Neural Progenitor Cell Transplantation and Proneurogenic Compound Administration Improve Outcomes After Trauma: Neuroprotection and Neurogenesis for the Treatment of Traumatic Brain Injury

Meghan O. Blaya

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NEURAL PROGENITOR CELL TRANSPLANTATION AND PRONEUROGENIC COMPOUND ADMINISTRATION IMPROVE OUTCOMES AFTER TRAUMA: NEUROPROTECTION AND NEUROGENESIS FOR THE TREATMENT OF TRAUMATIC BRAIN INJURY

By

Meghan O’Connell Blaya

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

May 2014
NEURAL PROGENITOR CELL TRANSPLANTATION AND PRONEUROGENIC COMPOUND ADMINISTRATION IMPROVE OUTCOMES AFTER TRAUMA: NEUROPROTECTION AND NEUROGENESIS FOR THE TREATMENT OF TRAUMATIC BRAIN INJURY

Meghan O’Connell Blaya

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Traumatic brain injury (TBI) represents a serious public health problem as there are no clinically-available treatments to mitigate the functional complications and societal burdens endured by patients and their caregivers. In addition to the primary mechanical insult, deleterious secondary injuries contribute to the progressive atrophy and long-term histopathological changes that impair functional and cognitive outcomes. The studies carried out in this dissertation project assessed two treatment strategies designed to engage and enhance endogenous neurorestorative responses in the injured brain. We postulated that the protection of vulnerable cortical neurons and perilesional parenchyma together with the promotion of endogenous hippocampal neurogenesis would confer histological and behavioral improvement after brain injury.

The first series of experiments evaluated the effects of transplanting syngeneic neural progenitor cells (NPCs) with or without genetic modification to secrete a synthetic multineurotrophin (MNTS1) with multifunctional, multitargeting, neurotrophic capacity. NPCs were obtained from Sprague Dawley fetuses at embryonic stage E15 and transduced with either MNTS1 and GFP constructs (MNTS1-NPCs) or with GFP and blue fluorescent protein (BFP) constructs (control GFP-NPCs). Adult Sprague Dawley
rats received a moderate fluid percussion-induced insult over the right parietal cortex or underwent sham surgery. Animals were transplanted pericontusionally 1 week later with either control GFP-NPCs, MNTS1-NPCs, or injected with saline (vehicle). Five weeks after surgery, groups were evaluated for hippocampal-dependent spatial memory and then sacrificed for immunohistochemical analyses.

Six weeks after TBI (5 weeks after transplantation), there was significant survival and neuronal differentiation of MNTS1-transduced NPCs, as well as injury-activated targeted migration towards contused brain regions. NPCs displayed long processes with spine-like formations that extended into many cortical and subcortical brain structures, including the hippocampus and contralateral hemisphere. All transplanted NPCs, irrespective of transduction profile, conferred significant preservation of pericontusional host tissues and enhanced hippocampal neurogenesis in the posttraumatic brain. Furthermore, NPC transplantation significantly improved spatial memory capacity on the hippocampal-dependent Morris water maze (MWM) cognitive task. Transplant recipients exhibited escape latencies approximately half that of injured vehicle controls, performing on par with sham uninjured animals.

The second set of experiments was conducted to assess histological and functional outcomes with administration of a recently-discovered proneurogenic compound, the highly-active aminopropyl carbazole, P7C3-A20. Sprague Dawley rats were subjected to moderate fluid percussion brain injury or sham surgery. Treatment with 10 mg/kg of P7C3-A20 or vehicle was initiated intraperitoneally 30 min post surgery, and twice per day everyday thereafter for 7 days. Administration of P7C3-A20 significantly reduced overall contusion volume, preserved vulnerable NeuN-positive pericontusional cortical
neurons, and improved sensorimotor function 1 week after trauma. P7C3-A20 treatment also significantly increased both 5-bromo-2'-deoxyuridine (BrdU)-positive and doublecortin (DCX)-positive cells within the subgranular zone of the ipsilateral hippocampus 1 week after TBI. Five weeks after TBI, animals treated with P7C3-A20 showed significantly increased BrdU/neuronal nuclei (NeuN) double-labeled neurons in the ipsilateral dentate gyrus and improved cognitive function in the MWM compared to TBI-vehicle animals. These results suggest that P7C3-A20 is neuroprotective and promotes endogenous reparative strategies, such as hippocampal neurogenesis, after brain trauma. The chemical scaffold represented by P7C3-A20 may provide a basis for developing new pharmacological agents for protecting patients against the early and chronic consequences of TBI.

Neural progenitor cell transplantation and treatment with a highly-active proneurogenic compound both resulted in significant neuroprotection, enhanced hippocampal neurogenesis, and preservation of cognitive capacity in an experimental model of TBI. Collectively, the experiments carried out in this dissertation project suggest that exogenous interventions that target and strengthen endogenous reparative processes, such as NPC-mediated trophic support and enhanced hippocampal neurogenesis, may be effective at restoring and protecting histological and functional outcomes after traumatic brain injury.
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<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALDH1</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BFP</td>
<td>blue fluorescent protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis area 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis area 3</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP-response element binding</td>
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<tr>
<td>CsA</td>
<td>cyclosporine A</td>
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<td>cerebrospinal fluid</td>
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<td>cardiotrophin-1</td>
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<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>DCX</td>
<td>doublecortin</td>
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<td>dentate gyrus</td>
</tr>
<tr>
<td>DGCs</td>
<td>dentate granule cells</td>
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<td>dorsal root ganglion</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>E-LTP</td>
<td>early phase LTP</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>ESCs</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FdUrd</td>
<td>5-fluoro-2'-deoxyuridine</td>
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<td>fibroblast growth factor</td>
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<td>FGF receptor 1</td>
</tr>
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<td>FPI</td>
<td>fluid percussion injury</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>granular cell layer</td>
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<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
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<tr>
<td>GDNF</td>
<td>glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-coupled protein receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
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<td>HD</td>
<td>Huntington’s disease</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HI</td>
<td>hypoxic-ischemic</td>
</tr>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>Iba1</td>
<td>ionized calcium-binding adapter molecule 1</td>
</tr>
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<td>ICM</td>
<td>inner cell mass</td>
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<td>ICD-10</td>
<td>International Classification of Diseases (10th revision)</td>
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<td>ICV</td>
<td>intracerebroventricular</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>NT-4/5</td>
<td>neurotrophin 4/5</td>
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<tr>
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<td>neuronal nuclei</td>
</tr>
<tr>
<td>OPCs</td>
<td>oligodendrocyte precursor cells</td>
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<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>P7C3</td>
<td>Pool #7 Compound #3</td>
</tr>
<tr>
<td>PCSK</td>
<td>proprotein convertase subtilisin kexin</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>PKC</td>
<td>protein kinase-C</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>phospholipase C&lt;sub&gt;γ&lt;/sub&gt;</td>
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<td>PO</td>
<td><em>per os</em></td>
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<tr>
<td>PTE</td>
<td>post traumatic epilepsy</td>
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<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
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<tr>
<td>RGCs</td>
<td>radial glia-like cells</td>
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<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
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<td>RMS</td>
<td>rostral migratory stream</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S1</td>
<td>Swap 1</td>
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<td>SAR</td>
<td>structural activity relationship</td>
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<td>SCI</td>
<td>spinal cord injury</td>
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<tr>
<td>SDF-1α</td>
<td>stromal-derived factor-1α</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
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<tr>
<td>She</td>
<td>Src homologous and collagen protein</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>STAP</td>
<td>stimulus-triggered acquisition of pluripotency</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------</td>
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<tr>
<td>Sup</td>
<td>supernatant</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>T3</td>
<td>triiodothyronine</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TGF-b</td>
<td>transforming growth factor-b</td>
</tr>
<tr>
<td>TH</td>
<td>T helper (cell)</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin tyrosine kinase</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential cation channel C</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vasculature endothelial growth factor</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
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Chapter 1

Introduction

1.1 Traumatic Brain Injury

Definition

Traumatic brain injury (TBI) is a multifaceted, progressive disorder that is often difficult to define due to the complexity and heterogeneity of the human brain. Most physicians, epidemiologists, and researchers define TBI as an external force that comes into contact with the head of an individual and results in neurological damage. Kraus et al. (1984) characterized TBI as “physical damage to, or functional impairment of, the cranial contents from acute mechanical exchange.” TBI can occur by different mechanisms such as rapid acceleration-deceleration of the brain, blast waves, penetration by a foreign object, or a direct crush, all of which can result in compression and shearing of brain parenchyma causing temporary or permanent impairments in cognitive, physical, and psychosocial functions (Maas et al., 2008). According to the most recent edition of the International Classification of Diseases (ICD-10) published by the World Health Organization, the etiology of TBI-associated neurological damage includes concussive injuries, cerebral contusions and lacerations, diffuse and focal brain injury, and traumatic hemorrhages (epidural, subdural, subarachnoid), or a combination of two or more of these events (Reilly and Bullock, 2005).

Prevalence

TBI is a significant cause of morbidity and mortality with approximately 1.7 million occurring annually in the United States. Strikingly, TBI occurs more frequently than any disease, including AIDS, Parkinson’s disease, breast cancer, and multiple
sclerosis (Prins et al., 2013) and is the number one cause of death and disability for people under the age of 45 (Langlois et al., 2006). In 2013, the Centers for Disease Control and Prevention (CDC) reported that TBI contributes to a third of all injury-related deaths in the U.S.; however, this figure does not take into account those individuals who suffered a TBI while serving abroad in the military or those who did not seek medical care (Coronado et al., 2012). Epidemiological studies carried out in recent years have estimated that 57 million (still living) people have experienced a TBI and approximately 3 to 5 million people live with long-term disabilities as a direct result of the trauma (Langlois et al., 2006, Coronado et al., 2012).

In addition to debilitating biomedical consequences to the patient and caregivers, the societal and economic burdens are also serious concerns. Coronado and colleagues (2012) determined that the direct and indirect costs associated with TBI in 2000 were estimated to be $76.5 billion in the U.S. Thus, brain trauma has a severe impact on society as a whole. Falling incidents and motor-vehicle-related accidents are the leading causes of moderate to severe TBI. The next most frequent causes are strikes and blows to the head (such as assaults, sports-related injuries, etc.), followed by blast-induced TBIs, such as those incurred during combat (Faul et al., 2010). Blast injuries have become much more commonplace in the last two decades and remain a critical area of study.

Pathophysiology

The complexity of the cerebral pathophysiology makes TBI one of the most difficult disorders to treat. TBI is not an isolated, single event, but rather a progressive disease process with complex sequelae. Posttraumatic injury mechanisms can be divided into two classes: primary and secondary. The primary injury is defined as the direct
mechanical insult and immediate disruption of brain tissue, which varies considerably according to force, acceleration, direction, and severity of the trauma. Characteristically, there is a central core of necrotic tissue surrounded by a pericontusional penumbra of degenerating cells and diffuse axonal injury. Traumatic damage to brain tissue initiates a cascade of secondary injuries that commence within minutes after the direct insult and can continue chronically for months or years after the initial trauma (Bramlett et al., 2007). Secondary injuries evolve with aberrant metabolic and cellular events, as well as with the release of antagonistic (and sometimes beneficial) biochemical and inflammatory factors, all which alter the local milieu (Cox, 2011). After TBI, this cascade of events ultimately leads to more cell death and neurological dysfunction (Bramlett and Dietrich, 2004). Primary injuries can only be prevented (through the use of seatbelts, helmets, etc.); thus, deleterious secondary injury mechanisms are those targeted for therapeutic intervention after TBI.

One of the most acute (< 1 h) neurochemical alterations that occurs after brain trauma is the aberrant release of excitatory neurotransmitters, including glutamate and aspartate. Elevated extracellular glutamate and influx of sodium and calcium disrupt the ionic equilibrium on postsynaptic membranes and induce massive neuronal depolarization. Posttraumatic excitotoxicity activates enzymes that catalyze the decomposition of adenosine triphosphate (ATPases), depleting cellular energy supply. Furthermore, excitotoxic events generate harmful molecules including free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals are highly reactive as a result of having unpaired electrons. They are normally produced in the brain under homeostatic conditions and levels are well managed by cellular
antioxidant defense systems. Under traumatic conditions, ROS production overwhelms cellular antioxidant systems, leading to significant oxidative damage (Prins et al., 2013). Furthermore, elevated intracellular calcium activates phospholipases, proteases, and endonucleases that degrade lipids, proteins, and nucleic acids, and break down the cellular membrane causing it to become more permeable. (Bullock et al., 1995).

Additionally, there are acute metabolic alterations observed in patients after TBI. Work by Casey (2008) and colleagues showed acute increases in lactate/creatinine ratios in rat cerebral tissue, which correlated with decreased oxidative metabolism and higher levels of glycolysis as early as 24 h after injury and sustained for up to 7 days. Acute cerebral hyperglycolysis was also observed in human patients following severe TBI (Bergsneider et al., 1997). The acute period of hyperglycolysis is followed by a prolonged period of global glucose metabolic depression, which has been shown to correlate with injury severity in both experimental and clinical settings (Bergsneider et al., 2000; Chen et al., 2004). The precise mechanisms underlying decreased glucose uptake remain unknown. If the energy needs of the brain are not met, metabolic-associated negative downstream effects can significantly exacerbate histopathological and functional outcomes. The disruption of oxygen consumption and sugar production after brain injury leads to the depletion of ATP stores and subsequent failure of ATP-reliant ion pumps. When these pumps stop working, there is a massive influx of calcium and sodium and cells become further depolarized, causing subsequent release of glutamate within the synaptic cleft and a continuation of the cycle through positive feedback loops (Conti et al., 1998; Bramlett and Dietrich, 2004; Reilly and Bullock, 2005). High levels of extracellular potassium, due in part to the failure of ATPase
sodium/potassium pumps, exacerbate disruption of the ionic homeostasis necessary for maintaining membrane resting potentials.

Mitochondria play a critical role in the pathophysiology of brain injury. An overload of calcium sequestration in mitochondria compromises mitochondrial membrane integrity, causes energy disturbances, generates high levels of ROS, induces oxidative stress, and initiates apoptotic and necrotic cell death pathways (Keane et al., 2001; Prins et al., 2013). One of the most detrimental and pervasive secondary injuries that occurs with brain trauma is pathologically high levels of widespread apoptosis. Apoptotic mechanisms have been reported to contribute to the pathogenesis of neurological impairment, and cortical and hippocampal neurons are particularly vulnerable after TBI (Yakovlev et al., 1994; Conti et al., 1998). Initiator caspases, including caspase-8, -9, and -10, cleave and activate effector caspases, which are the primary mediators of apoptotic processes. Increased levels of these caspases are seen in vulnerable cortical and hippocampal regions as early as 1 h after moderate TBI (Keane et al., 2001). Effector caspases, such as caspase-3, -6, and -7, participate as the “executioners” of programmed cell death and mediate the biochemistry of apoptosis. Our lab and collaborators have shown that areas susceptible to cell loss have higher levels of apoptotic-associated proteins in the acute phase after TBI (Keane et al., 2001). More recent data indicate that oligodendrocytes also undergo cell death by caspase-3-mediated processes after TBI (Lotocki et al., 2011). Other forms of cell death are also prevalent after brain trauma, including necrosis, necroptosis, and pyroptosis, each of which can be defined by distinct morphological characteristics and by the enzymatic molecules
involved (Kroemer et al., 2009). Further understanding of these pervasive secondary injury mechanisms would allow for the identification of novel targets to treat TBI.

As a result of primary and secondary injury mechanisms, there are significant disruptions in cellular function, axonal injury, cerebrovasculature changes, and widespread cell death, which contribute to the structural and histopathological outcomes observed after TBI. While aggressive early monotherapies (such as hypothermia, controlling intracranial pressure, surgical decompression, and maintaining cerebral perfusion) in the acute stage after injury have shown some improvement in functional outcomes (Bramlett et al., 1997; Chatzpanтели et al., 2000), the ability to reverse neuronal injury at the cellular/subcellular level has proven difficult. Currently there are no reliable treatment strategies to fully attenuate the deleterious outcomes associated with this significant clinical problem.

*Functional deficits: sequelae of TBI*

TBI is a heterogeneous affliction with varying degrees of symptomology. Impaired functional outcomes in TBI patients may manifest in a variety of ways. After brain injury, functional outcomes can be assessed using the Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974) or other neurological assessment batteries. Patients are scored on a variety of clinical symptoms and the resulting overall numerical value classifies their TBI as mild (score of 13-15), moderate (9-12), or severe (< 9). It is well established that TBI leads to residual and chronic impairments beyond the period of clinical care. These include psychosocial, emotional, physical, and cognitive issues that can significantly affect quality of life. In the acute stages after injury (0 – 6 months), spontaneous functional recovery may occur, but not sufficiently and equally for all
symptoms. Motor improvement occurs more quickly than cognitive recovery, which is one of the most lingering and debilitating deficits expressed by patients affected by TBI (McAllister, 2011).

