

All cells were grown in a standard humidified incubator at 37 °C. The state of cell differentiation from tertiary neurospheres was monitored by immunodetection of Ki-67 and nestin, both markers for proliferating and immature cells (Fig. 1Aa-h), class III-β-tubulin (Tuj-1; neuronal marker, Fig. 1A i-l), and glial fibrillary acidic protein (GFAP; glial marker, Fig. 1Am-p).

*Induction of oxidative stress in vitro*

Cultures of NSCs and PNPs at comparable cell densities ($2 \times 10^4$ cells per well) were treated with 3-NP (0.05 mM) for 24 hrs and assayed for the next 5 days *in vitro* (5 DIVs, i.e., 120 hrs).
**Mitochondrial activity**

*The colorimetric 4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay* was used to measure mitochondrial functionality in cells [15]. Briefly, cells were incubated with 0.25 mg/ml MTT for 3 hrs at 37 °C and mitochondrial enzyme activity measured in culture supernatants in a spectrophotometer (Molecular Devices, Sunnyvale, CA) at 570 nm, with a reference wavelength of 630 nm.

**ROS levels**

*The 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) fluorescence assay* [16] was applied to measure the levels of free radicals in cells. CM-H$_2$DCFDA was added at 10 μM for 45 min to 3-NP treated cells (during the last 45 min of exposure) and to control cultures. Then, the cells were washed with phosphate buffer saline (PBS, 0.1 M, pH 7.2), and fluorescence recorded at a wavelength of 485 nm (excitation) and 535 nm (emission).

**Apoptosis**

Cultures exposed to 3-NP for 24 hr were fixed in 4% paraformaldehyde (PFA) and incubated with the Hoechst dye 33342 (10 μg/ml) for 3 min in the dark. Using UV illumination, a DAPI filter, and a 40x objective, fluorescent (apoptotic) cells were evaluated in 15-20 visual fields with a Zeiss Axioplan-2 microscope and the percentage of apoptotic cells determined.
Inhibition of antioxidant enzymes

In order to assess their direct relevance in the cellular anti-oxidative response, the enzymes UCP2, GPx, and SOD2 that showed significant differences in their expression levels at steady-state and after a 3-NP challenge were inhibited with guanosine 5' diphosphate (GDP) [17], mercaptosuccinic acid (MS) [18], and d ethylthiocarbamate (DETC) [19], respectively. The inhibitors were added to cultures 1 hour prior to 3-NP and left for the same duration as the toxin, or to 3-NP-free controls over the same culture period.

Immunocytochemistry in vitro

Cells grown on poly-L-lysine (0.1 mg/ml)-coated coverslips were fixed with 4% PFA and rinsed with PBS. Primary antibodies were diluted in blocking solution (5% goat serum supplemented with 0.2% BSA, 0.1% Triton X-100 in PBS) and preparations incubated overnight at 4°C. Specific binding was then revealed with the appropriate secondary antibodies conjugated to Alexa 488, 594 or 647 and diluted 1:500.

The following markers were used: For NSCs - nestin (1:1000), Musashi (1:500) and Ki-67 (1:500); for PNPs - Tuj-1 (1:500) as neuronal marker and GFAP (1:1000) as astroglial marker. Antibodies against UCP2, GPx, and SOD2 were diluted 1:200, 1:500, and 1:1000, respectively. Stains omitting primary or secondary antibodies and recordings through non-specific filters were used as signal-specificity controls.

Histology and immunohistochemistry of brain sections

Adult, 16-week-old C57BL/6 mice were perfused with 4% PFA under deep pentobarbital anesthesia. The brains were postfixed in the same fixative for 24 hours, processed for routine
cryostat sectioning, and 20-µm-thick serial coronal sections collected. Rehydrated sections were blocked and immunostained with antibodies against UCP2 (1:100), Gpx (1:250), nestin (1:500), Ki-67 (1:250) and Musashi (1:2500) using the same conditions as for the cell cultures described above. Stains occurred in coronal sections from levels 25-30 according to the stereotactic atlas of the mouse brain by Sidman et al. [20]. All animals were housed and maintained at Iowa State University and all animal procedures carried out were approved by the Iowa State University Committee on Animal care and adhered to NIH guidelines (Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002).

**Western blotting**

Cells were harvested, washed with ice-cold Ca\(^{2+}\)-free PBS and resuspended in 2 ml of homogenization buffer (20 mM Tris-HCl, pH 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, and 10 µg/ml leupeptin). Suspensions were sonicated for 10 s, centrifuged at 13,000g for 30 min, and the supernatants collected as whole cell lysate fractions. Samples containing equal amounts of protein were separated by 15% SDS-PAGE and blotted onto nitrocellulose membranes. After blocking of nonspecific binding sites with blocking reagent, antioxidant proteins were revealed by incubation of the membranes with antibodies against UCP2 (1:200), GpX (1:1000), SOD2 (1:2000), and CAT (1:1000) overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase (1:2000) were applied to visualize bound proteins in Amersham's ECL chemiluminescence assay. Equal protein loading was confirmed by re-probing of the membranes for β-tubulin.
**Densitometry**

Western blots were scanned and the NIH software Image J, version 1.34, used to quantify the densities of immunoreactive bands by calculating the area under the peak curves corresponding to UCP2, GPx, SOD2, and CAT.

**Chemicals**

3-NP, GDP, DETC, and MS were obtained from Sigma (St. Louis, MO). CM-H2DCFDA, MTT, and Hoechst 33342 stain were obtained from Molecular Probes (Eugene, OR). Dulbecco’s modified Eagle medium (DMEM), Neurobasal medium (NB), Dulbecco’s modified Eagle medium with F12 supplement (DMEM-F12), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, N2 supplement, B27 supplement, EGF, bFGF, and heparin were purchased from Invitrogen (Carlsbad, CA). Primary antibodies against nestin, UCP2, and GPx were received from Chemicon (Temecula, CA), anti-SOD2 from Upstate (Lake Placid, NY), anti-CAT from Genetex (San Antonio, TX), anti-Musashi from CeMines (Evergreen, CO), anti-Ki-67 from Dakocytomation (Carpinteria, CA), Tuj-1 from Covance (Berkeley, CA), and anti-GFAP from Sigma. All secondary antibodies were purchased from Molecular Probes.

**Data analysis and statistics**

All data are expressed as means ± S.E.M. and derived from at least 3 separate experiments. Data analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA). Data were first analyzed using one-way analysis of variance (ANOVA). Then, either Dunnett’s test was applied in the case of multiple comparisons with control, or Bonferroni’s
test for multiple comparisons between treatment groups and the statistical significance determined. Single comparisons were performed by Student's $t$ test or Welch-corrected unpaired $t$ test where appropriate. Differences were accepted as significant at $p < 0.05$ or less.

RESULTS

Steady-state characteristics of NSCs and PNPs related to redox state and cell survival in vitro

To obtain reference points for our study of 3-NP-induced oxidative stress in NSCs and PNPs, we first needed to assess basal levels of the evaluated cellular modalities, namely, mitochondrial activity (MTT assay), ROS production (CM-H$_2$DCFDA oxidation), and apoptosis (Hoechst-33342 staining).

In NSC cultures, about 80% of the cells proliferated (Fig. 1Aab and Bd, white columns) and expressed nestin (Fig. 1Aef), but no Tuj-1 and GFAP characterizing more differentiated cell types (Fig. 1Aijmn). After differentiation for 7 days, PNPs were characterized by less than 5% of dividing cells (Fig. 1Acd and Bd, hatched columns), had lost their nestin positivity (Fig. 1Agh) and expressed neuronal and glial markers (Fig. 1Aklop).

Striking differences in mitochondrial activity and intracellular ROS levels between NSCs and PNPs were recorded, whether primary or immortalized. NSCs (Fig. 1B, white columns) demonstrated a significantly ($p<0.05$) greater steady-state mitochondrial activity (Fig. 1Ba) than their postmitotic counterparts (hatched columns) and lower ROS production (Fig. 1Bc). Correspondingly, PNPs also counted higher numbers of apoptotic cells (Fig. 1Bb). These data were the first indicators supporting our hypothesis that NSCs and PNPs...
differ in their basal redox states, with the NSCs being better equipped to control intracellular ROS and resist oxidative stress.

A comparison of the analyzed parameters between the primary and immortalized cells revealed subtle cell type-specific differences (compare left and right plots in each panel a-d in Fig. 1B), although the same trend of their changes between NSCs (white columns) and PNPs (hatched columns) prevailed. Possible reasons for this finding will be discussed.

Response of NSCs and PNPs to 3-NP-induced oxidative stress

After collection of the data pertaining to steady-state ROS metabolism, apoptosis, and cell proliferation in NSCs and PNPs, we next investigated their response to the mitochondrial toxin 3-NP.

NSCs No significant changes in NSC number, proliferation, and apoptosis were observed at the time of the toxin removal (t=0), although the MTT readings had dropped about 20 - 30% below control values (Fig. 2, dashed lines). Cell behavior remained constant during the next 24 hours, after which mild deterioration became noticeable. The latter reached a peak at about 60-72 hrs post-3-NP, when a spontaneous recovery began, resulting in slightly different end values in primary and immortalized cells. While by the end of the 5th experimental day (120 hrs), primary NSCs had returned to values of the untreated controls (Fig. 2 acegi), immortalized NSCs remained at 75% in their MTT values (Fig. 2b) and also remained affected in their proliferative activity (Fig. 2j). Both translated to a reduction of 20% in their cell numbers (Fig. 2h) and suggested a less efficient control of intracellular ROS than in primary NSCs.
PNPs  The behavior of PNPs in response to 3-NP was radically different from that of the NSC cultures and appeared to perpetuate the initial steady-state difference between both culture types. Immediately after toxin removal (t=0), PNPs already exhibited obvious detrimental changes in all the measured parameters (Fig. 2, solid lines). In primary and immortalized PNPs, the considerable initial drop in mitochondrial viability quickly became a sigmoid decline bringing it to almost zero at 72 hrs post-3-NP (Fig. 2ab). This drastic decay of the cultures was mirrored in an exponential increase in ROS content and numbers of apoptotic cells (Fig. 2c-f). A corresponding decrease in cell numbers left hardly any surviving cells in the wells (Fig. 2gh), which made changes of intracellular ROS and apoptosis after 3 DIVs hardly measurable.

At all the evaluated times, PNPs were almost completely negative for the nuclear proliferation marker Ki-67, irrespective of their treatment with 3-NP (Fig. 2ij).

**Differential expression of antioxidant molecules between NSCs and PNPs during steady-state and after 3-NP intoxication**

The main enzymatic responses resulting in ROS detoxification are carried within the cells by molecules like GPx, SOD2, and CAT. UCP2, a mitochondrial proton transporter, can also help lowering ROS levels by reducing mitochondrial membrane potential [8,21]. Thus, part of the contributing reasons explaining the difference in the redox state values between NSCs and PNPs were likely to be found in unequal activities of their antioxidant defense mechanisms. To explore this hypothesis, we analyzed the expression levels of the above enzymes, either in qualitative immunostains of cell cultures (Fig. 3) or quantitatively from scans of Western blots (Fig. 4).
Steady-state expression of antioxidants

Initial qualitative comparison of antioxidant steady-state levels between NSCs and PNPs revealed a much stronger expression of UCP2 and GPx in the former, for both primary and immortalized cells (Fig. 3). In contrast, both types of cells showed similar levels of SOD2 and CAT activity (not shown). This result was subsequently confirmed and quantified in Western blots as illustrated in Figure 4 (U values).

3-NP-induced expression of antioxidants

Adaptive changes of antioxidant levels during the first 60 hours post-3-NP appeared less prominent in PNPs (dashed columns) than in NSCs (white columns). In neither of the evaluated enzymes did the PNPs show any significant upregulation, compared to their U control values, except in the case of UCP2 after 12 hours (p<0.05; Fig. 4a) and some increase in SOD2 at 60 hrs (p<0.06; Fig. 4e) in primary PNPs.

In contrast to PNPs, NSCs responded to 3-NP with considerable up-regulation of the enzymes UCP2 and SOD2 in primary (Fig. 4ae, white columns) and GPx in immortalized cells (Fig. 4d, white columns). Thus, in comparison to the steady-state levels (U values), 3-NP exposure could further increase existing enzymatic differences between NSCs and PNPs (Fig. 4a-d) and induced molecules such as SOD2, which did not show any differences at steady-state levels (Fig. 4e).

Inhibition of redox modulators in NSCs and its effects on their response to oxidative stress

In NSCs, the antioxidative enzymes UCP2 and GPx appeared to play a major role in steady-state-ROS metabolism and, together with SOD2, were the ones to be most prominently up-regulated in their response to oxidative stress. To test the direct functional relationship between the changing levels of these enzymes and the resistance of NSCs to 3-
NP, we inhibited the activity of these molecules with their inhibitors GDP, MS, and DETC, respectively. The resulting cellular behavior (Fig. 5, black lines) was then compared to that of non-inhibited NSCs and PNPs (Fig. 5, grey dashed and solid lines). In simultaneously processed 3-NP-free controls, the application of inhibitors alone did not produce any toxic effects (not shown).