The neurobehavioral sequelae associated with TBI correlate with the anatomical regions that are selectively vulnerable to injury, either due to tissue densities and inertial force mechanics or to bony proturbances on the inner skull, which make contact with certain brain regions during trauma. While there are individual variations (due to genetic differences, health status, age, etc.), there is a fairly predictable profile of high-risk brain regions that are often damaged in TBI (McAllister, 2011). This anatomical profile corroborates the clinical symptomology and neurobehavioral complications that are presented in the clinic.

High-risk brain regions include the orbital frontal cortices, frontal poles, anterior temporal poles, and lateral and inferior temporal cortices, including the hippocampi. (McAllister, 2011). These brain structures contribute to higher-order cognition including executive function, sustained attention, emotional and social responses, memory retrieval, and abstraction, among others. Cellular circuitry along the medial temporal regions plays an essential role in episodic memory and new memory formation. Vulnerable areas that are at risk for inertial injuries include the corpus callosum, gray/white matter interface, subfrontal white matter, and the rostral brain stem. After head trauma, these regions frequently show evidence of tissue shearing and compression, sliding contusions, and diffuse axonal injury (McAllister, 2011).

In addition to structural damage, behavioral deficits can also manifest with dysfunctional neurotransmission. After TBI, there is evidence of catecholaminergic
alterations, specific neurotransmitters that are critical for a broad spectrum of functions including memory and attention (McAllister et al., 2006). Dopamine (DA) is a catecholamine involved in numerous molecular interactions in several cortical and subcortical regions. DA systems contribute to movement, reward, attention, as well as to learning and memory functions. After TBI, perturbations in DA neurotransmission exacerbate cell death, neuroinflammation, and excitotoxicity (Bales et al., 2010). Both acute and chronic dopaminergic dysfunction has been reported after brain injury (Bales et al., 2010). Cholinergic systems have also been implicated in the genesis of mood disorders (Shytle et al., 2002; Arciniegas, 2003; Perry and Perry, 2004). Serotonin has been shown to increase in the injured brain as well, and these increased levels are associated with hypometabolism and depressed glucose usage (Tsuiki et al., 1995). Thus, neurotransmitter dysregulation is a potentially important target for therapeutic intervention after injury.

As mentioned previously, secondary injuries at the cellular and subcellular level evolve over a delayed period of time. Thus, in addition to primary structural damage, many TBI patients present with focal or diffuse cerebral edema, traumatic hematomas, elevated intracranial pressure, hypoxic-ischemic (HI) injury, and/or traumatic hemorrhages. Since TBI often occurs in the context of other injuries (polytrauma), additional systemic medical complications may arise, further contributing to posttraumatic mortality and morbidity.

Persistent cognitive and memory deficits are frequently described as some of the most debilitating functional consequences after neurotrauma (Whyte et al., 1996; Vakil, 2005; McAllister, 2011). Thus, the reversal of learning and memory deficits remains an
important target for clinical research. The hippocampi are high-risk for primary and secondary damage after TBI. The exact mechanisms as to why these structures are selectively vulnerable are not fully known. However, a region of the hippocampus, Cornu Ammonis area 1 (CA1), in humans is rich in glutamate receptors, which may make this region highly susceptible to excitotoxicity (McAllister, 2011). The hippocampi are also sensitive to intracranial pressure (Reilly and Bullock, 2005). Other non-motor behavioral consequences of TBI include problems with concentration and attention, delay of information processing, speech and language impairment, as well as increased irritability, impulsivity, affective disorders, and apathy (McMillan et al., 1997; Lehtonen et al., 2005; O’Jile et al., 2006). Many TBI patients are unaware of psychosocial and emotional changes (McAllister, 2011). These “personality changes” are encumbering for families and friends of patients affected by brain trauma.

TBI has also shown positive correlations with neuropsychiatric disorders, such as substance abuse, psychosis, posttraumatic stress disorder (PTSD), agoraphobia, depression, and panic disorders (Bryant et al., 2010). Furthermore, several studies have demonstrated a relationship between TBI and progressive dementia (Van Den Heuvel et al., 2007). TBI disrupts axonal transport, which can lead to a rapid accumulation of amyloid precursor protein (APP), a protein associated with Alzheimer’s disease (AD) and other neurodegenerative disorders (Mehta et al., 1999; Uryu et al., 2004).

The heterogeneity of TBI injury presents a significant challenge for developing successful treatment strategies. Patients who experience a traumatic insult may have comparable cognitive and behavioral issues due to the vulnerability of certain brain regions and selective neuronal loss, though the etiology of the insult is considerably
different. In contrast, patients may have analogous traumatic insults and similar GCS scores, but the injury functionally manifests in disparate symptomology (Prins et al., 2013). No two TBIs are exactly alike and the variables dictating outcomes and prognosis depend on many intrinsic and extrinsic factors, such as age, status of health prior to injury, medication, alcohol and drug use, genetics, among other factors, which further makes neurotrauma one of the most complicated disorders to treat. The pathological outcomes and persisting functional deficits are a result of the aggregate of primary and secondary brain injuries. Because of the wide spread nature of polytraumatic TBI, the development of combinatorial therapeutic interventions targeting several pathological events is critical.

Animal models of traumatic brain injury

As discussed by Xiong and colleagues (2013), TBI animal models can clinically reproduce the numerous cellular and functional alterations that are associated with brain trauma in a controlled experimental setting. Animal models provide insight into injury progression, vulnerable cell types, and allow for the investigation of various treatment strategies. Experimental animal models include those evaluating direct brain deformation (fluid percussion injury, controlled cortical impact model, penetrating ballistic bullet injury), non-impact injury (acceleration-deceleration model), and blast wave-induced neurotrauma, among others (Cernak, 2005; Xiong et al., 2013).

For the studies summarized in this dissertation, we investigated therapeutic interventions using a parasagittal fluid percussion injury (FPI) model of moderate TBI in the rat. This experimental TBI model generates both focal and diffuse injuries that are reproducible and reflect pathologies commonly seen in TBI patients. The insult is
inflicted with the release of a calibrated pendulum striking a saline-filled reservoir and generating a pressure pulse that rapidly travels down the length of the fluid-filled tube. The fluid pulse then comes into contact with the exposed, intact dura through a craniotomy, briefly (22 msec) displacing brain tissue without skull fracture. A pressure transducer unit located at the injury shaft where the animal is placed converts the pressure wave into a numerical value (in Atmospheres) indicating severity of the injury.

Parasagittal FPI generates a unilateral focal cortical contusion, shearing of gray and white matter tissues, and diffuse subcortical neuronal injury, reflecting the clinical histopathology observed in TBI patients. Progressive loss of selectively vulnerable cells in specific brain areas, including neuronal cell death in the ipsilateral parietal cortex, hippocampal dentate gyrus, and Cornu Ammonis 3 (CA3) regions, occurs with sustained activation of degenerative cascades that can persist up to one year after the initial insult (Smith et al., 1997; Bramlett and Dietrich, 2002; Bramlett and Dietrich, 2007; Nagamoto-Combs et al., 2007). Moderate parasagittal FPI also reproduces the neurobehavioral, sensorimotor, and cognitive impairments similar to those observed in human TBI patients, which allows for the evaluation of experimental treatments on functional outcomes (Xiong et al., 2013).

Loss of vulnerable neurons and disruption of cytoarchitecture contributes to the spectrum of negative outcomes after TBI. This dissertation project focuses on novel neuroprotective and reparative approaches to reduce histopathology and mitigate functional deficits. The following sections will provide background for the experiments and treatment strategies investigated in this dissertation project, the biological phenomena and relevant pathways, as well as endogenous responses under traumatic
conditions. By harnessing and augmenting clinically-relevant reparative and protective strategies through the investigation and application of single or combinatorial treatments, we strive to alleviate the significant functional burdens endured by TBI patients and their caregivers.

1.2 Neurotrophins

Neurotrophins are a family of neurotrophic factors that play a significant role in several aspects of nervous system development and function, including neuronal survival, cell differentiation, neurogenesis, regulation of synaptic activity, and apoptosis. Four neurotrophins have been identified in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). This family of secreted polypeptides is critical for the proper development and maintenance of the developing and adult nervous systems and plays an important role in preventing and/or reversing neuronal degeneration (Chao, 2003; Lu et al., 2005; Allen and Dawbarn, 2006). Neurotrophins are widely expressed in both the peripheral and central nervous systems (CNS), and expression patterns are highly regulated during development, adulthood, in an activity-dependent manner, and under pathological conditions (Lessmann et al., 2003).

Synthesis, processing and secretion:

Neurotrophins are originally synthesized as pre-pro-neurotrophin precursors. The pre-messenger RNA (mRNA) sequence directs the immature protein to the endoplasmic reticulum, where the pre-sequence is cleaved off to yield the proneurotrophin. Proneurotrophins translocate to the Golgi apparatus and accumulate within the membrane
stacks of the trans-Golgi network. Here the proneurotrophins are sorted into one of two types of secretory vesicles that are characterized according to their respective mechanisms of secretion. The pro-domain is thought to be important for the proper folding and intracellular sorting of the protein. The neurotrophin-containing vesicles are trafficked out to either the constitutive pathway or the regulated secretory pathway (Lessmann et al., 2003). Both pathways are prevalent in neurons, while some non-neuronal cell types lack the molecular machinery for regulated release and thus secrete neurotrophins only constitutively. In neurons, smaller secretory vesicles containing neurotrophins are trafficked to dendrites and spines and are secreted postsynaptically, while a second type of secretory vesicle, large dense core vesicles, are transported anterogradely to axon terminals (Lu et al., 2005). One of three things may occur at this stage: 1) the proneurotrophin is cleaved intracellularly and then secreted, 2) it is secreted first and then cleaved extracellularly, or 3) is secreted without subsequent cleavage (Lu et al., 2005). The mature 12 kDa neurotrophin results from proteolytic cleavage occurring intracellularly by proprotein convertase subtilisin kexin (PCSK) enzymes (i.e. furin), or extracellularly by plasmin or specific matrix metalloproteinases (MMPs). The amount of mature neurotrophin secreted from a cell is dependent on the amount and type of convertases, which are expressed differentially by cell type and physiologically regulated (Longo and Massa, 2013). After processing, mature neurotrophins exist as noncovalently-linked homodimers (Biebl and Barde, 2000; Lessmann et al., 2003). Proneurotrophins that do not get cleaved into a mature form can act as apoptotic-inducing signaling molecules in certain cellular contexts (Lee et al., 2001; Lu, 2003; Teng et al., 2005).
**Signaling**

Extensive work has demonstrated that the two forms of neurotrophins (pro- and mature) are both critical signaling molecules. Each form exhibits differential binding properties and activates unique signaling cascades to influence numerous cellular and subcellular events (Chao, 2003; Lu *et al.*, 2005; Figure 1.1). Neurotrophin signaling is initiated with the binding of two different classes of transmembrane receptors: the p75 neurotrophin receptor (p75NTR) and the tropomyosin tyrosine kinase (Trk) family of receptors, which includes TrkA, TrkB, and TrkC. (Dechant and Barde, 2002; Roux and Barker, 2002; Huang and Reichardt, 2003). All mature neurotrophins bind to p75NTR with a similar affinity, however proneurotrophins preferentially bind to this receptor (Lee *et al.*, 2001). During p75NTR interaction, the pro-domain of proneurotrophins concomitantly binds to the vacuolar protein sorting 10 (VPS10) family member, sortilin, which mediates cell death in neuronal and non-neuronal cell populations (Casaccia-Bonnefil *et al.*, 1996; Friedman, 2000; Longo and Massa, 2013). In contrast to non-selective p75NTR binding, each neurotrophin has a cognate Trk receptor: NGF preferentially binds TrkA, BDNF and NT-4/5 bind TrkB, and NT-3 binds TrkC, although some binding promiscuity does exist between NT-3 to TrkA and TrkB in certain cellular contexts.
Trk receptor expression is not ubiquitous, but rather highly regulated. Trk receptors are differentially expressed in neuronal populations, which is due to neuronal subgroups having separate and unique trophic requirements. In the CNS, TrkA is found in basal forebrain cholinergic neurons. In contrast, TrkB and TrkC are widely expressed in the CNS, including within the hippocampus, amygdala, cerebellum, and neocortex (Shelton et al., 1995). While there is overlap in Trk expression patterns and functions, certain neural mechanisms are regulated by specific neurotrophin-Trk interactions. For example, TrkA and NGF mediate basal forebrain cholinergic systems (Sofroniew et al., 2001), TrkB and BDNF mediate synaptic plasticity (Poo, 2001), and TrkC and NT-3 mediate the survival of several peripheral neuronal populations (Ernfors et al., 1994). Trk...
receptors are frequently coexpressed with p75NTR in several neuronal populations (Bibel and Barde, 2000).

Neurotrophin signaling through Trk receptors regulates neuronal survival, proliferation, differentiation, dendritic and axon growth, assembly and remodeling of cytoskeleton, membrane trafficking, as well as synaptic strength, plasticity, and memory formation (Birling and Price, 1995; Thoenen, 1995; Lewin and Barde, 1996; Chao, 2003; Huang and Reichardt, 2003). Activation of prosurvival Trk receptors induces a cascade of signaling events that initiates a two-step process: the ligand-mediated dimerization of Trk receptors at the cell surface followed by trans-autophosphorylation of their tyrosine residues (Barbacid, 1994). This phosphorylation of the cytoplasmic tyrosine residues mediates their conversion into anchors for binding downstream signaling molecules (Schlessinger and Ullrich, 1992). The recruitment of cytoplasmic signaling molecules involves distinct enzymes and adaptor proteins, and their interaction with activated Trk receptors initiates several intracellular signaling cascades involved in proliferative pathways (Schlessinger and Ullrich, 1992). After binding, ligand-receptor complexes are internalized and sorted into active signaling vesicles, which are retrogradely transported in a dynein-dependent manner along a microtubules network towards the cell soma. After reaching the cell body, signals are disseminated though a dismantling of the retrograde endosomal signaling complex and continue on to initiate various downstream events according to the specific signaling cascade that was activated (Zweifel et al., 2005).

The Src homologous and collagen-like (Shc) adaptor protein links activated Trk receptors to two separate pathways involved in survival, modulation of the cell cycle, differentiation, neurite outgrowth, and synaptic plasticity. Shc binding/phosphorylation
can induce phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling as well as activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling cascades. Furthermore, phospholipase Cγ (PLCγ) can also bind directly to activated Trk receptors and initiate signaling that results in the release of protein kinase C (PKC) and subsequent calcium interactions (Bieble and Barde, 2000). These signaling cascades influence transcriptional events including the activation of the cyclic AMP-response element binding (CREB) transcription factor, which can alter the neurotrophin-Trk-mediated physiological processes under homeostatic and pathological conditions (Lonze and Ginty, 2002).

All neurotrophins also bind with similar affinity to the p75NTR receptor, which mediates both antagonistic actions such as cell death and long-term depression (LTD), as well as positive actions including cell viability and survival (Lu et al., 2005; Woo et al., 2005; Charalampopoulos et al., 2012). Through a set of adaptor proteins, proneurotrophin-sortilin activation of p75NTR induces c-Jun N-terminal kinase (JNK) activity, which results in cell death or degeneration (Casaccia-Bonnefil et al., 1996; Friedman, 2000). On the other hand, mature neurotrophin-p75NTR binding activates both nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Ras homolog gene family, member A (RhoA) signaling, which results in cell survival and inhibition of neurite growth respectively (Carter et al., 1996; Yamashita et al., 1999). Furthermore, similar to Trk binding, mature neurotrophin interaction with p75NTR can engage Shc proteins and initiate PI3K/AKT and MAPK/ERK signaling activity, which contributes to cell survival and neurite outgrowth (Longo and Massa, 2013).
p75\textsuperscript{NTR}-neurotrophin binding interactions are also associated with increased proliferation of neuroblasts in the adult subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Young et al., 2007; Catts et al., 2008). These two zones house unique stem cell niches in which neurogenesis occurs throughout adulthood in several mammalian species, including humans (albeit it was recently shown that SVZ neurogenesis subsides by 18 months of age in humans; Sanai et al., 2011) (Altman, 1962; Erikkson et al., 1998; See Section 1.3). Young et al. (2007) found p75\textsuperscript{NTR} expression in a small subpopulation of neurogenically-active neural progenitor cells (NPCs) in the stem cell niche. Furthermore, neurogenic responses of p75\textsuperscript{NTR}-expressing NPCs were significantly enhanced with BDNF treatment (Young et al., 2007). In adult mice null for the p75\textsuperscript{NTR} receptor, there was a 70% reduction in adult hippocampal neurogenesis (Young et al., 2007). Recent work by Shi and colleagues (2013) showed that small-molecule activation of p75\textsuperscript{NTR}-dependent NF-\kappa B signaling significantly increased proliferation and survival of hippocampal NPCs and reversed spatial memory deficits after TBI.