After inhibition of UCP2 and GPx, the viability of primary and immortalized NSCs exposed to 3-NP was strongly reduced while a block in SOD2 activity did, interestingly, not produce any effects. In contrast to non-inhibited NSCs (Fig. 5, gray dashed line), the ones deprived of UCP2 or GPx activity (black solid lines) were unable to recover from the neurotoxic effects and they continued to deteriorate throughout the experimental period of 5 days. Not surprisingly, blocking of all three tested enzymes simultaneously led to the most severe deterioration of NSC viability, approaching it to that of the non-inhibited PNPs (compare the black and gray solid lines).

**Expression of redox modulators in the SVZ of the mouse brain**

In the *in vitro* studies described so far, we found that in their ROS household and tolerance of oxidative stress, NSCs are characterized by more active antioxidant defense mechanisms than more differentiated cell types. Since the largest steady-state differences were found in the expression levels of UCP2 and GPx (*U* values in Fig. 4a-d), we examined immunohistochemically the presence of both proteins in the germinative zones of the adult mouse brain [22] at coronal levels 25-30 according to reference 20.

While cells positive for both tested antioxidant molecules were dispersed throughout the brain parenchyma, strongly stained cells were predominantly localized within the antero-
lateral portion of the SVZ (about 5% of the resident cells; **Fig. 6, schema j**) and in the subgranular zone of the dentate gyrus (not shown). Both regions represent the two main stem cell niches of the forebrain [23].

To characterize these cells, we used the proliferation marker Ki-67 (**Fig. 6g-i**) and the stem cell marker Musashi (**Fig. 6a-f and details d1-f1**) [24]. Cells positive for these markers were consistently characterized by strong expression of UCP2 and GPx.

**DISCUSSION**

Oxidative stress due to excessive presence of ROS is a permanent threat to any cell with aerobic metabolism and accompanies many traumatic CNS pathologies and diseases like Parkinson’s disease and Huntington’s disease. Particularly in the brain, where 20% of the oxygen consumed by the body is utilized, cells have to rely on a variety of potent antioxidant defense mechanisms and a close interaction of glial and neuronal cells for constant removal of ROS [12,25-27]. Interestingly, while extensive studies have been performed on the mature cell types populating the CNS, almost no data exist about the effects of oxidative stress on NSCs and their postmitotic progeny. In the light of the present hopes to use these cells for therapeutic transplantation and/or take advantage of their endogenous counterparts, the investigation of this issue has become an important necessity and the present report is a first step in this direction.

To be able to realize their therapeutic purpose, grafted and endogenous NSCs have to resist the hostile pathological microenvironment as much as or even better than the other host cell populations. In the present study, we addressed this possibility by comparing NSCs and their PNP counterparts with respect to their steady-state ROS household, cell viability, and
expression levels of several key proteins with antioxidative functions, and their changes in response to 3-NP-induced oxidative stress.

By interfering with the mitochondrial electron transfer chain and leading to energy depletion, 3-NP exposure results in increased production of ROS like superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) [28]. To address both mitochondrial and cytoplasmic antioxidant detoxifier mechanisms controlling intracellular accumulation of ROS, we investigated the expression of the redox modulators UCP2, GPx, SOD2, and CAT.

UCP2 belongs to the family of mitochondrial $H^+$ transporters that regulate oxidative phosphorylation by increasing the uncoupling of electron transport and ATP formation [8]. Recently, UCP2 has been shown to be induced by acute brain injury like stroke and to participate in neuroprotection and cellular rescue during MPTP-mediated neuronal damage [13,29,30], which made us to include this redox modulator in our investigations. The other three antioxidant enzymes, GPx, SOD2, and CAT, are well characterized scavengers of free radicals; SOD2 is activated by high levels of superoxide and converts it to $H_2O_2$, while the cytoplasmic GPx and CAT convert the latter to $H_2O$ [27].

According to recent literature, it appears that the transcriptome of hematopoietic, neural, and embryonic stem cells includes a subset of genes, the products of which not only help to define “stemness” but can also provide the cells with higher resistance against oxidative stress [31,32]. Moreover, the intracellular redox state of NSCs also appears to regulate the cells’ balance between their self-renewal, cell differentiation, and apoptosis [9] and may therefore require special regulatory attention. The outcome of the present study is a concrete example of NSC behavior corroborating the first of these suggestions.
Steady-state characteristics  Cultured NSCs were found to be by default better equipped to control intracellular ROS than PNPs. This was reflected in their significantly higher mitochondrial activity, lower ROS content, reduced apoptosis, and in their comparatively higher basal levels of UCP2 and GPx. It has been reported that proliferating cells can actually produce substantial amounts of ROS endogenously [33]. Our results suggest that the maintenance of higher steady-state levels of UCP2 and GPx in stem cells is one of their strategies to offset this effect.

Response to 3-NP treatment  We observed that NSCs resisted evoked oxidative stress better than the PNPs and were able to recover from its effects within 5 days after the insult. In agreement with these findings, NSCs increased substantially their levels of antioxidant proteins while the latter remained almost unchanged in PNPs. The disparate response of both cell types to oxidative stress suggests, at least in part, an overall higher steady-state “vigilance” of redox control mechanisms in NSCs. Their stem cell characteristics, including proliferation, appear to be associated with a higher “alertness” of proteins like UCP2 or GPx to changes in intracellular ROS levels. Both proteins can decrease the probability of mitochondrial permeability transition [34], the release of apoptogenic factors [35], and the activation of caspase-3-mediated cell death [36]. GPx has also been shown to protect against 3-NP-induced apoptosis [37]. Hence, a possible interaction of apoptosis-modulating proteins, such as the members of the Bcl family, with antioxidant proteins like UCP2 or GPx and the ensuing suppression of apoptotic cell death can be one way for cells to counteract the effects of oxidative stress. The fact that we did not see any increase in CAT can be attributed to the role of GPx as the major source of protection against low levels of ROS and its greater affinity for hydrogen peroxide than CAT [27].
We propose that, intriguingly, a molecular network helping NSCs maintain stem cell status [9,31,32] may also be responsible for enzymatic “priming” in the control of cellular homeostasis. In the case of UCP2, GPx, and SOD2, this fact appeared to allow the NSCs to react quicker and more efficiently to external insults related to oxidative stress than PNPs which were marked by weaker expression and response of all three redox modulators. A similar principle, however, may also apply for other stem cell features helping NSCs to survive and interact with a pathological environment. Indeed, it has been recently reported that NSCs, by definition lacking differentiation markers and MHC molecules on their surface, show low immunogenicity and thus escape to a great extent the brain’s immune response [38,39].

In the present study, interestingly, primary and immortalized NSCs displayed some minor behavioral differences: In primary NSCs, the contents in UCP2 and SOD2 increased upon exposure to 3-NP, while both remained unchanged in the immortalized NSCs. The latter, on the other hand, up-regulated instead their GPx activity. It has been documented that overexpression of the myc gene can cause elevated ROS production in cells and can down-regulate NF-κB-mediated expression of redox regulators like SOD2 [40,41]. Such myc-dependent interference with the expression of antioxidants could explain the slightly worse values pertaining to cell viability and ROS household in immortalized NSCs when compared to those recorded from primary NSCs - both in their steady-state behavior and after a challenge with 3-NP. It could also play a role in the differential, 3-NP-induced expression patterns of the three tested redox modulators between primary and immortalized NSCs.

Irrespective of the induction patterns of antioxidants, both primary and immortalized NSCs – in comparison with PNPs - showed an equally high resistance to oxidative stress,
even if their levels of the tested antioxidants differed. This indicates that other existing defense mechanisms might help NSCs, in general, to stabilize their redox states. Examples of such mechanisms that we are currently investigating are enhanced adaptive controls of nitric oxide (NO) production and thiol pools due to proliferation- and myc-mediated permanently elevated ROS levels [42-44].

**Inhibition of the antioxidant response to 3-NP** To test the importance of the 3-NP-induced enzymes UCP2, GPx, and SOD2 for NSC survival, we inhibited their activity before and during the exposure of the cells to the mitochondrial toxin. The blocking of either UCP2 or GPx resulted in a substantial reduction in NSC viability and raised ROS levels and numbers of apoptotic cells. When all three enzymes were inhibited simultaneously, the NSCs died with rapidly increasing ROS levels and decreasing MTT values similarly to PNPs. Thus, although we can assume that additional signaling pathways contribute to the resistance of NSCs to oxidative stress, the *cumulative* control of membrane potential and efficient ROS clearance appear crucial for their survival and enhanced tolerance.

We found that the inhibition of SOD2 alone did not have any significant effects on the viability of NSCs. Although the steady-state levels of this enzyme were comparable between NSCs and PNPs, it was quickly and strongly induced in primary NSCs responding to 3-NP. No changes were observed in the case of immortalized NSCs, which could have been, as discussed before, the consequence of the inhibitory influence of myc over-expression in these cells. These results suggest that SOD2 is not involved in ROS control during steady-state homeostasis in these cells and that other additional antioxidant mechanisms may have compensated for its inhibition during the stress response. Also, UCP2, which is known to be
stimulated by mitochondrial superoxide, may have played an important role in quenching superoxide-induced oxidative stress in the NSCs [45].

*Steady-state expression of antioxidants in NSCs in vivo*  
Stem cells represent an important source of building material in both development and regeneration of the CNS and may very well take advantage of the “stemness-defining”, enhanced control of their redox state to be better prepared than the rest of the brain for the arrival of oxidative stress. This we found confirmed *in vitro*, where NSCs were characterized by more active antioxidant defense mechanism than their PNP counterparts. Since the largest differences in steady-state levels of antioxidant enzymes between NSCs and PNPs were those of UCP2 and GPx, we performed a pilot study of the expression of both enzymes in the stem cell niche [23] of adult mouse forebrain. Intriguingly, we found that endogenous NSCs localized in the SVZ and the subgranular zone of the dentate gyrus are also characterized by higher steady-state levels of UCP-2 and GPx when compared to neural cells in other brain areas. This finding, being in harmony with our *in vitro* results, adds a new and important characteristic to the already special nature of neural stem cell niches: they represent a source of cells that, by the nature of their stem cell transcriptome, are relatively well equipped to resist oxidative stress and, perhaps, other forms of pathological insults as well. A better understanding of such stem cell features is therefore likely to have an important impact on our views of CNS development, activation of endogenous NSCs during injury, and NSC transplantation.
ACKNOWLEDGEMENT

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Figure 1

Steady-state characteristics of NSCs and PNPs related to redox state and cell survival in vitro

[A] Microphotographs comparing the expression of stem cell and postmitotic cell markers in NSCs and PNPs. Most of the cultured NSCs (left group of panels) expressed the nuclear marker Ki-67, revealing all dividing cells (ab), and nestin, a prototypic marker for undifferentiated NSCs (ef). They were, however, negative for cell type-specific markers such as the neuronal Tuj-1 (ij) and the astrocytic GFAP (mn). PNPs (right group of panels), on the other hand, had lost their Ki-67 and nestin positivity (cd and gh) and began to express Tuj-1 and GFAP (kl and op). Bar: 40μm

[B] Quantitative evaluation of cell culture viability: Mitochondrial activity (MTT values, a), levels of free radicals (CM-H2DCFDA reaction, c), number of apoptotic cells (Hoechst 33342 staining, b), and cell proliferation (anti-Ki-67 immunostaining, d) were compared in cultured NSCs (white columns) and PNPs (hatched columns). Both primary and immortalized NSCs displayed stronger mitochondrial activity (a), lower ROS content (c) and less apoptosis (b) than the corresponding PNPs, indicating their better ROS buffering capacity. As expected, about 80-85% of the NSCs was proliferating and stained for the Ki-67 marker (d), while PNPs remained almost all negative. * p<0.05, *** p<0.001
A

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B

a. mitochondrial activity

b. apoptosis

c. free radicals

d. proliferation

Legend:
- NSCs
- PNPs
**Figure 2**

**Response of NSCs and PNPs to 3-NP-induced oxidative stress**  
After a 24-hr-treatment of the cultures with 0.05 mM 3-NP, NSCs (dashed curve) proved to be less susceptible to the resulting oxidative stress than PNPs (solid curve). Not only were the initial effects (t=0) less pronounced in the NSCs but they also were capable, after transient deterioration between 36-72 hrs, to recover. This was reflected in time-dependent changes of the cells’ mitochondrial activity (ab), increasing ROS levels (cd), and numbers of apoptotic cells (ef). Interestingly, while primary NSCs recovered completely within the 5-day experimental period, mitochondrial activity (b), cell numbers (h), and proliferation (j) of immortalized NSCs did not recuperate fully. PNPs (solid line) died within 72 hours (e-h), which was accompanied by their rapidly degrading mitochondrial activity and rising ROS levels (a-d). While in panels a-h, all values are expressed as percentage of the corresponding controls, in panels i and j, the control values referred to are those of Ki-67 labeling indices (percentage of labeled cells) in untreated NSC cultures represented by the horizontal lines.
Figure 3