Trk-neurotrophin signaling is modulated by p75\textsuperscript{NTR}. In the presence of Trk receptors, p75\textsuperscript{NTR} acts as a co-receptor, refining Trk affinity and specificity, as well as potentiating Trk signaling cascades (Culmsee et al., 2002; Huang and Reichardt, 2003; Kuruvila et al., 2004). It was further demonstrated that NT-3, which displays some Trk receptor promiscuity, was much more effective at activating non-cognate TrkA and TrkB receptors when they were coexpressed with p75\textsuperscript{NTR} on the same cell (Brennan et al., 1999; Esposito et al., 2001). Coexpression of p75\textsuperscript{NTR} and Trk receptors is dynamic and can vary accordingly under certain pathological conditions (Longo and Massa, 2013).
The diametrically opposed actions of mature and proneurotrophins on cell death and survival demonstrate the complexity by which these molecules exert their effects. Directionality is mediated by the ratio of receptor type expression and which form of neurotrophin (pro- or mature) is most prevalent in a particular cellular context, which may alter with age or disease state.

**Neurotrophins and synaptic plasticity**

In the early 1990s, a major advancement in the understanding of neurotrophic function came about when it was revealed that the synthesis of neurotrophins in the brain is increased in an activity-dependent manner (Thoenen, 1991). Present research has strongly implicated the involvement of neurotrophins, especially BDNF, in synaptogenesis, neural circuit function, synaptic plasticity, as well as learning and memory (Minichiello, 2009; Park and Poo, 2013).

Neurotrophins regulate the morphological and functional maturation of synapses. Evidence supporting this comes from both developmental and adult synaptogenesis studies (McAllister et al., 1995; Park and Poo, 2013). Work by Wang and colleagues (1995) showed that exogenous application of BDNF and NT-3 accelerated the maturation of developing neuromuscular synapses. Furthermore, administration of BDNF and NT-3 on immature spinal neurons from *X. laevis* tadpoles significantly increased synaptic activity within minutes, an effect that was attributed to greater exocytosis of synaptic vesicles at presynaptic terminals (Lohof et al., 1993). In hippocampal neurons isolated from embryonic rats, NT-3 promoted the formation of functional excitatory synapses, while BDNF application stimulated the development of both excitatory and inhibitory synapses, as well as increased axonal branching and total axon lengths (Vicario-Abejon...
et al., 1998). These observations are consistent with neurotrophin-induced expression of several synaptic proteins, neurotransmitters, and subunits of postsynaptic receptors (Vicario-Abejon et al., 2002; Park and Poo, 2013). The observation that BDNF can promote and potentiate the formation of both excitatory and inhibitory synapses may indicate the bidirectionality of neurotrophic action, which contributes to the coordination and synchronization of synapses with opposing actions, a phenomenon necessary for maintaining homeostasis and proper circuit function. Pro forms of neurotrophins are also important for synapse development and maturation. A recent study indicated that proBDNF was necessary for the formation of γ-aminobutyric acid (GABA)ergic synapses in an N-methyl-D-aspartate receptor (NMDA)-dependent manner (Langlois et al., 2013). Furthermore, Yang et al. (2009) showed that proBDNF promoted synaptic depression and retraction of synapses at nerve terminals. Recent work supported these findings and further demonstrated that neural activity stimulated the proteolytic cleavage of proBDNF to its mature form, which facilitated stabilization of the active synapse (Je et al., 2012). In addition to neurotrophic and proneurotrophic activity at local synapses, ligand-receptor complexes can be internalized via endocytosis and retrogradely transported back to the soma where their actions are further potentiated through the regulation of gene expression.

Neurotrophic mechanisms that mediate neural circuit function in the adult have also been extensively studied. Modulation of synaptic activity by neurotrophins can occur in several ways, and the differential kinetics of various neurotrophin-Trk interactions to influence synaptic activity are most likely occurring concomitantly. On the presynaptic side, Trk signaling can activate PLCγ and MAPK/ERK cascades, which can increase
neurotransmitter release via calcium increases or phosphorylation of synapsin, a protein that regulates the number of neurotransmitter vesicles that are exocytosed, respectively (Jovanovic et al., 2000; Park and Poo, 2013). Neurotrophin-Trk binding can also increase postsynaptic responses in several ways, including enhanced NMDA receptor conductance (Suen et al., 1997), increased PKC-mediated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor surface expression (Caldeira et al., 2007), decreased surface expression of GABA<sub>A</sub> receptors (Jovanovic et al., 2004), as well as differentially modulating additional ion channel conductance (Lesser et al., 1997). Additionally, there are changes in synaptic gene expression with increased CREB phosphorylation via Trk-induced MAPK/ERK signaling (Gaiddon et al., 1996; Blum and Dash, 2009) and PI3K/AKT-mediated phosphorylation of translation-regulating factors (Takei et al., 2004). Spine morphology is also altered with the activation of BDNF-TrkB signaling cascades. Activation of MAPK/ERK pathways (Alonso et al., 2004) as well as calcium increases via the transient receptor potential cation channel C (TRPC; Amaral and Pozzo-Miller, 2007) both promote dendritic growth and spine maturation in mature neural circuits.

Long-term potentiation (LTP) is a molecular correlate of learning and memory, and several studies have provided evidence for the considerable role neurotrophins, specifically BDNF, contribute to this complex process (Park and Poo, 2013). After intracellular synthesis, BDNF is sorted into the regulated pathway and secreted in response to neural activity (Mowla et al., 2001). LTP, which can be invoked experimentally by high frequency stimulation of a chemical synapse, synchronizes and strengthens signal transmission between two neurons, and enhances activity-dependent
BDNF gene expression. Korte et al. (1995) provided one of the first reports describing that BDNF is essential for LTP induction in CA1 pyramidal neurons. It was further shown that BDNF mediated hippocampal LTP by enhancing the efficacy of presynaptic neurotransmitter release (Figurov et al., 1996) and/or by a postsynaptic mechanism involving greater NMDAR- and voltage gated calcium channel (VGCC)-dependent calcium influxes (Kovalchuk et al., 2002). LTP was significantly impaired with inhibition of BDNF-TrkB signaling (Chen et al., 1999) and with deletion of *bdnf* and/or *trkb* (Minichiello et al., 1999). Importantly, LTP was restored to normal levels with exogenous application of BDNF (Patterson et al., 1996) or viral-mediated transfer of *bdnf* (Korte et al., 1996). Through genetic dissection analyses, Minichiello and colleagues (2002) showed that hippocampal LTP is dependent upon BDNF-TrkB-mediated PLCγ pathway activation that induces CREB phosphorylation. Activation of this critical transcription factor modulates gene expression leading to synaptic protein synthesis and neuroplastic changes (Sakamoto et al., 2011).

ProBDNF and p75NTR have been implicated in the induction of long-term depression (LTD) (Lu et al., 2005); however a direct correlation is still under debate. In contrast to LTP, LTD can be invoked by low frequency stimulation, and is associated with shrinkage of dendritic spines, potentially as a result of the loss of AMPA receptors (Zhou et al., 2004). Work by Woo and colleagues (2005) reported that under normal conditions, proBDNF binding to p75NTR facilitated hippocampal LTD at CA3-CA1 hippocampal synapses, and transgenic mice null for p75NTR had selective impairment of NMDA receptor-dependent LTD. Consistent with this, Rösch et al. (2005) also showed impairment of LTD in p75NTR-deficient mice in activity-dependent synaptic plasticity. In
contrast to these findings, work by Matsumoto et al. (2008) showed that LTD induction was normal in BDNF conditional knock-out mice, demonstrating that neither pro- nor mature BDNF were involved in LTD induction. Other recent studies published results showing that both BDNF and proBDNF were colocalized in presynaptic secretory granules in adult hippocampal neurons and indicated that proteolytic processing of proBDNF to its mature form occurs intracellularly and therefore would be unable to modulate LTD (Dieni et al., 2012). Given these conflicting findings, proneurotrophin-mediated actions on LTD are still unclear.

Neurotrophins and Learning and Memory

While the last few paragraphs discussed the subcellular and neuronal network correlates of learning and memory, neurotrophin-mediated effects on function can also be observed on a larger, behaviorally-relevant scale. BDNF has been shown to be crucial for both the hippocampal-dependent acquisition (learning) and retention (memory) of new information and certain learned behaviors (Lu and Woo, 2009). BDNF-induced hippocampal-dependent memory can be classified into short-term and long-term memory. Short-term memory resembles early phase LTP (E-LTP) and lasts between several minutes and a few hours, and does not require protein synthesis for its induction. On the other hand, long-term memory, parallel to late phase LTP (L-LTP), can last for hours to weeks to even longer, and does require mRNA and protein synthesis for its induction.

The role of BDNF signaling in memory acquisition and retention has been investigated with several relevant behavioral paradigms, including spatial assessments (Morris water maze (MWM) and radial maze; Falkenburg et al., 1992; Mizuno et al., 2000), contextual fear conditioning (Hall et al., 2000), olfactory memory (Broad et al.,
2002), and aversive conditioning (Kesslak et al., 1998). These studies demonstrated that hippocampal-dependent learning paradigms increased BDNF mRNA and/or TrkB expression. Consistent with these findings, inhibition of BDNF synthesis and/or blockade of TrkB signaling impaired contextual fear conditioning and spatial memory formation (Minichiello et al., 1999; Liu et al., 2004). Furthermore, Egan et al. (2003) showed that a single-nucleotide polymorphism (SNP) at codon 66 in human *bdnf* (val66met) resulted in abnormal activity-dependent BDNF sorting and secretion, which was associated with smaller brain volumes, compromised episodic memory, and hippocampal dysfunction in humans (Egan et al., 2003). More recent work has shown that this SNP also altered the prodomain of BDNF, resulting in retraction of growth cones in hippocampal neurons (Anastasia et al., 2013). In addition to memory dysfunction, the val66met polymorphism in *bdnf* predisposed patients to developing depression, anxiety, and PTSD (Chen et al., 2006; Frielingsdorf et al., 2010; Verhagen et al., 2010), three disorders that TBI patients are at greater risk of developing post trauma.

While the preceding studies have demonstrated that BDNF contributes to the formation of hippocampal-dependent memories, other studies have shown that disruption of BDNF can impair memory retrieval after the memory has been molecularly established (Lu et al., 2008). Work by Mizuno et al. (2000) showed that injection of antisense BDNF oligonucleotides disrupted a well-established spatial reference memory in rats that had been trained for 28 days in the radial arm maze. Furthermore, Bekinschtein and colleagues (2007) demonstrated that hippocampal infusion of BDNF antisense oligonucleotides 12 h after inhibitory avoidance training impaired memory retention 7 days later. These findings indicate that memory retrieval requires late protein
synthesis of BDNF long after the initial formation of the hippocampal-dependent memory. While the mechanisms involved in this phenomenon are still unclear, it has been suggested that repetition of the synaptic activity for a particular memory that occurs during the consolidation phase (i.e. during sleep) may be adequate to stimulate activity-dependent BDNF expression and result in persistent long-term memory (Foster et al., 2006; Lu et al., 2008).

Much work has been dedicated to investigating the positive correlation between neurotrophin expression and learning and memory, and how this process is modulated with exposure to external stimuli, such as environmental enrichment, dietary restriction, exercise, specific training paradigms, stress, and deprivation. Studies have shown that enriched environments increased expression of mature NGF, BDNF, and NT-3 in the basal forebrain, hippocampus, and cerebral cortex of adult rats. Upregulation of these neurotrophins facilitated neural plasticity and improved spatial memory capacity compared to rats in control environments (Ickes et al., 2000; Pham et al., 2002). In recent rodent studies, increased neurotrophin levels, mediated by positive external stimuli, enhanced neurotrophin-mediated synaptic plasticity and strengthened short- and long-term spatial memory, object recognition, and working memory performance (Birch et al., 2013; Callagan and Kelly, 2013).

**Neurotrophins and TBI**

Neurotrophin levels are altered after traumatic insults (Nieto-Sampedro et al., 1983). Work by Hicks and colleagues (1997; 1999) demonstrated differential expression patterns of neurotrophin mRNA in rat hippocampus after experimental brain injury. Using *in situ* hybridization, they showed that at various acute time points after injury (1,
3, 6, 24, and 72 h) BDNF mRNA expression was increased in the ipsilateral DG up to 72 h post trauma. Conversely, NT-3 mRNA levels were significantly decreased in the DG at 6 and 24 h post injury. Consistent with these findings, 2 and 10 min of ischemia caused a significant reduction of NT-3 in the DG, CA2, and CA3 regions of the hippocampus. Additional work by Lindvall et al. (1992) showed similar patterns of differential neurotrophin mRNA expression in the hippocampus after CNS injury. They reported that cerebral ischemia induced greater and sustained expression of hippocampal BDNF mRNA compared to sham animals, while NGF mRNA was increased 4 h after the insult and returned to baseline 24 h afterward. These findings suggest that brain trauma differentially modulates expression of certain neurotrophins in the hippocampus. The significance and mechanisms of these differential regulation patterns have yet to be clarified, but may contribute to the selectivity of certain neuronal populations that either die or survive following injury or the incomplete neuroreparative responses induced by the injured brain. This phenomenon may be critically important to the development of neurotrophin therapeutics for TBI and warrants further investigation.

In more chronic TBI cases, studies have suggested a dysregulation and reduction of BDNF expression and activity. Trophic deprivation can have severe consequences on the viability of vulnerable neurons that are dependent on neurotrophic activity for their survival. Preclinical studies have found high levels of chronic stress and elevated cortisol levels in animal models of TBI (Savaridas et al., 2004). These negative exposures can reduce BDNF levels and impair hippocampal function. Kaplan and colleagues (2010) reported that prolonged TBI-induced stress decreased BDNF and resulted in dendritic retraction and disruption of hippocampal circuitry. Additional work by Tsankova et al.
(2006) showed that a mouse model of chronic stress had increased histone methylation at important bdnf promoter regions, which suppressed transcriptional activity and decreased levels of mature BDNF. As discussed in the previous section, patients with TBI have higher incidence of neuropsychiatric complications including anxiety and depressive mood disorders, which are both associated with dysfunctional BDNF activity (McAllister, 2011).

In addition to alterations in mature neurotrophin expression, CNS pathophysiology also modulates proneurotrophin expression. Pathological upregulation of proneurotrophins can result in adverse consequences, such as aberrant basal dendritogenesis, erroneous innervation, and/or cell death (Lu et al., 2005; Volosin et al., 2006). Volosin et al. (2008) showed that proneurotrophins and activation of p75NTR-mediated apoptosis was increased in the hippocampus after initiation of temporal lobe seizure-induced injury. Furthermore, proNGF was upregulated after spinal cord injury (SCI) and mediated the death of oligodendrocytes via apoptogenic p75NTR signaling (Beattie et al., 2002). Harrington and team (2004) also showed that proNGF, p75NTR, and sortilin were all upregulated following acute neuronal damage and inflammation. They further demonstrated that disruption of p75NTR-proNGF binding resulted in near complete rescue of adult corticospinal neurons and considerable reversal of TBI pathology (Harrington et al., 2004).

With the dysregulation of mature neurotrophin expression and the upregulation of proneurotrophin death-inducing interactions after TBI, an important area of clinical research includes evaluating specific experimental manipulations to normalize levels and restore prosurvival signaling activity in the traumatized brain. Several studies have
investigated mature neurotrophin administration as a potential therapy for TBI given the numerous neurotrophin-mediated cell survival and viability properties (Sinson et al., 1997; Hetman and Xia, 2000; Royo et al., 2006; Aloe et al., 2012). The underlying hypothesis is that delivery of appropriate neurotrophic factors to the contusion site or more remote brain regions after CNS injury would reduce neuronal degeneration resulting from secondary injury mechanisms, such as apoptosis, and assist in stimulating the sprouting of spared neurons, thereby reestablishing disrupted networks. There has been some evidence of success with this technique, albeit it is somewhat limited as full recovery of function has yet to be observed after TBI.

TBI-induced alterations in neurotrophin levels can have considerable effects on a wide variety of physiological phenomena, such as pain, regeneration, memory, aggression, depression, neuropsychiatric status, and substance abuse (Chao et al., 2006, Gratacos et al., 2007; Alleva and Francia, 2009). Several studies have investigated the outcomes of neurotrophin administration after injury. Due to the profound effects of neurotrophins on the structural and functional integrity of the nervous system, there is a great amount of interest in the potential use of these proteins as therapeutic agents for neurodegenerative diseases as well as neurotrauma. Furthermore, the endogenous upregulation of certain neurotrophins after TBI may be an incomplete attempt by the brain to repair itself. This notion is supported by findings showing that administration of exogenous neurotrophic factors in the acute period after neurotrauma improved histopathological and functional outcomes (Sinson et al., 1997; Royo et al., 2006; Aloe et al., 2012). Exogenous application of neurotrophin 4/5 protected vulnerable hippocampal
neurons and facilitated motor recovery (Royo et al., 2006), while NGF infusion improved memory scores and reduced cholinergic cell death (Sinson et al., 1997).