Steady-state expression of the antioxidant molecules UCP2 and GPx in NSCs and PNPs 

in vitro Cultures of NSCs and PNPs were immunostained for their expression of the antioxidant molecules UCP2 and GPx. Most (~90%) of primary and immortalized NSCs stained intensively for both proteins (Aac and Bac) while their PNP counterparts were only weakly labeled (Abd and Bbd). Steady-state expression of SOD2 and CAT showed no differences between NSCs and PNPs (not shown). Bars: 20μm.
Western blot analysis of 3-NP-induced differential expression patterns of antioxidant molecules in NSCs and PNPs

Expression levels of antioxidant molecules (UCP2, GPx, SOD2, and CAT) in NSCs and PNPs were evaluated quantitatively in densitometry scans of Western blots prepared from whole protein extracts 12 and 60 hrs after treatment of the cultures with 3-NP. Quantification was achieved by integration of the area under the densitometric peak curve of each protein and the data are plotted in comparison to the untreated controls (U). The intensity of the 3-NP-induced changes in the levels of antioxidant molecules varied considerably not only between NSCs and PNPs but also between primary and immortalized cells. While NSCs (white columns) were characterized by strong upregulation of UCP2 and SOD2 (primary; ae), as well as GPx (immortalized; d), no striking response to the toxin was found in PNPs (dashed columns). See main text for more details. * p< 0.05, ** p< 0.01, *** p< 0.001.
**NSCs**
**PNPs**

- **UCP2**
  - Primary cells
  - Immortalized cells

- **DENSITY**
  - Primary cells
  - Immortalized cells

- **GPx**
  - Primary cells
  - Immortalized cells

- **SOD2**
  - Primary cells
  - Immortalized cells

- **CAT**
  - Primary cells
  - Immortalized cells
Figure 5

Inhibition of redox modulators in NSCs and its effects on their response to oxidative stress. A pharmacological block of the redox modulators UCP2, GPx, and SOD2 by GDP, MS, and DETC, respectively, led to a drastically increased sensitivity of the proliferating NSCs to the detrimental effects of 3-NP. Inhibition occurred either of individual antioxidants (A-C) or of all three of them simultaneously (D). In each of the 4 panels, the obtained data from the inhibited NSCs (solid black line) pertaining to their mitochondrial viability (ab), intracellular ROS levels (cd), and number of apoptotic cells (ef) were compared with 1) non-inhibited NSCs (gray dashed line) and 2) non-inhibited PNPs (gray solid line). While inhibition of UCP2 (A) and GPx (B) resulted in a significant (p<0.05) oxidative damage of the NSCs and their decreased viability, the block of SOD2 remained without obvious effects (C). The simultaneous inhibition of all three antioxidants led, on the other hand, to a marked (p<0.001) deterioration of NSC behavior and approached it to the values of the non-inhibited PNPs exposed to 3-NP. See main text for more details.
Figure 6

Expression of redox modulators in the subventricular zone (SVZ) of the mouse brain

Coronal, 20-μm cryostat sections of adult mouse forebrains were immunostained to reveal expression of UCP2 and GPx in neural cells. NSCs were monitored for the presence of the stem cell marker musashi (Mus; a-f and details in d1-f1) and the proliferation marker Ki-67 (g-i). Cells maintaining significant levels of UCP2 and GPx were found mainly within the antero-lateral regions of the forebrain SVZ and only rarely within the brain parenchyma (white crosses in schema j). Their location overlapped with that of NSCs labeled with Mus and Ki-67. See main text for more detail. LV – lateral ventricle, CP – caudoputamen, CC – corpus callosum, M – midsagittal line. Bars: a-i = 20μm, d1-f1 = 10μm
CHAPTER III. NEUROPROTECTION FROM OXIDATIVE STRESS IN THE STEM CELL NICHE: CELLULAR NETWORKS AND REGULATION OF ANTIOXIDANT EXPRESSION

LALITHA MADHAVAN, VÁCLAV OUREDNIK, AND JITKA OUREDNIK
A paper accepted with revisions by The Journal of Neuroscience

ABSTRACT

Recent studies indicate that transplanted neural stem cells (NSCs) can interact with the environment of the central nervous system and stimulate protection and regeneration of host cells exposed to oxidative stress. To inspect the cellular and molecular bases of this phenomenon, we applied 3-nitropropionic acid (3-NP) in vitro (co-cultures of NSCs and primary neural cell cultures) and in vivo (NSC-transplanted mice) to induce oxidative stress and to investigate NSC-dependent activation of antioxidant mechanisms and cell survival. In both models, proactive presence of NSCs significantly improved cell viability by interfering with production of free radicals and increasing the expression of neuroprotective factors. In vivo, animals grafted with NSCs and treated with 3-NP exhibited reduced behavioral symptoms and less severe damage of striatal vessels and cytoarchitecture than sham-transplanted controls. This amelioration correlated with the distribution pattern of donor cells in the host brain and expression of ciliary neurotrophic factor (CNTF) and vascular endothelial growth factor (VEGF) in a network of NSCs, local astrocytes, and endothelial cells. Intriguingly, both in vitro and in vivo, the enhanced secretion of both factors stimulated a robust up-regulation of the antioxidant enzyme superoxide dismutase 2 (SOD2) in neurons.
and resulted in their improved survival. Our findings thus reveal a so far unrecognized interaction between NSCs and surrounding neural and non-neural (including endothelial) cells accompanying neuroprotection. We illustrate how cellular networks in the niche may, through mutual stimulation of growth factor production and activation of antioxidant mechanisms, shield the local environment from the arriving impact of oxidative stress.

INTRODUCTION

When transferred into the recipient central nervous system (CNS), NSCs become tightly embedded within its microenvironment and a dialogue ensues between graft and host elements leading to dynamic cellular and molecular changes in both (Sheen et al., 1999; Ourednik and Ourednik, 2005). While grafted NSCs can integrate and mature in the developing or diseased CNS, a substantial number remains undifferentiated and can become important for the “rescue” of chronically impaired host neurons by, e.g., increased production of glia-derived neurotrophic factor (GDNF) (Ourednik et al., 2001; Ourednik et al., 2002). Therefore, although injury alone stimulates the host microenvironment to release important plasticity-modulating substances endogenously (Gall and Isackson, 1989; Hicks et al., 1999), their concentration and effects are likely to be substantially modified by interactions of donor and host elements (Ourednik et al., 2002; Park et al., 2002).

The aim of the present study was to examine in vitro and in vivo the nature of the interaction between NSCs and the surrounding cells in presence of oxidative stress evoked by 3-nitropropionic acid (3-NP), an inhibitor of the mitochondrial respiratory complex II (Beal et al., 1993; Beal et al., 1995). The central question was, whether NSCs, if added to the culture of primary neural cells or grafted into adult brain, could enhance 3-NP resistance in
both. In our in vitro studies, either primary neuronal/glial cells (PNGs) or primary neurons (PNs) were cultured alone or in the presence of NSCs prior to 3-NP treatment. Before and after the latter, we monitored cell viability, production of reactive oxygen species (ROS), apoptosis, and characterized cell types by immunocytochemistry. In the in vivo model, correspondingly, mock-grafted (vehicle or 3T3 fibroblasts) and NSC-grafted mice were intoxicated using a modification of the subacute 3-NP lesioning model by Femagut et al. (2002). Behavioral tests and immuno-histopathological analyses were used for evaluation in comparison to intact and intact/grafted animals.

Among the many aspects of interaction between NSCs and their cellular environment, we focused on intercellular signaling resulting in changed expression of growth factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or vascular endothelial growth factor (VEGF). Next, we asked how their presence may have contributed to the protective interaction of the participating cells and whether these factors influenced the production of a prototypic ROS detoxifier, the manganese-dependent superoxide dismutase (MnSOD or SOD2; Mates and Sanchez-Jimenez, 1999).

We found that NSCs in culture were more resistant to 3-NP than primary cells and that their presence prior to toxin exposure enhanced the resistance of postmitotic neural cells and enabled them to overcome the induced oxidative stress both in vitro and in vivo. In both cases, enhanced production of growth factors was associated with the activation of antioxidant mechanisms, as exemplified with SOD2. Particularly intriguing was the intimate dialogue between NSCs and their environment, in which not only the NSCs up-regulated their production of growth factors in response to 3-NP, but, importantly, they also
specifically induced a similar behavior in both the surrounding neural (neurons and glia) and non-neural (endothelial) cells.

**MATERIAL AND METHODS**

**In vitro studies**

*Neural stem cells* For our *in vitro* and *in vivo* studies, we used NSCs isolated from the SVZ of newborn C57BL/6 (Harlan, Indianapolis, IN) mouse and labeled with enhanced green fluorescent protein (eGFP) using retroviral transduction (Rappa et al., 2001). The cells were grown, under standard serum-free conditions in Neurobasal (NB) medium supplemented with B27, 50 units/ml penicillin and 50 µg/ml streptomycin, heparin (8µg/ml), basic fibroblast growth factor (bFGF; 20 ng/ml) and epidermal growth factor (EGF; 20 ng/ml).

*NIH 3T3 fibroblasts* Cells were grown in medium containing Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), and 50 units/ml penicillin and 50 µg/ml streptomycin.

*Primary neural cells (PCs)* PCs were derived from the striatum of embryonic day 17 (E17) C57BL/6 mouse embryos. After trypsin digestion and trituration of neural tissue, cells were resuspended in NB medium supplemented with B27, 10% horse serum, 500 µM L-glutamine, 25 µM L-glutamate, 200 units/ml penicillin, and 200 µg/ml streptomycin and seeded at 1x10⁵ cells/cm² into poly-L-lysine-coated wells and incubated under standard conditions to obtain mixed primary neuron-glia cultures (PNGs). Cultures were supplied with fresh medium by exchange of half of the culture medium every 4 days. To obtain primary neuronal cultures (PNs), 24 hr after plating, the medium was changed to serum-free NB medium supplemented with B27, 500 µM L-glutamine, 200 units/ml penicillin and 200 µg/streptomycin and treated
on day 4 with 10 μM cytosine 1-β-D-arabinofuranoside for 24 h to inhibit glial growth. Primary glial cell cultures (PGs) were grown as described previously (Jeftinija et al., 1996)

Co-cultures of NSCs with primary cells  NSCs were added at 5x10^3 cells/well either directly to 6-day-old cultures of PCs (PNGs or PNs; 10^5 cells/well) or co-cultured on a membranous polyester insert (Costar Transwell; 6.5 mm diameter and 0.4 μm pore size) above the primary cells. The co-culture medium consisted of Neurobasal (NB) medium supplemented with B27, 500 μM L-glutamine, 200 units/ml penicillin, and 200 μg/ml streptomycin, heparin (8μg/ml), bFGF (20 ng/ml) and EGF (20 ng/ml).

Induction of oxidative stress in vitro  Oxidative stress was induced in 7-day-old PCs by treatment of PNGs with 0.1 mM 3-NP, PGs with 0.25 mM, and PNs with 0.05 mM 3-NP for 48 hours according to our previously established dose/time-response curves. The use of three doses reflected the varying sensitivity of PNs, PNGs, and PGs to 3-NP and all treatments resulted in comparable oxidative stress in all three culture types. In the case when NSCs or 3T3 cells were added to the PCs, 3-NP was added 24 hrs later. After removal of 3-NP, the cells were rinsed and analyzed at three time intervals over 72 hrs.

To account for the possibility that some NSC-secreted factors might have inactivated 3-NP during the 48-hr treatment of the co-cultures, we removed their medium thereafter, treated it with neutralizing antibodies overnight to inactivate the secreted growth factors (see below), and added this medium back to fresh cultures of PNGs, PGs, and PNs. The 3-NP-mediated effects on mitochondrial activity, intracellular ROS levels, and apoptosis could still be recorded and occurred with undiminished strength.

Cell culture paradigms 1) PCs only (no 3-NP), 2) NSCs only (no 3-NP), 3) PCs + 3-NP, 4) NSCs + 3-NP, 5) co-culture of NSCs with PCs before the addition of 3-NP, 6) co-culture of
NSCs with PCs (no 3-NP) 7) co-culture of 3T3 cells with PCs before the addition of 3-NP and 8) co-culture of 3T3 cells with PCs (no-3-NP). Co-cultures of NSCs with PCs occurred either with or without separating polyester inserts (see above for specification).

*Inhibition of growth factor signaling* A titration was first conducted with different concentrations of the neutralizing antibodies and a dose-response curve was generated to determine the optimal concentrations to be used. Then, neutralizing antibodies against VEGF, CNTF, and BDNF were added to the co-cultures 24 hrs prior to the addition of 3-NP, at concentrations of 1, 2 and 0.5 µg/ml respectively. Their presence was maintained also after 3-NP was removed.

*MTT assay* Cell viability assessment during the co-culture studies was made using the standard colorimetric 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Hansen et al., 1989).

*Production of ROS* The presence of ROS was evaluated in a 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) fluorescence assay as described (Ohba et al., 1994; Shimazawa et al., 2005).

*Detection of apoptosis* Apoptotic cells *in vitro* were detected in a standard nuclear stain with the Hoechst dye 33342 and cells with apoptotic (condensed and/or fragmented) nuclei were quantified as described (Madhavan et al., 2003).

*Immunocytochemistry* Cells were fixed with 4% paraformaldehyde (PFA), washed 3 x 5 min. with 0.1M PBS, and blocked for 2 hr in 5% goat serum in PBS supplemented with 0.2% BSA, 0.1% Triton X-100. Incubation with primary antibodies (Abs) diluted in blocking solution occurred overnight at 4°C. After washing, the appropriate fluorescent secondary antibodies were applied for ½ hr at room temperature (RT). Coverslips mounted in
Vectashield medium were analyzed using a Zeiss Axioplan 2 microscope and images captured with a Zeiss AxioCam digital camera and Axiovision 4 software. The following cell markers and factors were detected: nestin (Ab dilution 1:500) and Ki-67 (1:500) for detection of NSCs and proliferating cells; class III-β-tubulin (Tuj1; 1:300) and microtubule-associated protein 2ab (MAP2ab; 1:500) as neuronal markers; glial fibrillary acidic protein (GFAP; 1:1000) and S-100β (1:1000) as astroglial markers; 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP; 1:500) as marker for oligodendrocytes; monitored was also expression of CNTF (1:250), BDNF (1:1000), VEGF (1:250), GDNF (1:500), NT-3 (1:500), and of the antioxidant SOD2 (1:500).

Protein quantification by ELISA Medium was collected from the various co-cultures, and its content in growth factors determined using an R & D Quantikine ELISA Immunoassay kit following the recommendations of the manufacturer (R & D systems, Minneapolis, MN). Pure growth factor proteins provided in the kit were used to establish standard curves for quantification.

Western blot analysis Forty-eight hours after 3-NP treatment, PCs were harvested, washed with ice-cold Ca^{2+}-free PBS, and resuspended in 2 ml of homogenization buffer (20 mM Tris- HCl pH 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 10 μg/ml leupeptin). Suspensions were sonicated and supernatants collected as whole cell lysate fractions. Equal amounts of protein were then loaded on a 15% SDS-polyacrylamide gel and the size-separated proteins transferred to a nitrocellulose membrane by electroblotting. The presence of SOD2 was revealed by incubating the membranes with specific antibodies using Amersham's ECL
chemiluminescence kit. Equal protein loading in blots was confirmed by their re-probing with a monoclonal Ab against α-tubulin.

**In vivo studies**

**Animals** 16-20-week-old male C57BL/6 mice (Harlan, Indianapolis, IN) were housed and maintained at Iowa State University and all animal procedures carried out were approved by the Iowa State University Committee on Animal care and adhered to NIH guidelines (Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002). The experimental groups and the number of animals in each group were as follows: 1) mice grafted with vehicle (Hank’s Balanced Salt Solution; HBSS) and 3-NP injected (n=22); 2) mice grafted with NSCs and 3-NP injected (n=17); 3) mice grafted with 3T3 cells and 3-NP injected (n=10); 4) intact mice (n=10); 5) intact mice grafted with NSCs (n=15). Mice from 1) and 3) were collectively defined as “mock-grafted”, since both groups presented similar behavior and histology throughout this study. The mice were sacrificed at 3 days or 3 weeks post-3-NP treatment.

**Transplantation** Grafting occurred unilaterally into the intact right striatum of trained mice (see below). Anesthetized mice (0.5 mg of pentobarbital (Nembutal) per g.b.w) received NSCs, 3T3 cells (both in 1.5 μl of HBBS, 75,000 cells/μl), or an equal volume of vehicle at the following stereotactic coordinates: 2 mm anterior to bregma, 1.5 mm lateral to the midline, and 2 mm along the dorso-ventral axis. For more details see Ourednik et al. (2002).

**3-NP treatment** One week after surgery, animals were subject to subacute striatal damage by 3-NP obtained by modification of the strategy employed by Fernagut et al. (2002). Briefly, the mice received systemic injections of the neurotoxin twice a day, 12 hrs apart, for
six days. The number of injections and the dosing were as follows: 4 x 20 mg/kg b. w.
injections followed by 8 x 40 mg/kg b.w. injections, resulting in a total dose of 400 mg/kg
b.w. Behavioral testing of the mice started with the first 3-NP injection.

**Experimental Time-Table**

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<td>grafting</td>
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<td>0 1 2 3 4 5 6 7 weeks</td>
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<td>behavioral training</td>
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*Behavioral training and tests* All mice were trained daily in pole and string tests according
to (Lalonde et al., 1992; Femagut et al., 2002) for two weeks prior to surgery. The behavioral
testing started from the first day of the 3-NP administration and continued for 3 weeks after
cessation of the neurotoxin injections. In addition to the pole and string tests, the following
set of behavior assessments was used for monitoring of motor deficits: 1) general locomotor
activity, 2) hind limb dystonia, 3) truncal dystonia, 4) hind limb clasping and 5) postural
challenge (see Table 1).

*Monitoring of the grafted-NSCs* Grafted cells were monitored by direct GFP fluorescence
or by immuno-detection of GFP.

*Histological analyses* Mice were sacrificed either 3 days or 3 weeks after the last 3-NP
injection. Brains were removed, fixed in 4% PFA in 0.1 M PBS, and processed for routine
cryostat or polyester wax (Ourednik et al., 2002) sectioning. Serial, coronal, 20-µm-thick
sections were collected from both striata between coronal levels 151 and 221 as defined in the atlas of the mouse brain and spinal cord by Sidman et al. (1971).

**Immunohistochemistry** Rehydrated sections were blocked and immunostained over night at 4°C with Abs against calbindin (Calb; 1:500), neuronal nuclear antigen (NeuN; 1:300), MAP2ab (1:250), GFAP (1:1000), S100β (1:1000), CNP (1:100), nestin (1:250), endothelial marker (CD146; 1:100), BDNF (1:250), CNTF (1:100), VEGF (1:100), 4-hydroxy-2-nonenal (HNE; 1:500), ED1 (1:500) or SOD2 (1:200). The resulting signals were visualized in a 2-hr-incubation at RT with secondary Abs (1:500) coupled to the fluorochromes Alexa Fluor 488, Alexa Fluor 594, or Alexa 647.

**Quantification and data analysis**

**In vitro experiments**

All quantifications of cell cultures were done in a blind fashion, using a Zeiss Axioplan 2 microscope and images were captured through a 40x objective using Zeiss AxioCam digital camera and Axiovision 4 software. Counts were performed from three separate experiments and averaged over 15-20 visual fields.

**In vivo experiments**

**Behavioral tests** String and pole tests were evaluated by the number of falls per five trials (with 30s resting time between individual trials) as recorded every day for each animal and then averaged over each animal group during the testing phase. Animal motility and posture were monitored daily and each animal received a score of 0 (no effect), 1 (mild effect), or 2
(severe effect) according to the table adapted from Fernagut et al. (2002) in the five behavioral categories mentioned above (see Table 1).

**Histology** Morphological analyses were performed on blind-coded slides using an inverted Nikon Eclipse microscope, with a Hamamatsu C4742-95 digital camera controlled by Prairie Technologies software (Middleton, WI), with image relay to a desk-top computer and a motorized X-Y stage. The striatal area was first identified under a 2x objective and then sampled under a 40x objective by moving the counting frame systematically through the striatal region using the motorized X-Y stage. The Metamorph offline software (Universal Imaging, West Chester, PA) was used to automatically count and average cell numbers (only for NeuN counts).

In each section, cells were counted in both hemispheres in animals of all the experimental groups. Then, the values of left and right hemisphere were compared and expressed as mean percentage values ± S.E.M. of intact controls. All counts and measurements were performed on 3-4 adjacent sections using a 40x objective and within coronal level 2 according to Fernagut et al. (2002) and the *Atlas of mouse brain and spinal cord* by Sidman et al. (1971), page 25-28. We chose this area since it was there where maximal lesioning was expected (Fernagut et al., 2002) and indeed observed in sham-operated controls after 3-NP intoxication (common for all groups).

**Cell counts** – The expression of CNTF and VEGF in donor NSCs, host astrocytes, and host endothelial cells as well as host striatal neurons double-labeled for NeuN and SOD2 were determined in all grafted animals and cell numbers were calculated from 3 adjacent coronal sections (measured in the dorsolateral area as shown by circles in drawing in Fig. 8).
Counted were also host striatal neurons expressing NeuN only all over striatum as shown by slashed area in drawing in Fig. 8.

Blood vessel diameters – The blood vessel diameters were measured in the dorsolateral area (circles in the drawing in Fig. 8) using the morphometric tools provided by the Axiovision 4 software.

All counts were conducted in both the right and left striatum and in all the experimental animal groups.

Statistics  All data are expressed as mean ± S.E.M., and statistical significance is determined by analyses of variance (ANOVA) with Dunnett's post hoc test for multiple comparisons with the control or Bonferoni's multiple comparison tests for multiple comparisons between treatment groups. Significance of single comparisons was determined in a Student's t test or the Welch-corrected unpaired t test, where appropriate. A minimal probability of p<0.05 was considered significant.

Chemicals  
3-NP was obtained from Sigma (St. Louis, MO); CM-H$_2$DCFDA, MTT, and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). All the cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Antibodies against the following markers were purchased from: nestin, CNP, NeuN, MAP2ab, Calb, CD146, BDNF, CNTF, GDNF, and NT-3 from Chemicon (Temecula, CA); GFAP and S100β from Sigma (St Louis, MO); Tuj1 from Covance (Berkeley, CA); VEGF from Santa Cruz Biotechnology (Santa Cruz, CA); HNE from Alpha Diagnostic International (San Antonio, TX); ED1 from Serotec (Kidlington, Oxford, UK) and SOD2 from Upstate (Lake Placid, NY). Neutralizing
antibodies against VEGF and CNTF were obtained from R & D Systems (Minneapolis, MN) and against BDNF from Chemicon (Temecula, CA).

RESULTS

Co-culture with NSCs and the resistance of PCs to oxidative stress

In an initial titration of 3-NP concentrations on single-cell-type cultures, mixed cultures of primary neuronal and glial cells (PNGs) and cultures of primary glia (astrocytes, PGs) revealed themselves more resistant to the toxin than primary neuronal cultures (PNs). Therefore, in order to elicit the same effects in all three culture types, PNGs were treated with 0.1 mM 3-NP, PGs with 0.25mM, and PNs with 0.05 mM 3-NP. Treatment of NSCs grown alone, consequently, had to be tested with the same three 3-NP concentrations before their co-culture with PCs.

While in NSCs cultured alone, only the highest dose of the toxin, 0.25mM, resulted in moderate deterioration of cell viability [Fig. 1A-C, dashed lines], all 3-NP concentrations induced severe oxidative stress in PCs [Fig. 1D-F]. This was reflected in a rapid decay of their mitochondrial viability [Fig. 1D] and in increasing levels of ROS [Fig. 1E], both leading to massive apoptosis and cell death [Fig. 1F].

The survival capacity of the PCs increased substantially when they were co-cultured with NSCs prior to 3-NP intoxication [Fig. 1G-L; p<0.05]. This was reflected in increased initial resistance of the cells to 3-NP and in their induced recovery, in which PNGs and PGs regained almost control values after 72 hours. Notice that co-cultures with PNs [thin lines] were consistently and significantly (p<0.02) less resistant than those of PNGs [bold lines] and PGs [dashed lines]. This resulted in their noticeably higher initial MTT values [Fig.
1DGJ], lower levels of free radicals [Fig. 1EHK], and reduced numbers of apoptotic cells [Fig. 1FIL], all of which caused a shift of curves in the individual plots. Occasional value differences between PNGs and PGs in [G-L] turned out to be insignificant.

The higher resistance of NSC/PNG and NSC/PG co-cultures allowed them to fully recover their viability 72 hrs post-3-NP while co-cultures containing PNs remained affected. The main purpose of the cultures including astrocytes as the only PC type was to verify possible protective influences originating in neurons. Since, however, the sensitivity of these cells and their behavior in co-culture with NSCs were indistinguishable from that of PNGs, we focused, in the following, on PNGs and PNs only. Culturing PCs and NSCs in direct contact [Fig. 1G-I] or in separate compartments [Fig.1J-L] had no obvious effects on the experimental outcome. This suggested a major involvement of soluble factors in the mediation of the neuroprotective effect.

Effects of NSCs on neuronal cell death in co-culture with PNGs

Primary astrocytes and neurons were distinguished immunocytochemically by their expression of GFAP and MAP2ab and the glial/neuronal index (ratio) calculated over 72 hours post-3-NP [Fig. 2]. In untreated controls, the total cell number in the cultures increased, as did the G/N index [Fig. 2, column A]. Upon 3-NP treatment [Fig. 2, column B], a rapid cell death occurred and the concomitantly increasing G/N index revealed that this was mostly due to neurons dying during the first 48 hours. Within the next 24 hrs, most of the cultured cells had died. This neurotoxic effect of 3-NP on PNGs could be prevented by adding of NSCs prior to their intoxication, with or without direct contact between both cell types [Fig. 2, columns CD]. This was reflected in G/N indices remaining comparable to the
untreated controls and in restored survival of the cultures for at least 72 hours. The substantially higher vulnerability of neurons to 3-NP compared to that of glia made the formers’ involvement in NSC-mediated protective effects unlikely and lent support to the results represented in Fig. 1.