The administration of NGF has demonstrated interesting findings. The basal forebrain complex contributes to attention, arousal, motivation, and memory (Aloe et al., 2012), and is often implicated in AD pathology. This structure is also vulnerable after brain injury (McAllister, 2011). Clinical and preclinical studies have found abnormalities in cholinergic systems in TBI patients and a significant loss of basal forebrain neurons in animal models of FPI (Schmidt and Grady, 1995; Salmond et al., 2005). Both studies showed basal forebrain dysfunction correlated with relevant cholinergic-associated cognitive deficits. Previous studies have shown that exogenous administration of NGF protected these basal forebrain cholinergic neurons in the aged brain and after lesion-induced neuronal degeneration, and subsequently reduced memory impairment (Fischer et al., 1987; Tuszynski et al., 1991; Allen and Dawbarn, 2006). These findings have implications in neuropathological conditions in which atrophy and degeneration of cholinergic neurons is a prominent component. The mechanisms by which NGF exerts these protective and/or reparative effects are not fully understood. However, previous work by Tian and colleagues (2012) showed that administration of NGF acted as an anti-amyloidogenic factor after TBI, which is a risk factor for the development of AD later on in life. Additionally, infusion of NGF has been pursued in patients with HI injuries as well as TBI (Sinson et al., 1997; Chiaretti et al., 2008). The rationale for these studies was based on the correlation between greater NGF levels in cerebrospinal fluid (CSF) and positive neurological outcomes (Chiaretti et al., 2003; 2008).
However, conflicting findings demonstrate a lack of efficacy with neurotrophin therapy after brain trauma. Blaha and colleagues (2000) showed that BDNF-infusion in the posttraumatic brain did not protect rats from histopathological and behavioral deficits. Additional work demonstrated NT-4/5 infusion after TBI increased the survival of rat hippocampal pyramidal neurons, but produced no improvement on three different hippocampal-dependent behavior tasks relative to TBI controls (Royo et al., 2007). This lack of efficacy in certain contexts may indicate the incomplete capacity of single neurotrophin therapy to confer therapeutic effects, perhaps as a result of the selectivity and specificity of various cell populations for distinct neurotrophins.

Limitations of exogenous neurotrophin therapy

Although the potential of neurotrophin therapy after injury is compelling, there are several challenges that can impede clinical utilization. Neurotrophins have suboptimal pharmacological properties, including short half-lives and low protein stability (Bruno and Cuello, 2006). In the CNS, one of the greatest challenges is optimizing the means by which neurotrophins are delivered (Gao et al., 1997). Neurotrophins are unable to permeate the blood brain barrier (BBB: Pan et al., 1998) and, as a result, local infusion techniques directly into the CNS have been extensively explored in preclinical experiments and clinical trials. These methods include direct injection into brain parenchyma, intracerebroventricular (ICV) administration, and miniosmotic pump diffusion, among others (Sinson et al., 1997; Eriksdotter et al., 1998). Direct injection of neurotrophins relies on their adequate distribution and penetration within the parenchyma, which is typically poor (Lessmann et al., 2003). CSF delivery does not allow for achieving desired pharmacological concentrations at targets without affecting
non-target tissues. Other studies have investigated osmotic pump delivery systems, however inadequate diffusion, pump failure, protein stability issues, and risk of infection are all complications limiting the application of this approach (Tan and Aebischer, 1996). Thus, using a biologically-relevant cellular modality for constitutive and sustained delivery of therapeutic neurotrophins at target regions is a compelling alternative.

**Chimeric Neurotrophins**

Neurotrophins have valuable therapeutic potential for the treatment of CNS injury. However in many complex neuropathologies, including TBI, several distinct neuronal populations with different trophic requirements are affected. For example, several studies have demonstrated the significance of NGF for basal forebrain cholinergic neurons and NT-3 for locus coeruleus noradrenergic neurons, which are two neuronal populations affected in TBI and AD (Levin *et al.*, 1995; Schmidt and Grady, 1995). Reversal and repair of damaged neurons with differing trophic requirements may require the concomitant action of two or more neurotrophins. Thus, single neurotrophin therapy would not be as effective in such CNS pathologies where several heterogeneous neuronal populations are affected, such as TBI. In other words, due to the biological specificity of neurotrophins, one neurotrophin may not be suitable for a particular therapeutic application. Thus, it would be advantageous to have a single neurotrophic molecule with combined trophic activity and the ability to interact with several neuronal populations affected by a particular trauma or neurodegenerative disease. The synergistic combined actions of a multifunctional neurotrophic molecule may be more physiologically relevant in certain complex pathological contexts. In addition to affecting a broader spectrum of neurons, single chimeric molecules would greatly simplify the inherent problems
associated with using heterogeneous “cocktails” of neurotrophins, such as differences in diffusion rates and protein stability issues, which might affect their therapeutic efficacy.

*Multineurotrophin with Multiple Neurotrophic Specificities (MNTS1)*

Structural similarities between neurotrophins suggest that they evolved from a common ancestor, but have since diverged functionally (Ibanez, 1994). Much research has focused on the molecular structures of neurotrophins and the binding epitopes that direct specificity to their respective Trk receptors and p75NTR. In order to gain perspective on the structural basis of neurotrophic function and specificity, Urfer and colleagues (1994) determined essential binding sites, ligand-receptor interactions, and the functional consequences of various amino acid sequence alterations of human mature NT-3 to its receptors, TrkC and p75NTR. The significance of various amino acid residues was evaluated using alanine-scanning mutagenesis, in which native amino acids are replaced with alanine to determine their contributions to the endogenous activity of NT-3. The large screen of NT-3 mutants that were generated were evaluated with several assays, including receptor-ligand binding affinities, autophosphorylation capacity, neurite extension of PC12 cells, and survival of primary neuronal cultures from chicken embryos (Urfer et al., 1994). Through these experiments, Urfer and colleagues (1994) learned which residues were critical for binding interactions and necessary for neurotrophic action, and subsequently constructed the first human multifunctional neurotrophin possessing multiple neurotrophic specificities (MNTS1; Figure 1.2).
In order to confer multifunctional neurotrophic action to an endogenous mature human NT-3, the 6 amino acids of NT-3 (YAEHKS; sequence indicated by the one-letter code of amino acids) were substituted by the corresponding 7 N-terminus residues from human NGF (SSSHPIF). This NT-3 variant was called Swap 1 (S1). The authors determined that these first 7 residues on NGF are important for mediating affinity for TrkA. Thus, this mutation allowed the modified NT-3 to bind to TrkA with high affinity without altering binding to its cognate receptor, TrkC. Mutational analyses also revealed the NT-3 variant, D15A (mutants are designated by the one-letter code for the native amino acid, the residue number, and the substituted amino acid). Switching aspartic acid residue 15 for alanine conferred binding to TrkB with high affinity. The MNTS1 molecule consists of both the D15A mutation and the S1 mutation allowing for high affinity binding to all three Trk receptors. MNTS1 activity was determined by assessing ligand binding to the extracellular domains of human TrkA, TrkB, TrkC, and p75NTR receptors. Displacement curves showed MNTS1 had high affinity for all three Trk receptors and similar affinity for the p75NTR receptor. Furthermore, MNTS1 induced

*Figure 1.2. Schematic of the MNTS1 molecule, which recapitulates the biological activity of NGF, BDNF, and NT-3 neurotrophins (adapted from Urfer et al. 1994)*
autophosphorylation of all three Trk receptors on PC12 cells (Urfer et al., 1994). The authors also assessed the biological relevance of MNTS1. By evaluating several neuronal populations, including nodose ganglion sensory neurons, dorsal root ganglion (DRG) neurons, and sympathetic neurons, they demonstrated that MNTS1 induced neuronal differentiation, increased neurite extension, and promoted the survival of a similar number of neurons as effectively as when a cocktail of two or three NTs was administered simultaneously (Urfer et al., 1994; Figure 2.2).

Taken together, the biochemical and biological responses of these mutations on human NT-3, as assessed by receptor binding affinities, autophosphorylation of Trk receptors, neuronal survival, differentiation, and neurite outgrowth, allowed for the construction of a human multineurotrophin with multiple specificities. This chimeric molecule mimics the biological activity of NGF, NT-3, and BDNF, and thus possesses a broader spectrum of activity. Prosurvival MNTS1 may therefore have a definitive advantage over single neurotrophin administration, especially after a heterogeneous injury such as TBI that affects several distinct neuronal populations. Testing chimeric neurotrophins as experimental tools to help clarify mechanisms of cell death and repair or as clinical agents to restore function after neurotrauma is of significant interest.

1.3 Stem Cells

Stem cells are undifferentiated cells that are found in the tissues of all multicellular organisms and are essential for development and survival. Two basic criteria need to be met when defining and characterizing a stem cell: 1) the ability to generate a least one identical “daughter” cell, which is necessary to maintain the stem cell
population (this is known as *self-renewal*) and 2) the ability to *differentiate* into a certain cell lineage, acquiring the phenotypic and functional features of a tissue-specific specialized cell type. In other words, stem cells can divide indefinitely to produce identical stem cells (symmetric division), or give rise to lineage-restricted cells that can differentiate into various cell types (asymmetric division).

Stem cells can be derived from early embryos after blastocyst formation as well as from fetal, postnatal, and adult tissues. Stem cells differ with respect to potency, which is a measure of the ability to give rise to all or only certain cell types. Embryonic stem cells (ESCs) are distinct from other types of stem cells in that they are derived from pre-gastrula embryos, and can give rise to *all* cells of an organism, including germ cells that generate haploid gametes. Thus, these cells are totipotent. Somatic stem cells are found in various tissues during development as well as in adulthood. In addition to their self-renewal capacity, somatic stem cells can terminally differentiate into the full range of relevant cell types found within their specific tissue. These pluripotent cells mitotically generate diploid, tissue-specific cell types of all three germ layers (endoderm, mesoderm, or ectoderm). Progenitor cells are the direct descendants of stem cells and are less potent, but do self-renew and retain the ability to become at least one mature cell type. Under certain cellular contexts, progenitor cells have the capacity to generate additional mature cell types; thus these cells are multipotent.

*Therapeutic stem cells*

As mentioned previously, stem cells are found throughout various tissues for the duration of life of an organism. During development in the 3-5 day old blastocyst, inner cells located in the inner cell mass (ICM) give rise to every cell in the entire organism. In
adult tissues, stem cells serve as an internal repair and replacement system, and are located in several tissues including skeletal muscle, bone marrow, skin, peripheral blood and blood vessels, teeth, heart, intestine, gut, liver, ovarian epithelium, testis, spinal cord, and brain (Preynat-Seauve and Krause, 2011). The following paragraphs will primarily discuss stem cells with therapeutic potential for the treatment of CNS trauma and neurological diseases.

Embryonic stem cells

ESCs are derived from the ICM of early embryos called blastocysts. As mentioned previously, these cells can make up every other cell in the body, and thus have the greatest amount of potency and hence, broad clinical application. However, because the embryo is destroyed in the process of harvesting ESCs, ethical concerns with regard to the use of these cells for therapeutic purposes are still a significant source of debate. Previous ESC transplantation studies after TBI revealed enhanced neurological outcomes on relevant motor tests at several time points post injury. However 20% of transplanted animals developed tumors within 7 weeks (Riess et al., 2007), suggesting that ESCs may not be the optimal modality for cell transplantation into the injured brain. Thus, scientists and clinicians have looked to other sources of stem cells for use in cell-based therapies.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal stem cells found in distinct niches. They differentiate into non-blood cell types (unlike hematopoietic stem cells), including chondrocytes, adipocytes, osteocytes, and other types of connective tissue cells (Rompolas et al., 2013). An encouraging aspect of using MSCs in the clinic is the autologous component of this approach. MSCs can be readily harvested from the
patient’s own bone marrow, adipose tissue, umbilical cord, lung, or blood (Phinney and Prockop, 2007). Thus, the possibility of host rejection after cell transplantation is reduced and immunosuppressant drug therapy is often not essential (Pisciotta et al., 2013). MSCs transplantation after TBI has been reported to enhance endogenous hippocampal neurogenesis (Mahmood et al., 2004), ameliorate histopathological outcomes, increase BDNF expression and angiogenesis, as well as reduce motor and cognitive deficits (Jiang et al., 2012). However, other studies have shown that acute intravenously (IV) administration of MSCs did not mitigate motor or cognitive impairment after TBI and MSC presence in brain tissues was negligible (Harting et al., 2009).

**Induced pluripotent stem cells**

Induced pluripotent stem cells (iPSCs) have generated recent interest for therapeutic administration because their use alleviates some of the ethical concerns associated with using ESCs. iPSCs are artificially derived from adult somatic cells. Adult somatic cells lack the capacity for self-renewal as they have already committed and differentiated to a mature cell type. By genetically forcing the expression of specific genes that are found in ESCs, scientists have been able to “reprogram” murine somatic cells and return them to an immature stem-like state (Takahashi and Yamanaka, 2006). This groundbreaking discovery led by Yamanaka and colleagues (2006) resulted in the 2012 Nobel Prize in Physiology or Medicine. The critical factors required for generating genetically-induced iPSCs include Oct4, Sox2, c-Myc and Klf4. Subsequent research on human cells by James Thompson’s team replicated Yamanaka’s findings and showed that Nanog and Lin28 factors facilitated the reprogramming of somatic cells (Yu et al., 2007). Additional research has shown that iPSCs can be generated from skin, stomach, liver, and
blood cells. While this pioneering work has generated significant interest, there are important issues still being discussed concerning long-term effects. Delivery of stemness-inducing factors to adult somatic cells may require the use of retro- or lentiviral vectors, which integrate within the genome of the somatic cell permanently, leading to unpredictability concerns and potential tumorigenicity. iPSCs have recently been evaluated in rats and rhesus macaque models of CNS injury. The authors tracked human iPSCs \textit{in vivo} and reported targeted migration towards lesion sites and improved motor faculties within 30 days following administration (Tang \textit{et al.}, 2013).

\textit{Stimulus-triggered acquisition of pluripotency (STAP) cells}

In two recently published studies, Obokata and colleagues (2014a; 2014b) effectively reprogrammed committed adult somatic cells with exposure to a low-pH treatment for 25 min, which permitted acquisition of pluripotent stemness. This process was deemed stimulus-triggered acquisition of pluripotency (STAP) and could occur without transduction of specific transcription factors, such as those used to reprogram iPSCs. STAP cells are still very new and have not yet been assessed in models of neuropathology.

\textit{Neural stem/progenitor cells}

Neural stem cells (NSCs) are multipotent and give rise to an identical “daughter” NSC or differentiate into the main cellular phenotypes that make central and peripheral nervous systems. The mammalian brain was originally thought to be a postmitotic structure, however it has now been well established that NSCs not only exist in the developing CNS, but also within specific adult brain regions. These findings subsequently provided evidence for the existence of adult neurogenesis, or the birth of
newborn neurons in the postnatal brain. Newly-generated neurons actively integrate and participate within local networks, and provide distinct contributions to existing circuitry and brain function (Patton and Nottebohm, 1984; Gage 2000; Toni and Sultan, 2011). NSCs are considered to be a heterogeneous population comprised of several distinct stem cells of the same lineage coexisting together. Distinguishing characteristics of NSCs include their neurogenic state (active or latent) and contribution (to maintain homeostasis or participate in regeneration) (Göritz and Frisén, 2012). In the hippocampus, there are two types of glia-like stem cells that can give rise to hippocampal granular neurons: horizontal and radial NSCs, which are active and quiescent respectively. A recent paper reported that these morphologically distinct cells responded differentially to physiological and pathological stimuli, as well as to aging (Lungert et al., 2010). Much remains to be learned about the precise contributions and regulating-mechanisms of the various NSC types residing in the mammalian brain. In neuropathologies characterized by selective cell loss and dysfunction, such as degenerative disorders and CNS injury, NSCs/NPCs have spurred significant enthusiasm as potential regenerative agents to rescue cell loss, plasticity, and function.

One of the objectives of this dissertation project was to investigate NPC transplantation and hippocampal-dependent cognitive outcomes after moderate TBI. Thus, the following section will focus primarily on neural stem and neural progenitor cell characteristics as well as the phenomenon of adult hippocampal neurogenesis.

*Neurogenesis*

The process by which mature neurons are generated from neural stem/progenitor cells is called neurogenesis. Neurogenesis is most active during perinatal development;
however it also occurs in the adult mammalian brain within two discrete regions: the SVZ of the lateral ventricles and the SGZ of the hippocampal dentate gyrus. The SVZ is comprised of a subpopulation of astroglial-like cells (B1 cells) that function as the putative NSC and give rise to intermediate progenitor cells (IPCs; also known as transient amplifying cells or C cells). Neuronal maturation of these cells consists of C cell division to generate neuroblasts (A cells) that migrate via the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into granule and periglomerular interneurons (Ming and Song, 2011). The SGZ is the interface between the dentate hilus and dentate gyrus. Similar to SVZ neurogenesis, radial glia-like cells (RGCs; Type 1 cells) are the primary NSC in the adult SGZ. These cells can divide symmetrically or asymmetrically to give rise to additional multipotent cells, non-radial precursors and IPCs (Type 2a and Type 2b cells, respectively). Division of IPCs generates neuroblasts, which migrate through the granular cell layer (GCL), and eventually become functional mature granular neurons.

CNS stem cell development is highly spatially and temporally regulated. The timing of maturation is mediated by several factors including molecular cues from glial cells, chromatin configurations, and DNA methylation modifications (Jessell and Sanes, 2000; Pereira et al., 2010). Positional cues also contribute to the highly-regulated process of stem cell development, such as morphogenic gradients that pattern neural tissue (Temple, 2001). Furthermore, certain regions of the CNS can act as “organizers” and molecularly drive neuronal patterning through the release of diffusible signaling factors (Kiecker and Lumsden, 2012).
While much is known about developmental neurogenesis (Jessel and Sanes, 2000), adult neurogenesis is less fully understood. Adult neurogenesis occurs over a period of about 1-6+ months, a duration that varies in a species-specific manner. Rodent neurogenesis takes approximately 4-6 weeks (Gage and Temple, 2013), while adult neurogenesis in nonhuman primates can exceed 6 months (Kohler et al., 2011). At the end of the maturation period, newly-generated neurons become indistinguishable from perinatal-born neurons. There are five major processes that constitute neurogenesis: cell proliferation, fate specification, migration, neurite integration, and maturation (Ming and Song, 2005). Each stage is fundamental for the proper development of functional neurons.

Adult neurogenesis was first described over half a century ago by Joseph Altman, who demonstrated the presence of proliferating neurons in the adult mammalian brain. Using tritiated thymidine, which incorporates into the DNA of dividing cells, and autoradiographic labeling, he showed newly-generated neurons in the hippocampal DG of both adult rats and cats (Altman, 1962). Further investigations supported these findings, including those demonstrating that adult neurogenesis occurs in the olfactory bulb and that immature neurons migrate via the RMS (Altman, 1969), that neurogenesis exists in adult songbirds (Goldman and Nottebohm, 1983), as well as Paton and Nottebohm’s (1984) work showing the functionality of newly-generated neurons.

A later series of experiments demonstrated the impact of external environmental stimuli on the induction and degree of adult neurogenesis, specifically in the hippocampus. These studies elucidated how stress negatively affected NPC proliferation in the adult DG (Gould et al., 1992), how exercise substantially enhanced levels of
neurogenesis (Van Pragg et al., 1999), that environmental enrichment resulted in pronounced neurogenic effects (Kemerpmann et al., 1997), that learning paradigms could influence adult neuronal cell survival (Dobrossy et al., 2003), and that pharmacological exposures, such as antidepressant drug administration (Malberg et al., 2000), could also affect neurogenic processes. Age-related perturbations in neurogenesis were chronicled as well. Kuhn et al. (1996) showed that in the aged rat, adult neurogenesis was decreased due to a reduction of NPC proliferation. Most of the investigations up to this point were performed in adult rodent models; however in 1998, Eriksson and colleagues (1998) published the critically important finding that neurogenesis occurred in adult humans as well. More recently, an elegant investigative study carried out by Spalding et al. (2013) presented strong evidence for adult hippocampal neurogenesis in human brains. By measuring the concentration of nuclear-bomb-test-derived $^{14}$C in genomic DNA, they confirmed the presence of adult hippocampal neurogenesis in humans. Furthermore, the authors assessed the generation and turnover of adult-born hippocampal neurons and found that approximately 700 new neurons are added each day and that one-third of hippocampal cells are subject to exchange (Spalding et al., 2013)

Signaling mechanisms directing adult neurogenesis

The anatomical location and cellular/molecular constituents of the stem cell niche are fundamentally important for the generation and maintenance of NSCs/NPCs. In the adult brain, only NPCs located in the SVZ and SGZ can adopt a neuronal fate under normal physiological conditions. NPC proliferation does occur in other regions of the adult brain, however terminal differentiation into neurons is not observed. NPCs in these nonneurogenic regions remain in a precursor state or differentiate into glial cells (Gage
and Temple, 2013). That being said, if NPCs from these nonneurogenic regions are isolated and expanded in vitro, and treated with various differentiation-inducing factors, neuronal differentiation can indeed take place, indicating that extrinsic factors play a significant role in neuronal fate determination (Palmer et al., 1999). Consistent with these findings, if SVZ- or SGZ-derived NPCs are ectopically transplanted into nonneurogenic regions, they will adopt a glial phenotype (Seidenfaden et al., 2006). Hippocampal SGZ progenitor cells transplanted into the SVZ will adopt SVZ-NPC properties, migrating via the RMS and differentiating into olfactory bulb neurons (Sulhonen et al., 1996). In parallel, NPCs from the lateral ventricles will exhibit hippocampal neuron characteristics after transplantation into the SGZ (Gage and Temple, 2013). Furthermore, NPCs derived from spinal cord will become hippocampal granular neurons following transplantation into the SGZ (Shihabuddin et al., 2000). These findings support the notion that extrinsic cues from the stem cell niche are fundamental for creating and maintaining a permissive environment to support neurogenesis.

The cellular components that contribute to and promote neurogenesis in proneurogenic niches include astrocytes (Song et al., 2002), microglia (Sierra et al., 2010), and endothelial cells (Shen et al., 2004). Astrocytes in the hippocampus significantly influence adult neurogenesis. Work by Song and colleagues (2002) showed that hippocampal astrocytes promoted the differentiation and integration of adult-born neurons. Furthermore, blockage of Wnt signaling pathways inhibited the proneurogenic properties of hippocampal astrocytes, suggesting this signaling system is critical for their SGZ neurogenesis-inducing activity (Lie et al., 2005). The cerebrovasculature plays a critical role in neurogenesis as well (Palmer et al., 2000). The vasculature and nervous
systems have co-developed evolutionarily via common genetic pathways (Palmer et al., 2000; Carmeliet, 2003). Thus, coordinated functional interactions and anatomical associations between neurogenic regions and surrounding cerebrovasculature are not surprising. Unlike in the SVZ, there is active angiogenesis and vasculature remodeling occurring together with hippocampal neurogenesis. In one study, testosterone exposure induced expression of vasculature endothelial growth factor (VEGF). In response, endothelial cells surrounding the stem cell niche vasculature began producing high levels of BDNF, which led to the mobilization and integration of newborn neurons in the vocal centers of songbirds, and subsequently affected hippocampal-dependent memory (Cao et al., 2004). Furthermore, enhanced VEGF-induced SGZ neurogenesis was positively associated with improved performance of rodents on the MWM task, whereas inhibition of VEGF signaling abolished exercise- and enrichment-induced hippocampal neurogenesis (Cao et al., 2004).

Additional extrinsic factors that modulate NSC signaling include morphogens (such as Wnt, Notch, sonic hedgehog (shh) signals) and growth factors (BDNF, NT-3, fibroblast growth factor (FGF)-2, VEGF, etc.) (Faigle and Song, 2013). Neurotransmitters, including GABA, glutamate, serotonin, and DA, have also been implicated as modulatory factors in adult neurogenesis (Suh et al., 2009). Intrinsic developmental programs have been extensively investigated and established as major contributors to NSC/NPC proliferation and maturation (Fuentealba et al., 2012). These include transcription factors such as Sox2, Tlx, Ascl1/Mash1, NeuroD, Pax6, and CREB, among others. Epigenetics also play a role in regulating adult neurogenesis via histone modification and DNA methylation (Faigle and Song, 2013).
Relevant to the scope of this dissertation project, neurotrophin-Trk binding interactions also elicit important signaling cues that induce the survival and differentiation of neuronal precursor cells during maturation. In cortical NPCs, NT-3 binding selectively regulates cell cycle exit and neuronal differentiation (Lukaszewicz et al., 2002). BDNF and NT-3 are synthesized and secreted by NPCs themselves and signal in an autocrine/paracrine fashion to regulate their survival and neuronal differentiation (Barnabe-Heider and Miller, 2003). Work by Bartkowska and team (2007) showed that knocking down TrkB or using dominant-negative Trk transgenic mice resulted in significant decreases in progenitor cell proliferation, delayed generation of neurons (but not astrocytes), as well as ectopic migration of newly-generated neurons. Overexpression of BDNF reversed these abnormalities (Bartkowska et al., 2007).

The diversity of molecular signals and cellular interactions that regulate stem cell niches and influence neurogenesis has yet to be fully delineated. An understanding of the temporal and spatial contributions to adult neuron maturation may elucidate new therapeutic targets for stimulating and mobilizing endogenous neurogenesis after injury. 

*Functional significance of adult hippocampal neurogenesis*

Since the first publication of adult neurogenesis in 1962, numerous studies have extensively investigated the molecular components and signaling cues of adult neurogenic processes. Although it is now agreed that hippocampal neurogenesis in mammals continues throughout life, the exact functional consequences of these unique cellular events remain to be determined. In order to gain perspective on the functional contributions of adult-born neurons, is it important to understand their unique characteristics that allow them to effectively integrate within the existing hippocampal
circuitry. Immature adult-born neurons are unique to developmentally-born neurons in that they target *preexisting* synapses. After their birth, newly-generated neurons are not initially integrated within the local circuitry, however they are responsive to transmitters that “spill over” from local neighboring synapses (Song *et al.*, 2002). This transmitter spill over may act as a chemoattractant and encourage newborn filopodia to grow towards active synapses (Toni and Sultan, 2011). Additional differences between adult-born immature neurons and developmentally-born neurons include the unique electrophysiological characteristics exhibited by the former in the first few weeks after their birth. For example, newly-generated hippocampal neurons have a lower threshold for excitability, are more plastic than mature granular neurons, and more readily participate in LTP, a molecular correlate of learning and memory (van Pragg *et al.*, 1999; Snyder *et al.*, 2005; Ge *et al.*, 2007). Furthermore, immature neurons display high input resistance and are highly efficient at transducing ionic currents into corresponding membrane depolarization (Gage and Temple, 2013). Work by Toni *et al.* (2008) demonstrated structural and functional evidence showing axons of adult-born granular neurons forming synapses with interneurons in the hilus, mossy cells, and CA3 pyramidal neurons. These newborn neurons were integrated within the adult hippocampal circuitry and released glutamate as their primary neurotransmitter (Toni *et al.*, 2008).

The functions of the hippocampus are spatially regulated along the dorsal-ventral axis according to distinct circuits (Zhao *et al.*, 2008). The dorsal hippocampus preferentially contributes to learning and memory, while the ventral hippocampus plays a role in affective behaviors (Bannerman *et al.*, 2004). Newly-generated adult neurons contribute to both of these functions, however the precise associations are still not fully
understood. For the scope of this project, we will focus primarily on dorsal hippocampal neurogenesis and its influence on learning and memory.

A series of correlative studies revealed that enhanced DG neurogenesis is associated with improved performance in several hippocampal-related cognitive behaviors. As mentioned previously, external environmental exposures can stimulate or decrease adult neurogenesis, which in turn can enhance or impair hippocampal-dependent learning and memory, respectively. Voluntary running greatly enhances DG proliferation, whereas environmental enrichment promotes the survival of immature neurons (van Praag et al., 1999, Tashiro et al., 2007). Both of these external stimuli improved the performance of young and aged mice in the MWM (Zhao et al., 2008). Additional positive regulators of adult neurogenesis and memory function include exposure to specific learning paradigms as well as antidepressant drug administration (Gould et al., 1999; Anacker et al., 2011). The mechanisms by which these exposures enhance hippocampal neurogenesis and learning and memory are still being delineated, however BDNF has been shown to play a significant role in synaptic plasticity, as well as in the positive effects of exercise and antidepressants on neuronal survival (Schmidt and Duman, 2007; Bekinschein et al., 2011). A reciprocal correlation is observed in negative stimulus conditions. During periods of stress or deprivation, adult neurogenesis is compromised in the DG, which is concomitant with marked functional impairment on relevant hippocampal-dependent behavioral tests (Zhao et al., 2008; Lazarov et al., 2010). Aged animals also have depressed levels of hippocampal neurogenesis and subsequent poor performances on the spatial memory-dependent MWM task (Kuhn et al., 1996; Drapeau et al., 2003).
Hippocampal-dependent memory can refer to navigational and contextual (spatial) memory, olfactory memory, or aversive memory (Squire et al., 2004; Goosens, 2011). The hippocampus also contributes to new memory formation, recall, and sustainability (Gould et al., 1999; Shors et al., 2001). Recent computational network and theoretical perspectives suggested that newborn neurons in the DG act as mediators of “pattern separation” (Sahay et al., 2011). The DG is a sparse neuronal network, and the contribution of this network to hippocampal function is still being delineated. However, theoretical models suggest that the role of the DG involves both recoding and reorganizing cortical inputs into a specific orthogonal representation (Gage and Temple, 2013). By manipulating adult neurogenesis via altering the external environment or by using genetic models, researchers have found that an animal’s ability to recall familiar scenarios and/or discriminate between similar environments can be significantly altered. The supposition that neurogenesis and cognitive function have a direct association remains to be fully established, but an association does exist nonetheless. Taken together, these findings bring forth an important question: Can endogenous neurogenesis be harnessed and enhanced as a potential neurorestorative therapy for pathological conditions in which hippocampal neuronal loss results in debilitating cognitive impairment?

**Neurogenesis and TBI**

In the acute period after brain trauma, there are significant increases in NPC proliferation and neurogenesis in the SGZ of the hippocampus (Dash et al., 2001; Kernie et al., 2001; Chirumamilla et al., 2002; Urrea et al., 2007; Gao et al., 2009). A recent study showed a similar acute (time of tissue extraction averaged 6.5 hours post injury)
proliferative increase of NPCs in human brains after TBI (Zheng et al., 2013). Trauma-induced proliferation and neurogenesis have been reported in several models of CNS injury (Lie et al., 2004; Parent et al., 2006; Miles and Kernie, 2008). However, the relevance of injury-activated neurogenesis has yet to be fully clarified, but may represent a limited effort at self-repair (Blaiss et al., 2011).

IPCs in the SGZ are selectively vulnerable after brain injury. Studies have shown that 24 h following a moderate brain insult, 80% of the degenerating DG cells were newly-generated immature neurons (Gao et al., 2008). Yu and collaborators (2008) reported that DCX-positive Type 2b cells were selectively reduced 72 h after TBI; however early nestin-positive Type 1 precursors were significantly upregulated at this time point. Within 7 days, the Type 2b cell population had reestablished itself, which the authors attributed to acutely enhanced Type 1 precursor proliferation.

TBI-induced hippocampal neurogenesis appears to be a relatively transient phenomenon. Studies have demonstrated that DCX-positive immature neurons are chronically depressed to below baseline levels at 6, 8, and 12 weeks post trauma (Potts et al., 2009; Atkins et al., 2010; Blaiss et al., 2011). The mechanisms responsible for the chronic loss of immature neurons and decreased levels of neurogenesis are not clear, but studies have suggested that hippocampal progenitor pool depletion, reduced neuronal differentiation, ectopic migration patterns, aberrant synaptogenesis, and/or proapoptotic environmental conditions after TBI may be contributing factors (Monje et al., 2003; Hattiangady and Shetty, 2010; Blaiss et al., 2011; Sanchez et al., 2012; Shetty, 2014). Proper migration and synaptogenesis are two critically important stages for neuronal maturation in the adult hippocampus. Dentate hilar interneurons are selectively
vulnerable in the acute stages following TBI in both humans and experimental models (Lowenstein et al., 1992). Several of these interneurons express reelin, a secreted guidance cue which modulates NPC migration. Loss of these hilar interneurons and reelin expression leads to aberrant hilar-ectopic NPC chain migration (Gong et al., 2007). Several studies have showed aberrant synaptic reorganization and hippocampal network destabilization post injury (Hunt et al., 2011). Experimental models of brain injury have revealed hyperexcitability of dentate granular cells (DGCs), premature mossy fiber sprouting, and atypical occurrence of basal hilar dendrites from immature neurons in the posttraumatic DG (Santhakumar et al., 2000; Diaz-Cintra et al., 2009; Sanchez et al., 2012). These pathological events may contribute to perturbed adult hippocampal neurogenesis. Injury-induced modifications also facilitate the onset of seizure activity observed in TBI animal models (Golarai, et al., 2001) and patients who have experienced a TBI are predisposed to developing post traumatic epilepsy (PTE) at chronic time points post insult (Annegers et al., 1998). Thus, trauma-induced restructuring of hippocampal networks remains a critical area of research.

Hostile microenvironmental conditions induced by TBI, such as neurotransmitter level alterations, neuroinflammation, and prevalence of apoptogenic factors, may also contribute to fewer proliferating immature neurons integrating within the hippocampal circuitry. Neuroinflammation is prevalent in the traumatized brain and has been associated with deficits in cognition (Zhao et al., 2008). Several studies assessing neurogenesis levels in response to chronic inflammation showed that adult neurogenesis is depressed; however neurogenic decreases are reversible with anti-inflammatory treatment (Ekdahl et al., 2003; Monje et al., 2003). Perturbations in chronic adult
hippocampal neurogenesis may therefore contribute to some of the cognitive deficits following brain injury.

Research demonstrating that hippocampal neurogenesis is *acutely* enhanced in pathological conditions, such as CNS trauma, supports ongoing investigations assessing whether this process contributes to the degree of functional recovery sometimes observed after injury. Modifying the injured milieu to be more permissive for sustained neurogenesis may result in reduced levels of hippocampal memory impairment and improved functional outcomes. Taken together, this research demonstrates that acute injury-activated NPC proliferation/neurogenesis occurs, but this phenomenon may not be sustained and/or functionally relevant due to selective loss of immature hippocampal neurons, depletion of the progenitor pool, ectopic IPC migration and aberrant synaptogenesis, and/or TBI-induced proapoptotic environmental conditions. Additional interventions may be necessary to fully engage and sustain true functional neurogenic responses to trauma, and successfully repair the injured brain.