**3-NP-induced up-regulation of growth factors in vitro**

NSCs without 3-NP exposure and growing alone or in co-culture with PCs produced significant amounts of BDNF, CNTF, and VEGF. On the other hand, PCs, especially when growing alone, were marked by very low production of these factors. While PNGs still expressed measurable amounts of CNTF and VEGF, PNs remained essentially negative in the absence of 3-NP.

NSCs treated with 3-NP when cultured alone up-regulated their expression of CNTF, and VEGF as demonstrated by increasing numbers of immuno-positive cells [Fig. 3Aa-c and Ba, white columns]. Concentration changes of both factors in the culture medium were determined in parallel by ELISA [Fig. 3Bb]. No significant changes in BDNF expression were found.

In contrast, there was minimal expression of these factors in PCs alone, even after toxin treatment [Fig. 3Bc-f]. The same was true when the PCs were co-cultured with NSCs in the absence of 3-NP [Fig. 3Cbe, dashed columns]. Upon intoxication, however, a massive up-regulation of all three factors could be recorded in the stem cells [Fig. 3Cad, white columns] and of CNTF in the primary astrocytes in the NSC/PNG-co-cultures [Fig. 3Ad-f and Ch, white columns]. Correspondingly, the concentration of total secreted growth factors in the co-culture media was enhanced as well [Fig. 3Cef]. Almost no growth factors
were expressed by neurons [Fig. 3B, Fig. 3C] and the increase in their secretion measured by ELISA in NSC/PN co-cultures [Fig. 3C] was the result of NSC secretion only.

**Inhibition of growth factors in NSC/PC co-cultures and its effects on the response of the cells to oxidative stress**

To ascertain a functional relationship between the presence of BDNF, CNTF, and VEGF and the enhanced resistance of the NSC/PC co-cultures to 3-NP-induced oxidative stress, the activity of the growth factors was blocked specifically with neutralizing antibodies. The resulting behavior of such co-cultures [Fig. 4, columns B-E] was then compared to that of the non-inhibited ones [Fig. 4, column A] and to PCs cultured alone [Fig. 4, column F]. In simultaneously processed 3-NP-free controls, the addition of the neutralizing antibodies did not produce any toxic effects (not shown).

While an interference with BDNF activity had no significant effect on the viability of NSC/PC co-cultures exposed to 3-NP [Fig. 4, column B], both PNGs and PNs became strongly compromised after inhibition of CNTF [Fig. 4, column C] or VEGF [Fig. 4, column D]. The deterioration of the cultures was obvious from measurements of their mitochondrial activities [Fig. 4, row a], levels of ROS [Fig. 4, row b], and numbers of apoptotic cells [Fig. 4, row c]. All three parameters deteriorated progressively and cultured cells, both PCs and NSCs, were unable to recover from the neurotoxic effects as compared to non-inhibited controls [Fig. 4, column A]. Interestingly, a substantial number of NSCs persisted and survived this growth factor inhibition, indicating that other molecules and mechanisms must be contributing as well to their overall better tolerance of oxidative stress compared to PCs. A simultaneous neutralization of all three tested growth factors led, not
surprisingly, to the most severe deterioration of co-culture viability and approached it to that of the 3-NP-treated PCs cultured alone [compare columns E and F in Fig. 4]. In most measurements, interestingly, PNG viability [bold lines] appeared to deteriorate less severely (p<0.05) than that of PNs [thin lines].

**Growth factor production and up-regulation of antioxidant enzymes**

The reduced cell viability and increased ROS production in 3-NP-treated co-cultures after growth factor inhibition suggested that these factors may have directly participated in the up-regulation of cellular antioxidative defense mechanisms. To explore this possibility, we monitored by immunocytochemistry and in Western blots NSC-mediated changes of intracellular SOD2 levels. This enzyme represents an important member of the anti-oxidant defense system in mammalian cells (Mates and Sanchez-Jimenez, 1999) and has been shown to play an important role in quenching of oxidative stress in the striatum (Beal et al., 1995; Medina et al., 1996; Andreassen et al., 2001; Kim and Chan, 2002).

Using SOD2-specific antibodies, we found that the NSCs themselves reacted to 3-NP exposure by increased SOD2 expression [Fig. 5A]. This was in harmony with their initially found resistance to the toxin (compare Fig. 1A-C and D-F). Interestingly, a similar up-regulation of this enzyme was also induced in PNGs and PNs co-cultured with NSCs [Fig. 5Bab and Cab]. On the other hand, when growing alone, untreated and 3-NP-treated PCs did not show any change in SOD2 expression [Fig. 5Bb and Cb].

Since SOD2 is known to be inducible by VEGF (Abid et al., 2001; Abid et al., 2004) and in order to determine the latter's direct involvement in the expression changes of this enzyme, we decided to block the growth factor in a set of co-cultures prior to SOD2
detection. Indeed, this promptly abolished the up-regulation of SOD2 production in the cultured cells [Fig. 5Bc and Cc] and corroborated the VEGF-inhibition results depicted in [Fig. 4, column D], which had proven this growth factor to be directly involved in the enhanced survival of NSC/PC-co-cultures under oxidative stress. These data strongly supported the above suggestion of a direct relationship between increased VEGF signaling and up-regulation of SOD2 levels in 3-NP-exposed co-cultures.

Behavioral changes in NSC-grafted and non-grafted mice after 3-NP intoxication

Systemic exposure of mice to 3-NP, following the schedule modified from the work of Fernagut et al. (2002), evoked temporal behavioral changes such as akinesia, hind limb dystonia, kyphosis, and postural impairment. The expected spontaneous behavioral recovery occurred usually within one month. We asked, whether NSCs grafted one week prior to 3-NP intoxication could prevent or reduce the striatal damage and behavioral symptoms, or would accelerate the recovery from the latter. Data from these animals were compared with results obtained from vehicle- or 3T3-cell-grafted controls (together forming the mock-grafted group) and from intact mice [Fig. 6].

In all 3-NP treated mice (both grafted and non-grafted), no significant motor or behavioral signs appeared until the toxin reached a dose of 40 mg/kg b.w. (at the third to fourth day of 3-NP injection). After the first 2-4 injections, the animals became hyperactive and their scores in pole [Fig. 6A] and string [Fig. 6B] tests began to worsen. This was accompanied by progressive akinesia, inactivity, and motor deficits such as reduced exploratory behavior, hind limb clasping, and hind limb and truncal dystonia [Fig. 6C]. At the last day of 3-NP administration, four mock-grafted animals died and another one was
doing very poorly (score 9) and died during the next day. One out of the seventeen NSC-grafted mice was also strongly affected (score 9) and died within two hours after the last 3-NP injection. (This animal was perfused immediately for histotopathology. No donor cells could be found in its brain.)

The behavioral tests demonstrated significant differences between the mock-grafted, 3-NP lesioned controls [dashed lines with filled and empty triangles], the intact controls [solid lines with empty circles], and the NSC-grafted, 3-NP-treated [solid lines with filled circles] animals. The contrast was reflected mostly in the following parameters of motor deficits: peak values (the higher the value, the worse the symptoms), length of the recovery period, and degree of recovery. While animals without NSC implants [dashed lines] were significantly more vulnerable to 3-NP (much higher scores on deficit scale, longer and incomplete recovery), the NSC-grafted mice resisted the intoxication much more effectively in all three parameters.

Changes of striatal cytoarchitecture in NSC-grafted and control mice after systemic 3-NP intoxication

Mock-grafted controls

Three days after 3-NP treatment, characteristic pale “lesion spots” (with diameter range of about 350-450 μm) were seen in hematoxylin- and anti-Calb-stained dorso-lateral striata in mock-grafted control animals (an example of vehicle-grafted mice is shown in [Fig. 7Aa-d]). These weakly stained areas of degeneration were surrounded GFAP positive cells and presented ongoing neural cell loss with many pyknotic (dark shrunken nuclei indicating
early stages of apoptosis; [Fig. 7Bc], Harmon and Allan, 1988) and necrotic (swollen and pale cytoplasm; open arrow and insert in [Fig. 7Bc]) cells and significantly dilated [Figs. 7Ba and 8Aab, black columns], occasionally ruptured blood vessels [hematoma and red blood cells in Fig. 7Bb]. In order to assess the 3-NP-induced damage of striatal neurons in these controls, we quantified the change in NeuN-positive cell numbers and found them to be reduced by about 35% in both hemispheres [Fig. 8Bab, black columns]. The immunodetection of increased levels of HNE – a product of lipid peroxidation accumulating in cell membranes – revealed the presence of oxidative stress in these damaged areas (see Fig. 10Ab).

At three weeks post-3-NP, despite the behavioral amelioration of the mock-grafted control mice [Fig. 6], the bilateral reduction in their striatal neurons still persisted [Fig. 8Bab, white columns]. On the other hand, no pale “lesion spots” remained detectable and only some residual pycnotic cells. Dilation of blood vessels, although still noticeable, was significantly reduced in both hemispheres [Fig. 8Aab, white columns], as was the production of HNE (not shown).

**NSC-grafted animals**

*Monitoring of donor cells*  While in intact striata, NSCs did not migrate and remained close to the injection site during the entire experimental 3-week-period, 3-NP exposure stimulated their migratory activity. Three days after the last 3-NP injection (i.e., 2 weeks and 3 days after grafting; see “3-NP treatment” in M & M), GFP-positive cells were still mainly found unilaterally in the area of their deposit [see black dots in coronal slice drawings in Fig. 7A]. Three weeks post-3-NP, on the other hand, grafted cells had dispersed within the ipsilateral
striatum. Signaling from “tissue in distress” (Aboody et al., 2000; Ourednik et al., 2002) also induced weak contralateral migration of the grafted NSCs as well (see bottom schema in Fig. 7A). However, their delayed arrival and relatively low numbers prevented the NSCs from interfering with contralateral tissue damage, which resembled that of mock-grafted controls. Only rarely were donor cells found in other brain regions. Interestingly, many of these NSCs were situated close to blood vessels.

During the entire experimental time period, about 75% of the implanted NSCs remained undifferentiated and nestin-positive [Fig. 7Cf;g]; the others matured mostly into GFAP-positive astrocytes [Fig. 7Ch;i] and a minority into cell expressing NeuN [Fig. 7Bd] or CNP [Fig. 7Be]. No donor NSC progeny was found to express the endothelial marker CD146.

Response of host tissue When compared to 3-NP-treated controls [Fig. 7Aa–d], in animals grafted with NSCs, no initial (three days after toxin exposure) appearance of cell-depleted “spots” or areas of vascular damage were observed in the ipsilateral hemisphere [Fig. 7Aef and 8Ba, black columns]. Also, as in the intact controls, no elevated HNE positivity was found (see also Fig. 10 below). In contrast, the temporal emergence of “lesion spots” in the contralateral (non-grafted) hemisphere [Fig. 7Agh] appeared similar to that in the animals not receiving NSC-grafts prior to intoxication [Fig. 7Acd]. Correspondingly, the numbers of striatal neurons ipsilateral to the NSC deposit remained at almost intact values [Fig. 8Ba, black columns] while in the non-grafted hemisphere they became reduced similarly to the neurons in mock-grafted controls [Fig. 8Bb, black columns]. In NSC-grafted control mice not treated with 3-NP, neither changes in neuronal cell numbers nor vascular dilation were detected.
Three weeks post-3-NP, due to spontaneous tissue re-organization, no striatal “lesion spots” were found in any of the experimental animals. In contrast to the mock-grafted group, however, in the NSC-grafted animals, NeuN-positive cells were preserved in the grafted hemisphere [Fig. 8Ba, white columns] and only contralaterally, neuronal numbers were similarly decreased as in the controls [Fig. 8Bb, white columns].

Molecular and cellular processes accompanying NSC-mediated neuroprotection in vivo

Production of growth factors To investigate possible up-regulation of growth factors related to the neuroprotective phenomena described above, and based on our in vitro results, we immunostained the striatal sections for the presence of BDNF, CNTF, and VEGF.

At three days post-3-NP treatment, elevated expression of growth factors was observed in the grafted hemispheres and, interestingly, originated from both donor and host cells. Large subpopulations of undifferentiated GFP-positive donor cells expressed CNTF (~80%) and VEGF (~60%) [Fig.9Aa-c, Ag-i and plot Ba] while only a small fraction of their differentiated progeny, namely astrocytes, produced these growth factors as well. On the other hand, many of the host astrocytes (~75%; expressing S100β but not GFP) stained positively for CNTF [Fig. 9Ad-f and plot Bb] and about 60% of the endothelial cells produced VEGF [Fig. 9Ajk and plot Bc]. Besides their GFP-negativity, the host origin of these cells was supported by their mature morphology (post-grafting time was too short for the donor NSCs to fully differentiate and most of the donor NSCs remained undifferentiated, as mentioned above). Only weak staining of growth factors was detected in the brains of intact and mock-grafted, 3-NP-treated animals [Fig. 9, plots Bbc].
By 3 weeks after 3-NP exposure, donor and host expression of CNTF and VEGF in NSC-grafted mice had diminished and was again comparable to that of intact animals (not shown). BDNF remained weakly expressed in all the experimental animals at both time intervals.