*Exogenous neural stem/progenitor cell transplantation after CNS injury*

As discussed, stem cell therapy has emerged as a clinically-compelling option for regenerative medicine and the treatment of degenerative and traumatic injuries. Due to the inherent ability of stem cells to differentiate into relevant cell types, it was thought that under pathological conditions associated with significant neural cell loss, stem cells could replace dying and dysfunctional cells and integrate within cellular circuits to restore compromised tissues. Interestingly, several studies have now demonstrated neuroprotection and functional restoration *prior* to exogenous stem cell circuit integration (Martino and Pluchino, 2006). Thus, there has been a paradigm shift with regard to the
remedial effects of stem cells transplantation. The inherent salutary properties of NPC transplantation may not be restricted to cell replacement mechanisms, but also attributed to their secretory trophic properties and immunomodulatory activity. NSCs/NPCs release a potent combination of trophic factors that can alter the molecular composition of the local milieu and evoke favorable endogenous responses from host cells (Lu et al., 2003). The ability of transplanted stem cells to influence the surrounding host environment without direct integration and cell replacement is known as the “bystander” effect. As discussed by Baraniak and McDevitt (2010), transplanted stem cells can actively modulate the microenvironment through the release of various trophic factors and extracellular matrix (ECM) molecules. These secreted factors can act in an autocrine manner (on the secreting cell itself) or act to influence the behavior of neighboring cells (paracrine actions). Furthermore, through the constitutive release of certain factors, transplanted cells can reduce glial scar formation (Teng et al., 2002), as well as enhance survival and functionality of endogenous glial and neural progenitor cells after injury (Martino and Pluchino, 2006).

The observation that NSCs/NPCs retain an inherent capacity to secrete neurotrophic factors is relevant to the studies conducted in this dissertation project. As discussed in the previous section, neurotrophins are critical for the structural and functional integrity of the nervous system, and can repair and protect vulnerable neurons after traumatic insults. Work by the Tuszynski group has demonstrated that grafted C17.2 NSCs constitutively secreted NGF, BDNF, and GDNF after CNS injury (Lu et al., 2003). Their findings suggested that diffusive trophic support from exogenous NSC transplants promoted extensive axonal growth in injured host tissues. By harnessing and possibly
augmenting the salutary secreted factors released by stem cells, the implications for using cellular transplantation techniques for tissue protection and restoration after CNS injury are widespread.

Along these lines, much research has investigated the effects of NPC-secreted neurotrophins and growth factors on endogenous reparative activity after TBI. The brain is capable of considerable structural compensation and circuit reorganization after injury, which may contribute to the spontaneous, albeit limited, functional recovery that is sometimes observed after CNS injury (Kernie et al., 2001; Cramer, 2008). As discussed previously, TBI upregulates endogenous neurogenesis in both the SGZ and the SVZ. This response may be attributed to neurorestorative strategies evoked by the injured brain that are inadequate, as indicated by residual behavioral deficits and eventual decline of functionally-active hippocampal neurogenesis to below non-injured levels (Potts et al., 2009; Atkins et al., 2010; Blaiss et al., 2011). An emerging area of interest is the notion that exogenous cell transplantation has the capacity to evoke endogenous trophic-dependent reparative strategies, such as hippocampal neurogenesis, as well as provide trophic support to damaged and dying neurons after CNS injury (Jin et al., 2011). Recent work has reported that NSC transplantation elicited neurogenic responses in the injured hippocampus comparable to sham levels 2.5 months post injury. Injured non-transplanted animals on the other hand exhibited a 37% reduction of endogenous DG neurogenesis compared to uninjured hippocampi in sham groups (Hattiangady and Shetty, 2010). These NSC graft-induced changes correlated with reversal of recognition and spatial memory deficits, as well as reduced depressive-like behavior. In addition to NPC transplantation-mediated rescue of endogenous neurogenesis, the authors further
attributed improved functional outcomes to constitutive secretion of neurotrophic factors, neuronal differentiation of grafted cells, and preservation of vulnerable neurons (Hattiangady and Shetty, 2010). Thus, cell-based treatment strategies that target and modulate neurogenesis, circuit plasticity, and other reparative processes may effectively promote functional recovery after TBI.

Another significant advantage of NSCs/NPCs transplantation is the tropic capacity exhibited by these cells. NSCs/NPCs retain an ability to migrate to various areas of abnormal histopathology by homing in on gradients of chemoattractants such as pro- and anti-inflammatory cytokines and chemokines that are present at the site of inflammatory lesions (Belmadani et al., 2006). NPCs also demonstrate extensive tropism towards gliomas in vivo. Aboody et al. (2000) showed NPCs migrated significant distances through normal tissue when transplanted distally from invading tumors.

There is significant neuroinflammation in the posttraumatic brain and several studies have elucidated the immunoregulatory properties of transplanted NSCs/NPCs after CNS injury (Pluchino et al., 2005). In a mouse model of chronic neuroinflammation, work by Pluchino and colleagues (2005) demonstrated that transplanted NPCs expressed immune-relevant cell surface receptors and utilized constitutively-activated integrins to enter the inflamed CNS. The authors found that these undifferentiated cells survived for long periods and through recurrent bouts of inflammation. T helper (T<sub>H</sub>) cells are part of the adaptive immune response and depending on the subtype, can confer anti- or proinflammatory effects within the traumatized brain (Romagnani, 2000). Transplanted NPCs favorably modulated the inflamed environment by inducing apoptosis of infiltrating proinflammatory T<sub>H1</sub> cells,
but not anti-inflammatory $T_H2$ cells (Pluchino et al., 2005). Several studies have reinforced findings showing immunomodulatory activity of NPCs. Transplanted NPCs contributed to the attenuation of proinflammatory factor expression, such as tumor necrosis factor alpha (TNF-\(\alpha\); Ryu et al., 2009), reduced proinflammatory interleukin (IL)-6 levels, and decreased IL-17-secreting $T_H17$ immune cells via NPC-mediated leukemia inhibitory factor (LIF) secretion (Cao et al., 2011). Hori et al. (2008) investigated the immunogenic and antigenic properties of CNS progenitor cells and found that transplanted NPCs did not express any detectable levels of major histocompatibility complex (MHC) class I or II molecules \textit{in vivo}, suggesting that NPCs are inherently immunoprivileged.

Another component of a successful therapeutic cell transplantation strategy is the ability of transplants to survive for extended periods, or at least long enough to confer positive effects to host tissues. The injured brain is a hostile environment with injurious degenerative cascades persisting for months to years after the insult (Bramlett and Dietrich, 2002). Thus, due to the progressive nature of TBI, it is crucial to employ sustainable therapeutic strategies that confer beneficial effects for long periods. Encouragingly, studies have shown that NPCs transplanted directly into brain parenchyma promoted long-term functional improvement after TBI. Shear \textit{et al.} (2004) evaluated the safety and efficacy of NPC transplantation for extended periods after TBI. They found that NPCs survived, migrated, differentiated, and functionally integrated within the injured brain. Behavioral assessments showed improvement in motor skills as early as one week, most likely due to secreted trophic factors and bystander effects, and
cognitive improvement at 1 month. This functional recovery persisted out to 14 months (Shear et al., 2004).

An emerging focus of cell transplantation involves the ability of cells to act as “minipumps” for the delivery of therapeutic agents. The genetic modifiability of these cells increases their potential as an ideal modality for treatment of CNS injury and degenerative diseases. Numerous methods have been explored to deliver genetic material into NPCs, including transient methods such as transfection with calcium phosphate, as well as long-term sustained transduction mediated by viral vectors. For improving long term outcomes after the onset of progressive disorders such as TBI, it is important for the foreign DNA constructs to integrate within the NPC genome for stable, long-term transduction/transfection and for the foreign DNA constructs to be passed on to cell progeny. This can be achieved using viral-mediated transduction techniques. An additional advantage of using viruses to transduce cells is the efficiency with which infection is achieved. Furthermore, using lenti- or adenoviruses allows for the infection of both dividing and non-dividing cells (Jandial et al., 2008). This is an important factor to consider when engaging NSCs/NPCs (which continue to divide) as the cellular modality for drug delivery.

Several studies have revealed enhanced reparative effects when NSC/NPC transplantation was coupled with gene therapy, such as genetic overexpression to release certain prosurvival factors. In recent work it was demonstrated that NSCs overexpressing BDNF ameliorated neurological motor deficits and increased the expression of SYP and ProSAP1/Shank2, two synaptic proteins (pre- and post-, respectively) involved in neural circuitry and brain function, compared to naïve-NPC grafts (Ma et al., 2012). Additional
research found that transplanted NSCs genetically modified to secrete NT-3 exhibited significant survival and neuronal differentiation 4 weeks post brain injury relative to non-transduced NSCs (Park et al., 2006). Furthermore, sustained overexpression of glial cell-derived neurotrophic factor (GDNF) in NPCs significantly enhanced survival, neuronal differentiation, and preservation of cognitive capacity after TBI compared to NPC transplantation alone (Bakshi et al., 2006).

Of note, Lu et al. (2003) showed that NSCs genetically modified to overproduce NT-3 caused a reciprocal effect, downregulating the endogenous expression of GDNF and BDNF by transplanted NSCs in vivo. This decrease in production of BDNF and GDNF also reduced local motor axon growth (Lu et al., 2003). These data suggest that neurotrophic factor expression in stem cells is dynamically modulated and influenced by the production of other growth factors in the cell. This potential interaction between specific cells and multiple factors is critically important to consider and assess when designing and investigating genetic overexpression studies for therapeutic applications.

In summary, NSCs/NPCs are potentially advantageous for TBI in that they inherently secrete salutary factors that mitigate cellular dysfunction and hostile environmental conditions, as well as evoke endogenous trophic and neurogenic responses. They exhibit tropism and can migrate to compromised tissues, are immunoprivileged and immunomodulatory, and can survive for long periods in the injured brain. They can also be genetically altered to deliver therapeutic molecules after CNS injury. The clinical implications of harnessing and employing these remedial progenitor cells to develop novel strategies for tissue preservation and regeneration after TBI are of significant interest. As previously described, MNTS1 is a chimeric
neurotrophin that retains the ability to bind to all Trk receptors and engage in prosurvival
signaling pathways that are critical for the maintenance and repair of the CNS. We
hypothesized that transplanting NPCs genetically modified to secrete MNTS1 would be
effective in alleviating neuropathological outcomes. A multitargeted combinatorial
approach using NPCs and MNTS1 will not only engage affected neurons in prosurvival
Trk signaling pathways, but possibly also restore endogenous reparative strategies, such
as adult hippocampal neurogenesis, to preserve vulnerable mature and immature neurons,
and contribute to functional and cellular recovery after neurotrauma.

1.4 Pharmacotherapeutics

With the advent of reliable screening techniques, novel small molecules are being
discovered with significant clinical potential for various pathological conditions. Certain
pharmacotherapies may be advantageous for treating CNS trauma over natural,
physiologically-occurring interventions in that they can be systematically manipulated to
assess dosing and potency. Controlled dose-response assessment would allow one to
determine the minimal amount of drug needed to achieve therapeutic efficacy and thereby
decrease the possibility of unwanted side effects on non-target tissues during
administration. Pharmacotherapies can be designed to have favorable metabolic profiles,
low toxicity, and to readily cross the BBB. In order to assess experimentally-enhanced
neuroreparative strategies for the treatment of TBI, this dissertation project additionally
investigated the therapeutic potential of a small proneurogenic compound that was
previously shown to rescue neurogenesis in animal models with pathologically high
levels of cell death of newborn neural precursor cells in the adult hippocampus. As
previously described, disruption and depression of adult hippocampal neurogenesis and
subsequent hippocampal-dependent memory impairment are some of the most debilitating pathological consequences observed after neurotrauma.

In an elegant experiment by Pieper and colleagues (2010), 1,000 compounds were selected from a library of 200,000 drug-like chemicals for use in a target-agnostic, unbiased *in vivo* screen to identify new agents that augment the net magnitude of hippocampal neurogenesis in 12 week old wild type mice. Candidate molecules were selected according to the following criteria: 1) molecular weight of 350-650; 2) at least two hydrogen bond donors; 3) at least three hydrogen bond acceptors; 4) more than five degrees of unsaturation; 5) net charge not to exceed +2 or −2; and 6) chirality with 1-3 asymmetric centers (Pieper *et al.*, 2010). These criteria were designed to include chemicals that were readily able to form hydrogen bonds and electrostatic contacts with endogenous receptors. Compounds were randomly designated into one of 100 pools (10 compounds per pool). In order to assess neurogenic potential, 10 μM of each compound was dissolved in artificial cerebrospinal fluid (aCSF; 100 μM total solute concentration) and infused at a constant rate (0.5 μl/h) for 7 days into the left lateral ventricle of two living mice via subcutaneously-implanted Alzet osmotic minipumps. FGF-2 and aCSF were employed as positive and negative controls, respectively. During the 7-day infusion period, mice received daily intraperitoneal (IP) injections of BrdU (50 mg/kg) to label proliferating precursor cells in the SGZ of the dentate gyrus. Because 40% of newborn neural precursor cells in the hippocampus die within one week (Pieper *et al.*, 2010), the assay was designed to identify agents that enhance the net magnitude of hippocampal neurogenesis by either stimulating proliferation of these cells or by blocking their death. After 7 days of compound administration, 10 pools were found to be associated with
significantly enhanced DG neurogenesis to levels that approximated what was seen with direct infusion of FGF-2 (Pieper et al., 2010). In order to verify proneurogenic effects, positive pools were first retested in two additional mice. If proneurogenic outcomes were validated, pools were broken down into their individual compound components, verified by high-performance liquid chromatography (HPLC) and by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and then reassessed in 4 additional animals at a 10 μM concentration (Pieper et al., 2010). Compound #3 from Pool #7 (deemed P7C3), an aminopropyl carbazole, exerted robust neurogenic activity in the SGZ and held the highest potential for favorable pharmacokinetics as assessed by in silico prediction of ability to penetrate the BBB. Furthermore, P7C3 could be readily formulated for IP, IV, or per os (PO; oral) administration, which permitted evaluation for prolonged periods of time for more extensive studies. The authors determined that P7C3 had sufficient oral bioavailability, a half-life of 6.7 h after IP administration, and good BBB penetration. Encouragingly, the authors also observed desirable toxicity profiles, good blood/brain distribution (AUC_{brain}/AUC_{plasma} = 3.7 oral, 0.61 IP; AUC: area under the curve), and excellent metabolic stability in rodents (MacMillan et al., 2011). Dose-response assessments also revealed that maximal neurogenic efficacy was observed at oral doses at or above 5 mg/kg.

P7C3 is a synthetic small molecule with unique chemical and structural properties. In order to determine the essential 3D structural elements and optimize biological activity, the authors conducted a structural activity relationship (SAR) study using 37 P7C3 chemical derivatives. They employed the same unbiased, in vivo screening paradigm (7 days, ICV-lateral ventricle infusion) as in the previous studies and found that
one variant in particular, P7C3-A20, had robust neurogenic potency (Pieper et al., 2010). The P7C3-A20 analog (hereafter referred to as A20) differs from P7C3 chiefly by replacement of the hydroxyl group at the chiral center of the linker region with a fluorine and the addition of a methoxy group to the alanine ring. Furthermore, in addition to enhanced efficacy, the A20 molecule maintained a lower toxicity profile and more favorable pharmacokinetics (Pieper et al., 2010; MacMillian et al., 2011; Naidoo et al., 2013).

After the discovery and characterization of this class of potent neurogenic molecules, the authors next determined whether P7C3 was eliciting robust proliferative responses and neuronal differentiation in a greater number of DG NPCs or whether P7C3 was enhancing neurogenesis levels by protecting newborn neurons from cell death during the maturation process. Using short- and long-term BrdU pulse-chase assays, Pieper and colleagues (2010) found that P7C3 exerted its proneurogenic effects during the neuronal maturation process, subsequent to the initiation of NPC proliferation.

To further characterize the proneurogenic capacity of P7C3 in naïve mice, the authors next assessed hippocampal neurogenesis rescue in two rodent models with pathologically high levels of DG apoptosis known to result in impaired hippocampal neurogenesis. The first experiments were carried out in neuronal PAS domain protein 3 (NPAS3) transcription factor-deficient mice. Npas3<sup>−/−</sup> knock-out mice exhibited reduced expression of the hippocampal FGF receptor 1 (FGFR1), which resulted in an 84% decrease of adult DG NPC proliferation (Pieper et al., 2005). Hippocampal FGFR1 expression is critical for growth factor signaling and the survival of newly-generated granular neurons in the DG (Ohkubo et al., 2004). Thus, this rodent model with
pathological levels of neuronal apoptosis was fitting to test the neurogenic potential of
the newly-characterized class of aminopropyl carbazoles. In addition to defective adult
hippocampal neurogenesis, npas3\(-/\) mice also displayed abnormal granular neuron
hyperexcitability, reduced dendritic arborization, decreased spine density, and marked
thinning of the DG granular layer (Pieper \textit{et al.}, 2005). Prolonged P7C3 administration
rescued DG and dendritic morphological deficits, and reversed aberrant
electrophysiological responses in npas3\(-/\) mice. Immunohistochemical analyses also
revealed that P7C3 administration restored the impaired expression of critical synapsin
phosphoproteins involved in synaptic vesicle exocytosis and neurotransmission, as well
as decreased the presence of proapoptotic cleaved caspase-3 protein in the DG (Pieper \textit{et
al.}, 2010).

As previously discussed, the rate of hippocampal neurogenesis strongly correlates
with performance on specific hippocampal-dependent behavioral tasks. Mice lacking the
npas3 gene are unable to swim, thus the authors sought to evaluate P7C3 in a separate
animal model of impaired neurogenesis. Aged Fischer-344 rats have reduced DG
neurogenesis compared to their younger counterparts, a condition attributed to either less
NPC proliferation/greater quiescence and/or increased levels of immature neurons
undergoing apoptosis (Kuhn \textit{et al.}, 1996; Artegiani and Calegari, 2012). 18-month old
Fischer rats were administered daily doses of P7C3 (10 mg/kg) or vehicle delivered IP for
2 months, and subjected to hippocampal-dependent spatial memory assessment via the
MWM. Pieper and colleagues (2010) found that prolonged administration of P7C3
enhanced endogenous DG neurogenesis in aged rats, prevented neuronal death, and
preserved hippocampal-dependent learning that normally declines with aging.
Unexpectedly, the authors also found that treatment with P7C3 maintained stable body weight compared to vehicle-administered animals, independent of food intake. The body weight of vehicle-control animals steadily declined with aging (Pieper et al., 2010). The peripheral mechanisms by which this weight stabilization occurred remain unclear. The rescue and enhancement of hippocampal neurogenesis in two different animal models and species underscores the potency of this important class of molecules.

The precise mechanisms by which the P7C3-class of molecules enhance the survival of newborn neurons and confer antiapoptotic effects have not yet been fully delineated. However the authors did report that P7C3 and its active derivatives preserved mitochondrial membrane potential in the presence of a calcium challenge. In collaboration with the laboratory of Xiaodong Wang, Pieper et al. (2010) evaluated the survival of human osteosarcoma U2OS cells under toxic conditions that result in mitochondrial dissolution. These cells were loaded with tetramethylrhodamine methyl ester (TMRM) dye, which is sequestered by active mitochondria. After exposure to a calcium ionophore, U2OS cells release the dye, which is indicative of pathological mitochondrial membrane permeability. Mitochondrial membrane potentials were fully disrupted within 15 min after exposure to the ionophore. Administration of P7C3 and its derivatives however preserved mitochondrial membranes from dissolution in a dose-dependent manner (Pieper et al., 2010). This is significant in that an intrinsic pathway leading to apoptosis is initiated in mitochondria, which play a critical role in the pathophysiology of brain injury (Keane et al., 2001, Zhang et al., 2005). After TBI, there is extensive apoptosis of vulnerable neurons in several brain regions, including within the dentate gyrus (Colicos and Dash, 1996). Thus, stabilization of mitochondrial membrane
integrity may have significant therapeutic value for the treatment of secondary injuries after TBI.

More recently, this group has also reported the beneficial effects of the most active variants of P7C3 in adult models of neurodegeneration, including protection of dopaminergic neurons in the substantia nigra in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-model of Parkinson’s disease (PD; De Jesús-Cortés et al., 2012), and protection of spinal cord motor neurons in the G93A-SOD1 transgenic mouse model of amyotrophic lateral sclerosis (ALS; Tesla et al., 2012). In addition, others have independently demonstrated efficacy of P7C3 in protecting retinal neurons from cell death in the transforming growth factor-b (TGF-b) ligand Growth Differentiation Factor 6-deficient zebrafish model of Leber congenital amaurosis (Asai Coakwell et al., 2013).

The chemical scaffold represented by A20 and the P7C3-family of aminopropyl carbazoles contains three-ring heterocycles (Pieper et al., 2010). An analogous configuration is also found in Dimebon (latrepirdine), an FDA-approved drug developed as an allergy medication in Russia in 1983. Dimebon was administered as an antihistamine, but was found to confer antiapoptotic effects and mitigate cognitive impairment in experimental animal models of AD and Huntington’s disease (HD) (Bachurin et al., 2001; Bharadwaj et al., 2013). As a result, Dimebon has been the subject of extensive clinical studies in AD and HD patients. In initial studies carried out in 2008, Dimebon exhibited efficacy in a phase II trial for AD (Doody et al., 2008), however it recently failed in two independent phase III trials (The CONNECTION (2010) and CONCERT (2012) studies sponsored by Pfizer and Medivation, Inc.; Sweelove, 2012). Importantly, Dimebon continues to be well-tolerated in patients and without adverse side
effects. Wu et al. (2008) showed that in high concentrations, Dimebon effectively stabilized adverse calcium levels and blocked initiation of the mitochondrial permeability transition pore (PTP) in a cellular model of HD. However, therapeutic levels were achieved only at very high doses. Wu and colleagues (2008) observed that a 50 μM concentration was required for neuroprotective effects. In AD clinical trials, patients received 20 mg of Dimebon to be taken orally, which most likely did not result in concentrations greater than 0.6 μM, assuming optimal BBB permeability (Doody et al., 2008; Wu et al., 2008).

The P7C3-class of drugs was shown to be more efficacious than Dimebon under neuropathological and neurotoxic conditions. During the calcium challenge experiments in U2OS cells, Pieper et al. (2010) showed that at least 10 μM of Dimebon was required to protect mitochondria for up to 40 min after toxic calcium exposure. On the other hand, a 0.001 μM dose of A20 resulted in significant mitochondrial preservation that persisted for at least 40 min (Pieper et al., 2010). The parallel studies of aminopropyl carbazoles in ALS and PD animal models supported previous findings showing that Dimebon displayed significantly diminished levels of potency and efficacy relative to P7C3-derived compounds (Jesús-Cortez et al., 2012; Tesla et al., 2012). Thus, the chemical carboline scaffold represented by the P7C3-class of compounds may provide a basis for optimizing and advancing new pharmacological agents for protecting patients against the early and chronic consequences of TBI.

Given the vulnerability of newly-born DG neurons to brain injury and their important role in hippocampal-dependent memory, as well as widespread mitochondria-mediated apoptosis initiated after TBI, we hypothesized that administration of A20, the
most potent of the P7C3-class of compounds, would enhance hippocampal neurogenesis, protect against hippocampal-dependent spatial memory deficits, and preserve vulnerable mature neuronal populations. This newly-discovered class of proneurogenic, neuroprotective agents might promote clinically-relevant functional recovery in patients after TBI by preserving mature brain structures and helping to promote endogenous reparative processes within the hippocampus.
Chapter 2

Genetically-Modified Neural Progenitor Cell Transplantation for the Treatment of Traumatic Brain Injury

2.1 Introduction to the Experiment

As discussed in Chapter 1, TBI is a significant clinical problem with long-lasting morbidity. Functional deficits result from a combination of pathological events that occur with the initial insult as well as more progressive changes that transpire over long periods after the initial trauma. These injury processes include selective neuronal loss in vulnerable cortical and hippocampal areas, damage to the microenvironment and cerebrovasculature, circuit disruption, as well as decreased levels of hippocampal neurogenesis, which is crucial for hippocampal-dependent memory.

Neurotrophins contribute to the development and functional integrity of the CNS through regulation of neuronal survival, differentiation, repair, neurite outgrowth, synaptic plasticity, neurotransmission, and apoptosis (Chao, 2003; Huang and Reichardt, 2003; Lu et al., 2005). Each neurotrophin has a cognate Trk receptor. Through these specific interactions, prosurvival neurotrophin-Trk signaling cascades, including PI3K/AKT, MAPK/ERK, and PLCγ pathways, increase the expression of survival-promoting genes, prodifferentiation genes, as well as other substrates involved in synaptic plasticity and synaptogenesis. (Figurov et al., 1996; Bibel and Barde, 2000; Reichardt, 2006).

Due to their intrinsic reparative and survival-promoting properties, neurotrophin-Trk interactions have clinical potential after injury. However, there are some clinical limitations to using neurotrophins therapeutically. They have suboptimal
pharmacological properties, such as short half-lives, low stability, and negligible BBB permeability. Furthermore, within the CNS parenchyma there is limited diffusion and the possibility of infection with the implantation of osmotic pumps. Delivery issues are further complicated by the fact that Trk receptors are expressed differentially by various cell types and subpopulations of neurons may be responsive to only one neurotrophin. Thus, due to the biological specificity of neurotrophins for their cognate Trk receptors, single-neurotrophin administration as a therapy may not be sufficient to interact with all neuronal populations affected by a heterogeneous injury such as TBI. The generation of novel multineurotrophin chimeras possessing a broader spectrum of binding specificity may therefore be more effective than endogenous neurotrophins for promoting protection and recovery after TBI.

As discussed in Chapter 1, the transplantation of NPCs may be a promising strategy to mitigate some of the detrimental histologic and functional outcomes after CNS injury. NPCs are well-suited for therapeutic intervention in that they possess tropic migratory properties, maintain multipotency through several passages, can be isolated from several sources and expanded in vitro, and are easily genetically modified (De Feo et al., 2012; Gage and Temple, 2013; Shetty, 2014). It was initially believed that the efficacy of stem cell transplantation was likely attributed to transplanted cells integrating within local compromised circuits and replacing lost or dysfunctional cells (De Feo et al., 2012). Several studies have since elucidated that the benefits of stem cell therapy are more extensive than previously thought. Research suggests that NPCs can ameliorate the injured milieu by providing and provoking trophic support to and from host tissues, as well as by modulating host immune responses (Cossetti et al., 2012). The trophic and
immunomodulatory effects conferred by transplanted NPCs are collectively known as “bystander” mechanisms and may potentially be prominent factors underlying the therapeutic effects of NPC transplantation after CNS injury (De Feo et al., 2012). Important to the scope of this study, transplanted NPC-mediated trophic secretion can mobilize endogenous stem cells, thus enhancing neuroregenerative responses, such as hippocampal neurogenesis, within the injured milieu.

Hippocampal neurogenesis occurs continually throughout the life of most mammals, including humans (Altman, 1962; Eriksson et al., 1998). Active NPCs residing in the adult DG continually divide and give rise to IPCs, which in turn generate DCX-positive neuroblasts. These newly-born immature neurons migrate from the SGZ to the GCL, where they eventually integrate within specific neural circuits and mature into fully-functional hippocampal granular neurons, a process that occurs over a period of 4-8 weeks in rodents (Ge et al., 2007). Newly-generated immature neurons have distinguishing characteristics that set them apart from developmentally-born granular neurons (Ge et al., 2007; Piatti et al., 2013). Immature neurons are highly excitable, retain a lower threshold for LTP induction, and have enhanced plasticity at both input and output synapses (Piatti et al., 2013) These distinct properties allow immature DG neurons to make unique contributions to specific aspects of hippocampal memory, such as pattern separation (Deng et al., 2010; Gage and Temple, 2013). The full spectrum and relevance of adult hippocampal neurogenesis is not fully known and warrants future investigation.

CNS injury induces acute proliferative and neurogenic responses, which have been shown to contribute to some degree of cognitive and sensorimotor recovery after TBI (Blaiss et al., 2011). However, this endogenous neurorestorative response is
incomplete as residual cognitive deficits persist long after the injury. Exogenous therapeutic strategies that enhance endogenous neuroreparative responses may potentially augment recovery processes and thus remain a critical area of study.

In the present study, we sought to enhance the inherent salutary effects of NPCs through genetic modification. We investigated the potential benefits of transplanting NPCs that have been transduced to continually secrete MNTS1, a multineurotrophin with multiple neurotrophic specificities. Through the exchange and mutation of 8 amino acid residues on a mature human NT-3 construct, Urfer et al. (1994) generated a human multifunctional, multitargeting molecule that retains the capacity to bind all prosurvival Trk receptors and effectively supports the survival of NGF-, BDNF-, and NT-3-dependent neurons, without altering intracellular neurotrophin-Trk signaling cascades.

Previous work in our laboratory has demonstrated the significant prosurvival capacity of MNTS1, including experiments showing extensive neurite outgrowth of dorsal root ganglia (DRG) explants with application of MNTS1-rich medium (Figure 2.1).
In order to determine if MNTS1 recapitulates the biological activity of endogenous neurotrophins, the survival of DRG neurons was assayed in a dose-response experiment. It was revealed that MNTS1 was as potent in promoting DRG survival as a cocktail of several neurotrophins (NGF, BDNF, and NT-3) administered simultaneously (Figure 2.2).

**Figure 2.1.** MNTS1 generates extensive neurite outgrowth in DRG explants. Cell culture medium from MNTS1-transduced HEK-293 cells was collected and added to whole DRG explants from rat E15.5 embryos. Three days after application of media, cells were fixed and stained with neuronal tubulin, a marker for neurite outgrowth. DRG explants exposed to MNTS1-rich medium had extensive neurite outgrowth relative to supernatant (sup) controls and explants exposed to several growth factors.
Furthermore, after transduction, MNTS1 secretion was consistent and sustained over several experiments, and secretion levels could be manipulated in a lentiviral infection-dependent manner (Figure 2.3, A, B). It was also established that NPCs transduced with MNTS1 remained indistinguishable from control NPCs in vitro and exhibited intact differentiation programs. Addition of differentiation-inducing molecules, such as cardiotrophin-1 (CT-1) and triiodothyronine (T3) induced NPC differentiation into the appropriate astrocytic and oligodendritic phenotypes, respectively (Figure 2.3, C).
Figure 2.3. Characterization of transduced NPCs in vitro prior to transplantation.

A) Four separate transduction experiments are shown, demonstrating consistent, reliable levels of secreted MNTS1 from NPCs as measured by ELISA. B) Levels of secreted MNTS1 as a function of the amount of lentivirus used for the transduction. (N.D., not detectable). C) Images of NPCs after transduction with the lentivirus containing the GFP construct or the lentivirus containing the MNTS1 construct, 8 days after transduction. NPCs remained positive for nestin (green) while no GFAP-immunostaining was seen (red). The nuclei were stained with DAPI (blue). Addition of CT-1 (20 ng/ml for 6 days) differentiated the NPCs into astrocytes (GFAP, red) and administration of T3 (30 ng/ml for 6 days) differentiated the NPCs into oligodendrocytes (O4, green). This differentiation occurred regardless of the presence of MNTS1. Scale bars 20 µm.
The objective of the present study was to assess histological and functional outcomes with transplantation of control- (NPCs transduced with GFP and BFP constructs) or genetically-modified NPCs (transduced with MNTS1 and GFP constructs) in an experimental model of brain injury. We hypothesized that engaging several Trk-survival pathways in multiple neuronal populations via MNTS1, together with the intrinsic advantages of NPC transplantation, would result in significant rescue of neuropathological outcomes and augmentation of endogenous reparative responses, such as hippocampal neurogenesis, after moderate TBI.

2.2 Materials and Methods

Construction of the MNTS1 Lentivirus

The chimeric MNTS1 molecule was generated via selective point mutations and amino acid residue exchanges on a mature human NT-3 backbone as discussed previously (Chapter 1; Urfer et al., 1994). MNTS1 complementary DNA (cDNA) was produced synthetically by GeneArt® Gene Synthesis (Life Technologies, Carlsbad, CA) and subcloned into the lentiviral vector pRRLsinPPT-CMV-MCS-WPRE as previously described (Dull et al., 1998). The lentiviral particles were generated by The Miami Project Viral Vector Core of University of Miami Miller School of Medicine. Lentiviruses were produced using the four-plasmid method (Follenzi and Naldini, 2002). Viral titers, shown as transducing units (TU) from $10^7$ to $10^8$, were determined by a p24 ELISA assay (Perkin Elmer, Waltham, MA) used to quantify p24 core protein concentrations. Purified lentiviral particles were stored at -80°C until use.
Isolation and Preparation of Neural Progenitor Cells

NPCs were isolated from Sprague Dawley rat fetuses at embryonic stage E15. Frontal cortical tissue was microdissected in Lebovitz’s L-15 medium (GIBCO/Life Technologies, Carlsbad, CA). Cortical tissue was transferred to a conical tube, titrated to obtain a single-cell solution, and counted using trypan blue (Life Technologies). Between 600,000 to 700,000 cells were placed on sterile, polystyrene 10 cm tissue culture dishes (Corning, Corning, NY) in NeuralCult proliferation medium solution (StemCell Technologies, Vancouver, BC) that was selective for neurospheres and supplemented with penicillin-streptomycin, 10 µg/mL of basic fibroblast growth factor (bFGF), 10 µg/mL epidermal growth factor (EGF), and 0.2% Heparin. Two days later, neurospheres were infected with lentiviral vectors expressing MNTS1 and GFP (pLV-eGFP) constructs, or GFP and BFP (pLV-EBFP2-nuc) constructs, and 2-3mL of fresh medium was added to dishes. After two days of proliferation, NPCs were assessed for positive lentiviral transduction and cell viability (Figure 2.4).