*Up-regulation of SOD2 and prevention of oxidative stress* Together with our findings *in vitro*, the above data describing enhanced growth factor production and decreased oxidative stress effects in the NSC-transplanted striata suggested a possible up-regulation of antioxidative enzymes by these factors. We explored this possibility by monitoring the levels of SOD2 in the areas of increased growth factor expression.

In mock-grafted animals, SOD2 production was insignificant at 3 days after 3-NP treatment and the strong HNE-specific staining revealed persistence of oxidative striatal damage [Fig. 10Ab and Bb]. In NSC-grafted, 3-NP-treated animals, on the other hand, we observed a strong expression of SOD2 in graft and host cells, the latter including neurons [Fig. 10 Ac and C]. The resulting reduction in oxidative stress was reflected in down-regulation of the NHE signal, which now was of much lower intensity and comparable to that in intact animals [compare Fig. 10 Ba and Bc] Since cytokines secreted by activated microglia were shown to induce SOD2 (Wong and Goeddel, 1988) as well and could have blurred the role of the donor NSCs in the grafted animals, we also stained sections from all animal groups for the presence of the microglial marker ED1. We found, however, no evidence indicating a significant participation of this cell type in the neuroprotective networks.

The up-regulation of SOD2 disappeared by 3 weeks post-3-NP and regained the intensity found in intact animals.
All the collected data in this study support our hypothesis that cellular networking of NSCs, astrocytes, and – in vivo – endothelial cells is capable of controlling homeostasis in a niche challenged by oxidative stress. This control appears to be based on a network-induced, elevated production of growth factor in the three participating cell types, which ultimately results in the activation of effective anti-oxidant defense mechanisms in neurons.

DISCUSSION

The mode of action of growth factors in the intricate dialogue between stem cells and their microenvironment is complex and does involve many parallel signaling pathways. Depending on the environmental context, one or the other of them may become the dominant one and determine the final cell behavior. How this is controlled and which environmental input selects which pathway is, however, still poorly understood.

The present study provides evidence that, under oxidative stress, growth factors produced by NSCs can directly regulate the activation of anti-oxidant defense mechanisms. In a newly established network of growth-factor-producing NSCs, astrocytes, and even non-neural cells such as the endothelial cells, this collaborative effort to re-establish homeostasis ultimately leads to up-regulation of antioxidants like the superoxide detoxifier SOD2, and provides a molecular basis for cellular protection and rescue.

Recently, it has been demonstrated that exogenous stem cells possess an inherent ability to create host micro-environments that favor recovery or preservation of damaged or imperiled cells (Ourednik et al., 2002; Park et al., 2002; Teng et al., 2002; Lu et al., 2003; Ryu et al., 2004; Yan et al., 2004; Pluchino et al., 2005). It soon became evident that this return to homeostasis is many times accompanied by the intrinsic production and secretion of
a variety of donor NSC-derived growth factors, including GDNF, NT-3, BDNF, and NGF (Ourednik et al., 2002; Teng et al., 2002; Lu et al., 2003; Maurer et al., 2003; Imitola et al., 2004; Ryu et al., 2004; Brusselmans et al., 2005). As demonstrated in this study, the baseline expression of growth factors in NSCs is already substantially higher than in mature neural cell types and also increases more vigorously upon arrival of oxidative stress. This “stemness feature” would appear crucial for the usefulness of NSCs in neurotransplantation since they need to resist the hostile environment of the diseased nervous system in order to exert their beneficial influence. The latter, as documented by the here adduced in vitro and in vivo data, becomes particularly effective when exogenous NSCs are present already before an insult and can prevent cell damage upon its arrival. Thus, in the grafted, 3-NP-treated mice, ipsilateral to the site of a NSC deposit, host striatal neurons were protected from oxidative stress and their numbers did not decline.

We observed that NSCs, in contrast to PCs, produced significant amounts of BDNF, CNTF, and VEGF and could induce astrocytic production of CNTF in co-cultures with PNGs (mixed primary neurons and glial cells) and PGs (primary astrocytes) exposed to 3-NP. This resulted in higher resistance of both PNGs and PGs to the toxin when compared to PNs. The fact that NSC/PG co-cultures appeared to be slightly less protected than cultures of NSCs with PNGs (even though the differences proved statistically insignificant) may have reflected the greater struggle of the NSCs themselves in presence of the elevated 3-NP concentration required in the co-cultures with PGs. Similarly, in vivo, an intimate interaction between grafted NSCs and host astrocytes was associated, after a subsequent exposure of the recipients to oxidative stress, with up-regulation of astrocytic CNTF and with sparing of neurons and neural tissue integrity. As described recently, astrocytes possess a variety of
homeostasis-regulating properties and represent important and influential components in the
stem cell niche (Svendsen, 2002; Doetsch, 2003; Horner and Palmer, 2003). Here, we may
assign to them a new capability, namely, responsiveness to NSC signaling in presence of
injury, reflected in their enhanced secretion of neuroprotective factors.

Intriguingly, astrocytes were not the only host cell type found to interact with grafted
NSCs in the presence of 3-NP. We observed that, under oxidative stress, an interaction
between grafted NSCs and endothelial cells can stimulate the latter to increase their VEGF
synthesis. Several recent reports suggest a role of this factor in increased neurogenesis
accompanying reciprocal communication between endothelial cells and the
microenvironment of the neural stem cell niche during development (Palmer et al., 2000; Cao
et al., 2004; Schanzer et al., 2004; Shen et al., 2004; Wurmser et al., 2004). It also appears
that, e.g., in adult brain plasticity, endothelial cells do not only participate in angiogenesis but
also have a neurotrophic and neurogenic impact on neural cells, including stem cells (Jin et
al., 2002; Cao et al., 2004; Rosenstein and Krum, 2004; Schanzer et al., 2004; Storkebaum et
al., 2004; Wurmser et al., 2004). Since regeneration in the CNS often induces cellular and
molecular processes that, to a certain degree, “revisit” events guiding neural development
and plasticity, the NSC-mediated endothelial up-regulation of VEGF observed in this study
could have been such a “review” of neuro-morphological events, now contributing to the
neuroprotective/regenerative processes.

An important quality of VEGF and CNTF is their ability to promote cellular
resistance against oxidative stress. CNTF can stimulate the JAK/STAT and MAPK pathways
(Bonni et al., 1997; Kuroda et al., 2001; Ozog et al., 2004) and VEGF can up-regulate SOD2
(Abid et al., 2001; Abid et al., 2004), both of which may in part, be responsible for their
antioxidant functions. Since NSCs, neurons, and glial cells have all been shown to express CNTF and VEGF receptors and the latter receptor type is also found on endothelial cells (Sondell et al., 2000; Jin et al., 2002; Emsley and Hagg, 2003; Maurer et al., 2003; Rosenstein et al., 2003; Alberch et al., 2004; Storkebaum et al., 2004), these factors were likely to contribute to the observed NSC-mediated prevention of 3-NP-induced striatal damage.

Since the improved NSC-mediated resistance of mature neural cells to oxidative stress was associated, both in vitro and in vivo, with enhanced cellular production and secretion of growth factors, it suggested their direct control of the cellular antioxidant response. It appears that VEGF can be regulated by cellular redox processes and oxidative stress (Maulik, 2002; Schafer et al., 2003; Eyries et al., 2004) and can control the production of SOD2 in non-neural (endothelial and pulmonary) cells (Abid et al., 2001; Abid et al., 2004; BelAiba et al., 2004). Thus, growth-factor-mediated changes in SOD2 expression might be, at least in part, directly responsible for the increased cellular resistance of NSCs and other neural cells to 3-NP-induced oxidative stress described in this report.

Indeed, we found an involvement of SOD2 in the cellular response to 3-NP-exposure in cultured NSCs and in their co-cultures with PNGs and PNs, as well as in the vicinity of grafted cells in 3-NP-treated mouse striata. Here, not only did the grafted NSCs strongly express SOD2, but their presence was associated with an induction of the antioxidant in the resident cells, including neurons, and in efficient maintenance of local tissue homeostasis apparent by the reduced striatal HNE staining. Corroborating this finding, a critical involvement of superoxide in 3-NP-induced excitotoxic damage in the brain and its increased vulnerability in SOD2-knockout mice has recently been suggested (Andreassen et al., 2001;
Kim and Chan, 2002). Since we did not detect any significant presence of a local inflammatory response recruiting activated microglia, it is unlikely that SOD2 might have been up-regulated by cytokines.

We could test our hypothesis of growth-factor-mediated control of stress-related redox processes by blocking of the action of VEGF with neutralizing antibodies in vitro. Intriguingly, the resulting elimination of cell survival capability (a similar effect was also observed after blocking the action of BDNF and CNTF) was accompanied by a marked loss of SOD2 expression and demonstrated a direct relationship between VEGF signaling and up-regulation of this superoxide detoxifier. Together with CNTF, the VEGF produced by NSCs and endothelial cells in vivo was therefore likely to have contributed efficiently to the protection of host striatal neurons against oxidative stress and their subsequent survival.

In conclusion, this study provides evidence that direct activation and control of antioxidant defense mechanisms through growth factor signaling appears to accompany NSC-mediated neuroprotection. NSCs can thereby act as cellular “probes” and initiate an appropriate network of growth factor-producing cells, including themselves and resident elements like astrocytes and even the non-neural endothelia. A deeper understanding of this novel “recruiting” characteristic of NSCs might add another important element to the kaleidoscope of their beneficial features, making these cells a potentially powerful tool for preventive treatment of neuropathologies.

ACKNOWLEDGEMENTS: We thank Nada Pavlović and Heidi Gabel for technical assistance. The work was supported by ISU grants to VO and JO.
REFERENCES


unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci USA 99:3024-3029.


Figure 1

Co-culture with NSCs increases the resistance of PCs to oxidative stress  Cell cultures were exposed to 3-NP and the effects on mitochondrial viability, levels of ROS, and apoptotic cell death measured. Treated were either NSCs alone [A-C], PCs alone [D-F], or their co-cultures in absence [G-I] or presence [J-L] of a separating, porous insert. Three types of PCs were evaluated: primary neurons and glial cells [PNGs; bold lines in D-L], primary glial cells [astrocytes, PGs; dashed lines in D-L] and primary neurons [PNs; thin lines in D-L]. For reasons of different sensitivity to the toxin (see M&M), each type of PCs was exposed to its specific concentration of 3-NP and NSCs grown alone to all three concentration [A-C; see M & M]. Measurement of all parameters started 24 hrs after 3-NP removal. While MTT values and changes in ROS levels are expressed in % of untreated controls, apoptosis is expressed as percentage of apoptotic cells in culture. NSCs alone [A-C] were significantly less sensitive to the toxin than PCs alone [D-F]. The vulnerability of the PCs decreased, however, substantially when they were co-cultured with NSCs prior to 3-NP intoxication [G-L; p<0.05]. This was reflected in increased initial resistance of the cells to 3-NP and in their induced recovery, in which PNGs and PGs regained almost control values after 72 hours. Separating NSCs form PCs by inserts had no significant effects.
Single-cell type cultures

NSCs + 3-NP

PCs + 3-NP

NSCs/PCs + 3-NP

cell contact

no cell contact

Mitochondrial activity (MTT [%])

Free radicals (CM-H2DCFDA [%])

Apoptosis (Hoechst [%])

24 48 72 hrs

24 48 72 hrs

24 48 72 hrs

0 20 40 60 80 100 120

0 200 400 600 800 1000 1200 1400

0 10 20 30 40 50

0.1 mM

0.05 mM

0.25 mM

PNGs

PNGs

PNGs
Figure 2

3-NP-induced changes in cell numbers and glial/neuronal ratios (G/N indices) of PNGs are modulated by co-culture with NSCs. Cell numbers [upper graphs] and G/N indices [bottom graphs] in untreated [A] and 3-NP treated [B] PNGs alone were compared with those of PNGs co-cultured with NSCs in absence [C] or presence [D] of a separating porous insert. Cells were monitored during the first three 24-hour-intervals post-intoxication and their numbers normalized to a hypothetical standard culture of 100 cells. NSCs prevented 3-NP-induced cell death in PNGs as judged from the difference in cell numbers between co-cultures [C and D] and the 3-NP-treated PNG controls [B]. The latter died rapidly, with their G/N index rising explosively at 48 hrs, which suggested that 3-NP affected primary neurons more severely than glia and that the presence of NSCs provided their protection. At 72 hours, virtually all cells in the PNG-only controls were dead and the remainder made measurement of a meaningful G/N index impossible [hence n/a].
Figure 3

Expression of growth factors by NSCs and PCs cultivated alone or in co-cultures

Single-type cell cultures and co-cultures were grown with or without exposure to 3-NP and the percentage of cells expressing BDNF, CNTF, and VEGF quantified immunocytochemically two days after 3-NP treatment (or after two days in vitro for the controls). The content of growth factors in the culture media was quantified in parallel in an ELISA assay. [A] microphotographs with examples of the expression of all three growth factors in NSCs [a-c] and of CNTF in primary astrocytes in PNGs/NSCs co-cultures responding to 3-NP-intoxication [d-f]. When cultured alone, significantly more NSCs expressed CNTF and VEGF after 3-NP treatment than in untreated controls [Bab]. In contrast, PCs alone produced minimal amounts of growth factors, irrespective of toxin exposure [Bc-f]. When NSCs co-cultured with either PNGs or PNs were treated with 3-NP, their numbers now expressing all three factors increased substantially [Cad], while only one of the factors, CNTF, was up-regulated in PCs and this only in primary astrocytes [Ad-f and Cb]. Increasing numbers of factor-expressing cells were always reflected in higher concentrations of the secreted proteins in the culture media [Bb and Cef]. *p<0.05, **p<0.01, ***p<0.001 when compared to untreated controls. Bars: 20 μm [Aa-c], 40 μm [Ad-f].
**A**

Neural stem cells

PNG astrocytes

**B**

<table>
<thead>
<tr>
<th>Cell numbers</th>
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<tr>
<td>PNs</td>
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**C**

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<th>Cell numbers</th>
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<tr>
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<tr>
<td>PNs</td>
<td><img src="image" alt="Graph" /></td>
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* 3-NP absent  □  3-NP present
Figure 5

Growth factor-dependent up-regulation of SOD2 in NSC/PC co-cultures under oxidative stress Changes in SOD2 expression and its regulation by secreted growth factors 48 hours post-3-NP were monitored by immunocytochemistry [Ba and Ca] and in Western blots [A, Bbc, and Cbc]. In the first case, an SOD2-specific antibody revealed an up-regulation of this enzyme in both PNGs [Ba; red combined with blue nuclear DAPI stain] and PNs [Ca; red, merged image with double-stain against NeuN - green]. Western blots revealed that NSCs cultured alone up-regulated SOD2 in response to 3-NP [A]. Similar activation of the enzyme was revealed in primary cultures [Bb and Cb] but only when NSCs were present before 3-NP treatment while untreated co-cultures and 3-NP-treated PCs cultivated alone always showed significantly lower SOD2 expression. The up-regulation of SOD2 was effectively inhibited when VEGF activity was blocked with neutralizing antibodies [Be and Ce]. Equal protein loading in Western blots was verified by detection of α-tubulin. Bars: 10 μm.
Figure 4

Inhibition of growth factors and its effects on NSC-mediated neuroprotection. The activity of BDNF, CNTF, and VEGF was blocked specifically with neutralizing antibodies in NSC/PNG [bold line] and NSC/PN [thin line] co-cultures exposed to 3-NP [columns B-E]. Mitochondrial activity [row a], ROS content [row b], and numbers of apoptotic cells [row c] in these co-cultures were then compared to those measured in non-inhibited co-cultures [column A] and PCs cultivated without NSCs [column F]. Blockade of VEGF and CNTF activity led to a significant (p<0.01) decrease in the NSC-mediated protection while inhibition of BDNF did not have any important effect. When all three factors were inhibited, the NSC-mediated protective effect was virtually abolished [column E] and cell behavior comparable to that measured in 3-NP-treated non-inhibited PCs cultured alone [column F].
A. NSCs
- SOD2-24 kDa
- α-tubulin

B. PNGs
- NSCs
- no 3-NP
- no PCs
- 3-NP
- no PCs

C. PNs
- NSCs
- no 3-NP
- 3-NP
- 3-NP

No VEGF inhibition
- NSCs
- no NSCs
- NSCs
- no 3-NP
- 3-NP
- 3-NP

VEGF inhibition
- NSCs
- no NSCs
- NSCs
- no 3-NP
- 3-NP
- 3-NP

SOD2-24 kDa
- α-tubulin

SOD2-24 kDa
- α-tubulin
Figure 6

Proactive effects of NSC grafts on behavioral changes in 3-NP-treated mice

Prior to grafting, animals were trained in the climbing pole [A] and suspended string [B] tests and monitored in a set of five motor behaviors (C; see M & M and Femagut et al., 2002). After 2 weeks of trainings, four groups of animals were prepared: NSC-grafted (solid lines with filled circles), vehicle-grafted (dashed lines with filled triangles), 3T3-grafted (dashed lines with open triangles), and intact NSC-grafted (without subsequent 3-NP treatment; solid lines with open circles). During and after 3-NP treatment of the mice, motor tests were resumed and performed daily until sacrifice of the mice three weeks after the last 3-NP injection. The higher the score, the more pronounced were the animal’s deficits. While the motor activity of the intact animals remained unchanged, vehicle- and 3T3-grafted mice deteriorated progressively until days 7-8 when they slowly began to recover. In contrast, the motor deficits of NSC-transplanted animals were substantially weaker, which was reflected in lower peak values, shorter recovery period, and higher degree of remission. ** p<0.01 in comparisons of vehicle-injected and NSC-grafted animals.
Figure A: Pole test number of falls over 25 days with 3-NP treated groups compared to controls.

- NSCs + 3-NP
- Vehicle + 3-NP
- 3T3 cells + 3-NP
- NSCs, no 3-NP

Figure B: String test number of falls over 25 days with 3-NP treated groups compared to controls.

Figure C: Total score on motor tests over 25 days with 3-NP treated groups compared to controls.
Figure 7

Striatal cytoarchitecture in grafted and non-grafted mice three days and three weeks post-3-NP intoxication

Three days and three weeks after the last toxin injection, the animals were sacrificed and brain sections stained with hematoxylin or reacted with antibodies against cell type-specific antigens (calbindin, nestin, NeuN, CNP, and GFAP). Structural changes in left and right dorsolateral striatum [red circles in the drawings] were compared between animal groups. At three days post-3-NP, the striatal tissue of the vehicle-grafted mice displayed the characteristic, weakly stained spots in both hemispheres [arrows in Aa-d], enlarged blood vessels [arrows in Ba], hemorrhage [arrow in Bb, red blood cell in insert], pycnotic cells [filled arrow in Bc], and necrotic cells [open arrow and insert in Bc]. In contrast, these defects were not detected in the striata protected by NSCs grafted prior to 3-NP exposure of the host [Aef]. Contralaterally, on the other hand, weakly stained spots remained detectable [arrows in Agh]. At three weeks post-3-NP, even though the striatal tissue spontaneously reorganized [Ai-1], a neuronal deficit remained in the non-grafted hemisphere (see Fig. 8). While the majority of the grafted NSCs (GFP-positive) remained undifferentiated and nestin-positive [arrowhead in Cfg; note a rare mature, GFP-positive but nestin-negative cell marked by an arrow in Cg and absent in Cf], the remainder matured mostly into GFAP-positive astrocytes [arrowheads in Chi] and only a minority expressed NeuN [Bd] or CNP [Be]. No endothelial cells were observed to differentiate from donor NSCs. Bars: 300 μm [A], 50 μm [Ba], 20 μm [Bbc], 10 μm [Bde], 10 μm [Cf-i].
Figure 8

Assessment of striatal blood vessel diameters and numbers of NeuN-positive cells
Data were collected comparing left and right striata in the four experimental animal groups (3T3-grafted mice represent both mock-grafted groups) and related to numbers of intact controls (100%). Evaluations were performed within the dorsolateral striatum [circles in drawing] at coronal level 2 as defined by Fernagut et al., 2002. In animals sacrificed at three days after the last 3-NP injection [black columns], only the NSC-grafted hemispheres were protected from serious vascular dilation and decrease of neuronal numbers [AB]. The contralateral side and control-grafted hemispheres, on the other hand, always showed significant (p<0.01) structural changes and loss of neurons. Similar histology was found also in mice sacrificed three weeks post-3-NP (white columns). p<0.01 or p<0.05 when compared to intact [** and *, respectively] or vehicle-injected animals [# and #, respectively].
A Grafted hemisphere  Non-grafted hemisphere

**Blood vessel diameters [%]**

- NSCs
- Vehicle
- 3T3 cells
- NSCs
  - no 3-NP
  - 3-NP

**Number of NeuN+ cells [%]**

- NSCs
- Vehicle
- 3T3 cells
- NSCs
  - no 3-NP
  - 3-NP

- 3 days after 3-NP
- 3 weeks after 3-NP
Expression of growth factors in NSC-grafted striatum. Three days after the last 3-NP injection, the majority of GFP-labeled (donor) cells expressed CNTF [Aa-c, Ba] and VEGF [Ag-i, Ba] as visualized by double filters merging green (GFP-positive) and red (CNTF- or VEGF-positive) fluorescence signals to become yellow-orange in the merged image [Ac]. The mutual interaction between graft and recipient is reflected in simultaneous up-regulation of CNTF and VEGF in host cells [red cells in Acijk and Bbc], which was absent in intact and vehicle grafted controls [Bbc]. Intensive immunostaining for the factors was detected in host astrocytes [S100/CNTF-positive but GFP-negative; Ad-f, violet staining results from merging red and blue colors] and host endothelial cells [VEGF/CD146-positive, GFP-negative; Ajk]. ***p<0.001, when compared to intact animals. Bars: 50 μm [Aa-c] [Ag-i], 80 μm [Ad-f], 10 μm [Aj], 5 μm [Ak].
A

CNTF/GFP

a b c

CNTF/S100β/GFP
d e f

VEGF/GFP
g h i

VEGF/CD146
j k

B

<table>
<thead>
<tr>
<th>Donor (GFP⁺)</th>
<th>Host (GFP⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>CNTF</td>
</tr>
<tr>
<td>intact</td>
<td>Vehicle</td>
</tr>
<tr>
<td>+3-NP</td>
<td>+3-NP</td>
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</table>
Expression of SOD2 in NSC-grafted striatum  In comparison to intact [Aa] and vehicle-grafted-3-NP-treated controls [Ab], three days after grafting, an NSC-mediated up-regulation of the anti-oxidant enzyme SOD2 was observed in both host [red in Ac] and double-stained donor cells [yellow in Ac]. About 60% of NeuN-positive host cells within the grafted area also expressed SOD2 [red in C]. While striata in intact animals remain negative for HNE (marker for lipid oxidation during oxidative stress; Ba), 3-NP exposure of vehicle-grafted animals led to elevation of oxidative stress and HNE levels [Bb]. In contrast, in animals receiving NSC grafts prior to the intoxication, the up-regulation of SOD2 appeared to be associated with lower but still noticeable levels of HNE [Bc]. Bars: 80 μm [Aa-c], 40 μm [Ba-c], 10 μm [C].
<table>
<thead>
<tr>
<th>Scale used for the scoring of motor symptoms</th>
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</thead>
<tbody>
<tr>
<td><strong>General locomotor activity:</strong></td>
</tr>
<tr>
<td>0 - normal locomotion, rearing, and grooming</td>
</tr>
<tr>
<td>1 - decreased locomotion, rearing, and grooming</td>
</tr>
<tr>
<td>2 - no locomotion; mouse is sedentary and exhibits almost no rearing or grooming behaviour</td>
</tr>
<tr>
<td><strong>Hindlimb dystonia:</strong></td>
</tr>
<tr>
<td>0 - no hind limb dystonia</td>
</tr>
<tr>
<td>1 - transient or permanent increase in hindlimb spread, crouched posture with no changes in gait</td>
</tr>
<tr>
<td>2 - wide spread of hindlimbs, poor hindlimb movements and coordination, pronounced crouching posture and impaired gait</td>
</tr>
<tr>
<td><strong>Truncal dystonia:</strong></td>
</tr>
<tr>
<td>0 - no truncal dystonia</td>
</tr>
<tr>
<td>1 - visible kyphosis and flexed posture</td>
</tr>
<tr>
<td>2 - severe kyphosis and flexed posture</td>
</tr>
<tr>
<td><strong>Hind limb clasping (tested by suspending the mouse in mid-air)</strong></td>
</tr>
<tr>
<td>0 - hindlimbs are fully extended, spread out, and moving about; mouse attempts to straighten up</td>
</tr>
<tr>
<td>1 - intermittent clasping of hindlimbs; hindlimbs are not fully spread out and the mouse shows weak attempts to straighten up</td>
</tr>
<tr>
<td>2 - hindlimbs are fully drawn up to the abdomen and there is virtually no attempt by the mouse to straighten up</td>
</tr>
<tr>
<td><strong>Postural challenge:</strong></td>
</tr>
<tr>
<td>0 - Normally, the mouse cannot be tipped on its side</td>
</tr>
<tr>
<td>1 - the mouse can be made to fall on its side but also can slowly get back on its feet</td>
</tr>
<tr>
<td>2 - the mouse can be tipped on its side and cannot get back on its feet by itself</td>
</tr>
</tbody>
</table>

Table 1
CHAPTER IV. GENERAL CONCLUSIONS

We have investigated and characterized NSC interactions with its microenvironment leading to neuroprotection from oxidative stress, in vitro and in vivo. The two investigations in this dissertation (chapters II and III) present novel data describing the mechanisms underlying the neuroprotective potential of NSCs and provide important information for the field of stem cell biology.

The major findings of our research have already been deliberated upon in the discussion sections at the end of each chapter. The overall conclusions derived from our studies, and some suggestions for future research will be presented here.

NSCs and resistance to oxidative stress

In this dissertation, we presented the first report (Chapter II), which analyzed sensitivity and reaction of NSCs to oxidative stress in comparison with post-mitotic neural cells, and the extended implications in terms of CNS regeneration and repair.

Recent reports from the group of Noble M indicate that in stem/precursor cell populations, intracellular redox state is an intrinsic property which regulates self-renewal/differentiation and responsiveness to environmental factors (Noble et al., 2003). Additionally, a couple of other groups have shown, by expression profiling of stem cells, that there is a subset of genes, enriched in hematopoietic, neural, and embryonic stem cell lines, which provide resistance to stress, including oxidative stress (Ivanova et al., 2002; Ramalho-Santos et al., 2002). These enriched genes control the core stem cell properties ("stemness") that underlie self-renewal and differentiation.
The outcome of our studies, considered in conjunction with these works, permits some general conclusions to be drawn:

The first is that NSCs, by default, appear to be better equipped to control intracellular ROS than post-mitotic neural cells, and hence may be inherently more capable of surviving in oxidatively stressed environments. This conclusion was substantiated in vitro by the measurement of significantly higher mitochondrial activity, lower ROS content, reduced tendency to undergo apoptosis, and presence of comparatively higher basal levels of important free radical detoxifiers such as UCP2 and GPx in NSCs. In vivo, we discovered that endogenous NSCs localized in the SVZ niche are also characterized by higher steady-state levels of UCP2 and GPx when compared to neighbouring neural cells in the brain parenchyma. This observation is in accord with our in vitro results, and shows another attractive property of neural stem cells, namely their natural ability to combat oxidative stress.

The second conclusion is that NSCs react more quickly and in a stronger fashion to oxidative stress, than post-mitotic neural cells, by up-regulating their proliferative activity and importantly, also critical anti-oxidant molecules. This was exemplified in our study by the greater NSC ability to up-regulate GPx, UCP2 and SOD2, in response to 3-NP. In further support of the key role played by these anti-oxidant molecules in the observed process, their inhibition reduced NSC resistance to 3-NP significantly and made the NSCs behave more "like" the post-mitotic neural cells. One may therefore speculate that an essential feature of NSCs which helps them survive in a hostile environment is their high expression of ROS metabolizing enzymes and ability to up-regulate them.
Finally, and interestingly, even though there were some variations in the “preparedness” and response of the immortalized and primary cells to oxidative stress, the cell behavior differences between NSCs and post-mitotic neural cells within these groups held true in the case of both these cell types, and also other NSC lines utilized (unpublished observations). Therefore, the third conclusion that arises from our studies is that “resistance to oxidative stress” is a very basic and fundamental characteristic of all neural stem cells.

In summary, the high expression of protective molecules and the resulting greater resistance of NSCs toward oxidative stress, compared with related neural cell types, may be part of their “stemness” feature. This attribute may help NSCs survive and be therapeutic under conditions of free radical-mediated injury. Further analyses of such stem cell features will significantly impact current concepts in CNS development, regeneration and repair.

**Soluble factors and NSC-mediated neuroprotection**

In the study presented in chapter III, we analyzed graft-induced host plasticity, specifically with regard to NSC-mediated neuroprotection from oxidative stress, and determined some of the contributing cellular and molecular mechanisms.

*In vitro*, we observed that the presence of NSCs before the 3-NP-induced oxidative stress resulted in a significant decrease in free radical production, and improved cell survival. This data indicates that NSC-mediated neuroprotection is plausible. Further, *in vivo* we observed that the NSC transplanted animals showed only a mild motor disorder on 3-NP intoxication when compared to the non-transplanted controls. Also, on histopathological examination, grafted mice showed increased neuronal cell counts, decreased oxidative stress, and striatal damage in the transplanted hemisphere. The NSCs when analyzed revealed that
they had remained mostly undifferentiated (Ourednik et al., 2001; Ourednik et al., 2002) and the surviving neurons were primarily of host origin. Also, in conjunction with our previous study presented in chapter II, the NSCs themselves had resisted well, *in vitro* and *in vivo*, the 3-NP induced oxidative stress and survived. Thus, this study for the first time demonstrates, both *in vitro* and *in vivo*, that preventive transplantation of NSCs, which are on their own quite resistant to ROS toxicity, may protect host elements from future oxidative stress insults.

Recent evidence suggests that undifferentiated donor NSCs can spontaneously express trophic factors (Lu et al., 2003; Toda et al., 2003; Imitola et al., 2004) and also exhibit neuroprotective potential due to their capacity to express these factors under stress (Ourednik et al., 2002; Park et al., 2002; Teng et al., 2002; Ryu et al., 2004). Our studies lend further support to these findings. *In vitro*, we found that a large percentage of NSCs secreted CNTF, VEGF and BDNF after co-culturing and treatment with 3-NP thereby providing a molecular basis for the observed neuroprotection. Similarly *in vivo*, we determined that a significant proportion of the NSC population expressed VEGF (demonstrated *in vivo* for the first time) and CNTF in NSC-transplanted animals which showed minimal 3-NP induced motor and pathological changes as compared to the vehicle-injected controls. Functional inhibition of these factors in culture resulted in a significant reduction of the NSC-mediated neuroprotection, supporting the crucial role of these factors in the observed phenomenon. Thus, a local elevation in the levels of these factors in areas prone to degeneration probably contributed to the NSC-mediated cell survival *in vitro* and *in vivo* by stimulating pathways counteractive to oxidative stress and cell death (Koh, 2002; Abid et al., 2004; Eyries et al., 2004; Kaur et al., 2005). These findings in particular point out that utilizing NSCs to achieve targeted delivery of such neuroprotective agents to areas at
risk to degeneration can help prevent neuronal dysfunction and atrophy. Also, these findings highlight the possibility that factors such as VEGF, traditionally not connected to the nervous system, may play an important role in NSC-mediated neuroprotection.

Interestingly, up-regulation of the aforementioned soluble factors after 3-NP intoxication occurred only when the NSCs were already present. In other words, NSC transplantation directly induced high expression of these growth factors. Introducing the NSCs may have helped modulate the rigidity of the mature CNS and led to neuroprotection by creating an environment more similar to that during embryonic development where regenerative and plastic occurrences are more common. Thus, one may hypothesize that the CNS might possess a set of inborn programs (such as those related to neurotrophic factor expression and neurogenesis) which not only ensure normal development but also help maintain homeostasis in pathological situations. NSC transplantation may help the re-emergence of such dormant mechanisms from within the host CNS. In consequence, future exploitation of stem cell biology, including enhanced release of therapeutic factors through genetic stem cell engineering or otherwise, might constitute promising approaches to treating diseases of the nervous system.

NSC-host interactions leading to neuroprotection

It is now known that stem cell interactions with localized space and time cues regulate their behavior, fate and plasticity in the nervous system. This socializing between NSCs and their neighbouring cells leads to dynamic cellular and molecular changes in both these entities. Although there exists a hypothesis (Doetsch, 2003; Alvarez-Buylla and Lim, 2004; Wurmser et al., 2004) with regard to events in NSC signaling with its
microenvironment, and the cell-types involved, none have addressed this issue in detail or shown any specifics of the interactions. The study presented in chapter III is the first to address and characterize some of the graft-host interactions in the context of NSC-mediated neuroprotection.

We found multiple evidences for the involvement of the cellular microenvironment, in the observed NSC-mediated protection. Firstly in vitro, our co-culture studies pointed out that the NSC-primary astrocyte communication increases the efficiency and success of the protection of primary neurons from oxidative stress-induced cell death. Specifically, the coordinated effort of both NSCs and primary astrocytes resulted in a significant increase in the production of the soluble neuroprotective factors VEGF, CNTF and BDNF (when compared to the condition when primary astrocytes were absent in the co-culture), and led to the activation of appropriate defenses against oxidative stress-induced cell death. These, astrocytes are known to be important regulators of the stem cell niche and have shown to promote the proliferative and neurogenic abilities of NSCs (Song et al., 2002; Doetsch, 2003). They are also known to be vast repositories of many different trophic and neuroprotective factors (Ridet et al., 1997; Petrova et al., 2004). Our results are in accord with these reports and we further explain their connection with respect to NSC-mediated neuroprotection.

Secondly, supporting our in vitro observations, NSC interactions with its microenvironment also played a crucial role in neuroprotection in vivo. Once more we found evidence suggesting an important role for the regional astrocytes and additionally, endothelial cells in the observed NSC-mediated neuroprotective process. Many host astrocytes were observed to be immunoreactive for the neuroprotective factor CNTF, in
NSC-transplanted animals in our studies. Also, a large proportion of the NSCs themselves were also found to be expressing CNTF. This elevation of CNTF expression in the NSCs and host astrocytes may have promoted the maintenance of both these cell populations (Ip and Yancopoulos, 1996; Emsley and Hagg, 2003). In turn, and more importantly, the increased levels of CNTF may have had direct and indirect (via astrocytes) neuroprotective effects on the host striatum and prevented it from degenerating. With regard to endothelial cell-NSC signaling, many NSCs as well as host endothelial cells in the transplanted animals were found to express vasculoprotective and neuroprotective factor VEGF (Storkebaum et al., 2004). Since these findings could not be made in the mock-transplanted controls, they were a direct response to the presence of NSCs and pointed to a cross-talk between NSCs and endothelial cells. Such NSC-endothelial cell interaction may have stimulated both these cell types to reciprocally secrete VEGF, which in turn, and in addition to its vasculoprotective effects, could have had a neuroprotective effect on the surrounding host neurons, and also neurotrophic and neurogenic effects on the NSCs themselves (Shen et al., 2004). In summary, NSC communication with host astrocytes and endothelial cells led to an amplification of VEGF and CNTF and later neuroprotection. These data indicate that NSCs can "interrogate" the host microenvironment and elicit plastic responses from within it in a pathological situation. Also, growth factors can be a critical medium through which NSCs and the host cells "talk" to each other.

Another question addressed in this study is how increased growth factor expression leads to neuroprotection. We observed up-regulation of the antioxidant enzyme, manganese superoxide dismutase (MnSOD/SOD2) and reduction of oxidative stress markers in the host neurons in the NSC-transplanted animals in vivo. Similarly, in vitro, the presence of NSCs
stimulated an increase in the expression levels of SOD2 after the 3-NP insult. As mentioned before, VEGF expression was observed during the neuroprotective process. A cause of the SOD2 up-regulation could have been VEGF as it has been previously shown to have this capacity (Abid et al., 2001; Abid et al., 2004). In further support of the causal role of VEGF in OD2 up-regulation, we observed that VEGF blockade \textit{in vitro} led to the inhibition of SOD2 and NSC neuroprotection. Therefore a combined graft-host effort, resulting in VEGF mediated up-regulation of SOD2 in concert with neuroprotective effects of CNTF, may have contributed towards the prevention of oxidative stress and host neuronal degeneration. These results point toward the direct involvement of growth factors in the observed antioxidant up-regulation and provide an example of how they can be neuroprotective against oxidative stress. The schematic in figure 1 summarizes a possible sequence of graft-host signaling events which lead to neuroprotection in our model of 3-NP induced oxidative stress and neurodegeneration. Of course, the specifics of such NSC-host signaling may change depending on the particulars of the pathological insult, age of host, nature of NSCs among other details.

In conclusion, this dissertation, for the first time, elaborates on the pivotal role of graft-host interactions in NSC-mediated therapy and sheds light on some of the molecular events which may be involved in these interactions which culminate in neuroprotection against oxidative stress. Specifically, this thesis illustrates how NSCs can network with neural and non-neural cells of the host CNS, such as astrocytes and endothelial cells, to prevent an impending neurodegenerative event.
Neural stem cells (equipped with robust antioxidant capacity) → Transplantation → Initiation of cellular interactions in the NSC niche → 3-NP intoxication (oxidative stress) → Astrocytes ↔ NSCs ↔ Endothelial cells → CNTF and VEGF production → SOD2 up-regulation and stimulation of other anti-oxidative stress pathways → Reduction of oxidative stress → Neuroprotection

Figure 1: Possible sequence of events underlying NSC-mediated neuroprotection from oxidative stress
Future directions

A number of further experiments can be suggested based upon questions raised by our results. In terms of the observed NSC behavior under oxidative stress, it would be important to understand the signaling pathways which are activated to allow the NSCs to be more resistant to oxidative stress. Also, the susceptibility of endogenous NSCs to oxidative stress should be further examined and ascertained in vivo. This thesis already examines this question in vitro.

Since NSCs showed the promise of neuroprotection in our in vitro and in vivo models in a certain time scale, they should be transplanted at other time points before and after the pathological insult, in other models of neurodegeneration and in various age-groups, in order to further explore the interplay between NSCs and their extracellular milieu. Also, NSC transplantation should be carried out and the resulting graft-hosts interactions analyzed in more complex species such as primates to test further their potential. In terms of the role of soluble factors such as VEGF and CNTF, which were found to play a crucial role in the observed neuroprotection in our studies, one could understand better their role in neuroprotection by utilizing NSCs which over-express these factors or through in vivo gene transfer techniques. Signaling pathways by which these factors can prove to be neuroprotective are still not clear, and would also need to be elucidated. On another note, the role of injury induced cytokines and microglial cells, ECM and cell adhesion molecules, which may also play an important role in the dialogue between the NSCs and the host environment, should be studied. Insight into their contribution and role in the repertoire of events which unfold after NSC-transplantation will significantly impact present views on NSC therapy.
References


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