If infection was positive, cells were plated on 10 cm fibronectin (Sigma-Aldrich, St. Louis, MO)-coated tissue culture dishes and allowed to adhere and colonize for an
additional 1-2 days. On day of transplantation, NPCs were trypsinized with Hank’s Balanced Salt Solution (HBSS; GIBCO/Life Technologies, Carlsbad, CA) and centrifuged at 2000 rpm for 4.5 min. Medium was carefully removed and pellet was resuspended in fresh medium. Cells were assessed for viability and counted using trypan blue in a hemacytometer, followed by microcentrifugation at 6500 rpm for 1.5 min. One million cells were resuspended in 10 μl of fresh medium (for a final volume of 100,000/μl) and kept on ice until transplantation (< 30 minutes).

Animals

58 adult male Sprague-Dawley rats were randomly assigned to 1 of 6 groups: Sham/Vehicle (n = 12), Sham/GFP-NPCs (n = 8), Sham/MNTS1-NPCs (n = 7), TBI/Vehicle (n = 15), TBI/GFP-NPCs (n = 8), or TBI/MNTS1-NPCs (n = 8). Animal care was in accordance with the guidelines set forth by the University of Miami Animal Care and Use Committee and the NIH Guide for the Care and Use of Laboratory Animals. Animals were housed in a temperature-controlled room (22°C) with a 12-h light/dark cycle. They had at least 7 days of acclimation before undergoing any experimentation. All animals had access to food and water ad libitum, except for a 24 h fast before the surgical procedure in order to maintain consistent glucose levels.

Fluid-percussion Traumatic Brain Injury

Rats were anesthetized with 3% isoflurane in 30% O₂/70% N₂O and placed in a stereotaxic frame. Animals received a 4.8 mm craniotomy over the right parietal cortex, 3.8 mm posterior to bregma and 2.5 mm lateral to midline. A modified plastic injury hub (3.5 mm inner diameter) was bonded to the skull over the exposed dura with cyanoacrylic adhesive. Twenty-four h after the craniotomy, animals were reanesthetized, intubated,
and mechanically ventilated with 0.5-0.75% isoflurane. Pancuronium bromide (1.0 mg/kg) was administered intravenously to facilitate ventilation. The tail artery was cannulated to ensure blood gases, pH, glucose, and mean arterial blood pressure (MABP) measurements were consistent among animals. Rectal and temporalis muscle thermometers measured body and brain temperatures using self-adjusting feedback warming lamps. All variables were maintained within physiological ranges from 15 min prior to TBI and for up to 30 min post injury.

A moderate (1.8-2.2 Atmospheres) fluid percussion-induced TBI was produced over the right parietal cortex. Sham animals underwent identical procedures minus the fluid percussion insult. A subset of animals (n = 28) received injections of a concentrated aqueous solution of 5-bromo-2'-deoxyuridine (BrdU) and 5-fluoro-2'-deoxyuridine (FdUrd) labeling reagent (10:1; 1 mL/100g of rat bodyweight administered IP; Invitrogen Life Technologies, Grand Island, NY) 24 h post TBI and daily thereafter for 7 days.

**Physiology**

All physiological variables including pH, pO₂, pCO₂ and MABP were within normal ranges before and after the fluid percussion insult (Supplemental Table 2.1). Animals displayed normal activity within 24 h after recovery from anesthesia as detected by visual inspection of grooming behavior, posture, and locomotion. There was no significant weight loss in any experimental group 7 days post surgery or at the time of perfusion-fixation (6 weeks post surgery).

**NPC Transplantation**

One week after surgery, animals were returned to a stereotaxic frame and reanesthetized with 3% isoflurane in 30% O₂/70% N₂O. Sutures were removed and
craniotomy was identified. Stereotaxic injection coordinates were as follows: -3.5 and -3.0 mm below dura, -4.3 mm posterior to bregma, and -5.0 mm lateral to midline. Four μl of medium containing 400,000 NPCs (100,000 cells per μl) were aspirated into a Gastight® 26 gauge microsyringe (Hamilton Co., Reno, NV). Using a motorized stereotaxic injector (Stoelting Co., Wood Dale, Illinois), medium + cells were slowly injected into the right parietal cortex at a rate of 1 μl per minute. 200,000 cells were transplanted at each depth. Before and after each injection, the syringe was allowed to remain in the parenchyma for 2 min for tissue acclimation. Cells were transplanted over the course of 12 min. After the final injection, animals were resutured and returned to their home cages. All animals displayed normal activity within 24 h after recovery from anesthesia. All animals received 10 mg/kg of the immunosuppressant, Cyclosporine A (CsA; Novartis Pharmaceuticals Corp., East Hanover, NJ). IP injections of CsA began 2 days prior to transplantation procedure and continued daily for 14 days post transplantation.

Cognitive Assessment

Five weeks after TBI or sham surgery, spatial memory was evaluated via the MWM, which consisted of a circular pool (122 cm diameter, 60 cm deep) filled with water rendered opaque by white, non-toxic paint. Submerged heaters maintained the water temperature between 22-25°C. A round platform (10 cm diameter) was submerged 5 cm beneath the water surface. The pool was divided into 4 equal-sized quadrants, one of which included the hidden platform. Prominent extramaze cues served as visual reference points. Swim patterns were recorded and analyzed via Ethovision (Noldus Information Technology, Leesburg, VA). MWM parameters evaluated included escape
latency (time to locate platform), swim speed, path length to platform (distance traveled), and percentage of total time spent in each quadrant.

Animals were tested for 3 consecutive days, each day consisting of 4 trials at 5-min intervals. For each trial, animals were released from the edge of the pool into a randomly selected quadrant. Release point order was identical for all animal groups. During each trial, the animals were allowed 60 sec to find the hidden platform. If they failed to locate it, they were guided to the platform. Animals were required to remain on the platform for an additional 30 sec for memory acquisition training (Experimental timeline, Supplemental Figure 2.1).

Histopathology and immunohistochemistry

At 6 weeks post TBI or sham procedures, animals were anesthetized with 3% isoflurane in 30% O₂/70% N₂O and transcardially perfused with saline (80 mL for 2 min) followed by 4% paraformaldehyde (4°C, 350 mL at a pressure of 100–120 mmHg for 28 min). Brains were immediately removed, placed in 4% paraformaldehyde for 48 h and then cryoprotected in 20% sucrose in phosphate-buffered saline. Brains sections (45 µm thickness) were cut with a frozen sliding microtome (LEICA SM200R, Leica Microsystems, Inc., Buffalo Grove, IL). Serial sections at 270 µm intervals were immunostained with hematoxylin and eosin (H&E), rat anti-BrdU (when applicable, 1:150), mouse anti-neuronal nuclei (NeuN; 1:400), chicken anti-GFP (1:2000), mouse anti-glial fibrillary acidic protein (GFAP; 1:2000), rabbit anti-Olig2 (1:250), rabbit anti-NG2 (1:500), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; 1:1000), or mouse anti-aldehyde dehydrogenase 1 (Aldh1; 1:800) as previously described (Atkins et al., 2010; Bregy et al., 2012). Development of immunofluorescent staining was
conducted with goat anti-rat Alexa Fluor 488 (1:200), goat anti-chicken Alexa Fluor 488 (1:200), goat anti-mouse Alexa Fluor 568/594/647 (1:200), or goat anti-rabbit Alexa Fluor 568/594/647 (1:200).

Volumetric and stereological analyses:

Antibody penetration was verified in all sections using 100x magnification. Cortical contusion volumes were determined by tracing the contused areas in serial H&E sections with a 5x objective on an Axiophot 200 M microscope (Zeiss Microscopy, LLC, Thornwood, NY) using Neurolucida software (MicroBrightfield, Inc., Williston, VT). Cortical contusion boundaries were well demarcated by hemorrhage and shearing at the gray/white matter interface between the cortex and external capsule on the ipsilateral hemisphere. Serial bregma levels were observed beginning at -1.0 mm posterior to bregma. At first indication of contusion, all subsequent bregma levels were included for volumetric analysis up until -6.8 mm posterior to bregma. Separate serial sections from -3.0 to -5.8 mm posterior to bregma were chosen to determine neuron survival within parietal cortical regions. NeuN-immunoreactive cells were quantified by a blinded observer in an unbiased manner using Stereoinvestigator software (MicroBrightfield, Inc.). The parietal cortex overlying the contused area was contoured at 4x. The epicenter of neuronal loss was identified and a border was defined 1000 μm to the right and to the left of the epicenter to give a total rectangular contour width of 2000 μm. A counting grid of 250 x 250 μm was placed over the contoured parietal cortex. Using a 70 × 70 μm counting frame, NeuN-positive cells were counted in 30-40 randomly-placed sampling sites with a 63x 1.4 NA objective.
Stereoinvestigator software was also used to conduct quantitative assessment of BrdU/NeuN-immunoreactive cells in the ipsilateral hippocampus. The DG cell layer was contoured using a BX51TRF Olympus microscope (Olympus America, Center Valley, PA, USA) at 4x magnification. Using a PlanApo N 60X/1.42 oil objective, a blinded observer analyzed 5 sections between bregma levels -3.8 mm and -5.0 mm posterior to bregma with a 100 x 100 μm counting frame and 115 x 115 μm counting grid in 40-55 randomly-placed sampling sites. To determine numbers of GFP- and GFP/NeuN-positive cells in transplanted animals, all sections positive for GFP-positive cell bodies in a single series were quantified.

Statistics

In all quantification and analysis procedures, observers were blinded to the nature of the experimental manipulation. Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using the Student’s t-test, one-way analysis of variance (ANOVA), or two-way repeated measures ANOVA, depending on the outcome measure. Post-hoc analysis was performed using Student-Newman-Keuls corrections. Significance level was p < 0.05 using two-tailed testing.

2.3 Results

Increased Survival and Neuronal Differentiation of MNTS1-Secreting NPCs

One week after TBI or sham surgery, animals were transplanted with either GFP-control NPCs, MNTS1-transduced NPCs, or injected with saline vehicle. NPCs were transplanted pericontusitionally into the right parietal cortex of the traumatized hemisphere at two different depths. The transplantation coordinates utilized in the present study were
selected after a series of optimization experiments. These coordinates were deemed most conducive for graft migration. Five weeks after transplantation, animals were sacrificed for immunohistochemical analyses.

NPCs express Trk receptors. Thus, based on our hypothesis that MNTS1 would engage a greater number of Trk-responsive neurons in prosurvival signaling pathways, we sought to determine whether transplanted NPCs secreting MNTS1 *themselves* displayed greater survival rates than control NPCs 6 weeks post TBI or sham surgery. Non-biased stereological analyses revealed significantly greater levels of internalized MNTS1-transduced NPCs in the traumatized brain relative to GFP-control NPCs (*Figure 2.5, A*). Consistent with our hypothesis, MNTS1-NPCs in sham animals also exhibited slightly elevated survival rates compared to GFP-control NPCs, albeit not significantly so.

Endogenous Trk signaling cascades also mediate neuronal differentiation. Thus, we sought to quantify levels of grafted NPCs that exhibited a neuronal phenotype. NeuN is a reliable marker to identify mature neurons. Quantification of double-labeled GFP/NeuN-positive cells revealed significant neuronal differentiation in TBI/MNTS1-NPC grafts compared to the GFP-control TBI group at 6 weeks post injury (*Figure 2.5, B, C*). Although brain trauma coupled with MNTS1-transduction induced greater neuronal differentiation of NPC grafts, levels of grafted NeuN-positive NPCs in the Sham/MNTS1 group trended toward significance relative to the injured control NPC-transplanted group.
Figure 2.5. GFP-positive cell survival 6 weeks post TBI. A) TBI/MNTS1 animals had greater GFP-positive cell survival compared to control GFP-alone NPC transplanted groups. B) The percentage of NeuN/GFP-double labeled cells was significantly increased in TBI/MNTS1 animals relative to TBI/GFP animals 6 weeks post injury. Data are expressed as mean ± SEM. C) Representative images showing numerous MNTS1-expressing NPCs positive for NeuN, indicating neuronal differentiation. NPCs migrated ventrally through parietal cortex and laterally via external capsule. Scale bar, 100 μm. D) Confocal micrographs of several NPCs positive for both GFP and NeuN expression 6 weeks post TBI. Boxed region in C demarcates area of high magnification in D. Arrows indicate NPCs with GFP/NeuN double-labeled immunoreactivity. Scale bar, 30 μm.
Additional cell lineage markers were utilized to detect non-neuronal phenotypes. Immunohistochemical findings demonstrated transplanted GFP-positive NPCs did not coexpress markers for mature astrocytes (Aldh1), oligodendrocytes or oligodendrocyte progenitor cells (OPCs; Olig2 and NG2, respectively), or activated microglia/macrophages (Iba1; Figure 2.6). Some grafted NPCs did express GFAP, a lineage marker indicative of astrocytes, however this expression is consistent with the immature glial-like NPC phenotype.

Figure 2.6. Cell lineage markers to determine transplanted NPC fate. Panels 1-4) Transplanted NPCs were negative for Aldh1 (a mature astrocyte marker), negative for NG2 and Olig2 expression (markers for oligodendrocyte precursor cells and mature oligodendrocytes, respectively), and negative for Iba1 expression (marker of activated microglia/macrophages). Panel 5) There was some colocalization of NPCs and GFAP (astrocytic marker), which is consistent with immature glial-like progenitor cells. Scale bar, 30μm.
Injury-induced Migration and Long-Distance Projections of Transplanted NPCs

Five weeks following transplantation, exogenous NPCs in TBI animals were observed to have moved ventrally away from the needle tract through the parietal cortex and, upon reaching the external capsule/white matter tract, migrated laterally towards sliding lesions at the gray/white matter interface and cortical contusions (Figure 2.7 A, B). Notably, several migrating NPCs exited the white matter tract and reentered the injured cortex ventral to the contused area (Figure 2.7 C).

Figure 2.7. Location and targeted migration of MNTS1-transduced NPCs 6 weeks post TBI. A) Montage showing transplant location. Box demarcates area of neuronal fallout (red, NeuN; green, GFP). Scale bar, 500 µm. B) Representative image showing migration of transplanted cells through cortex and towards lesion in external capsule (blue, DAPI). Scale bar, 100 µm C) NPCs migrate via external capsule and reenter cortex at contused areas. Box demarcates magnified image in last panel (blue, DAPI; green, GFP; red, GFAP). Scale bar, 100 µm.
We observed injury-induced tropism of transplanted NPCs in both GFP-control and MNTS1-transduced NPC groups, however migration appeared more robust in MNTS1 animals. NPCs in sham-uninjured animals had negligible migration patterns with the majority of neural progenitor cell bodies remaining within or directly proximal to the needle tract (Figure 2.8).

![GFP and NeuN images](image)

**Figure 2.8.** Representative image of limited migration in Sham/MNTS1 animals 6 weeks post surgery. Transplant cell survival was evident, which we attributed to MNTS1-transduction (blue, DAPI; green, GFP; red, NeuN). Scale bar, 100 µm.

All transplanted groups, irrespective of injury or transduction profile, displayed long-distance axonal-like projections from NPC grafts (Supplementary Figure 2.2). Projections were extensive and infiltrated several cortical and subcortical brain structures, including the hippocampus (Figure 2.9, 2.10).
Figure 2.9 GFP-positive processes and some GFP/NeuN-positive cell bodies infiltrated the hippocampus in both injured and non-injured animals 6 weeks post surgery. A) NPC grafts residing in external capsule extended GFP-positive processes (arrowhead) through CA1 of the hippocampus and into the stratum radiatum. Arrow indicates GFP/NeuN-positive cell. B) GFP-positive cell bodies, one of which was also NeuN-positive (arrow) extended processes into CA1 of the hippocampus (boxed region). C) GFP-positive processes located within stratum radiatum, which contains Schaffer collaterals projecting forward from CA3 to CA1. Scale bar, 200 µm.
GFP-positive processes were observed from bregma levels -2.8 to -5.3 and in both hemispheres, with several processes crossing the corpus callosum (Figure 2.11). In injured animals, the majority of processes appeared to specifically target areas of tissue pathology, including interface lesions and contused brain areas (Figure 2.12). Our qualitative observations showed that while both groups displayed extensive processes, they appeared more prominent and numerous in MNTS1-transduced animals. This observation supports our previous finding that MNTS1 transduction significantly augments NPC graft survival. Of note, the majority of GFP-positive processes in all transplanted groups exhibited spine-like formations throughout the length of the process, which may be indicative of host-donor cell interactions (Figure 2.10, 2.13).

Figure 2.10 GFP-positive cell bodies in ventral external capsule extending process into CA1 of ipsilateral hippocampus. Arrows indicate spine-like formations on GFP-positive processes. Box demarcates magnified image in last panel. Representative image from a Sham/GFP animal. Scale bar, 10 µm
Figure 2.11. GFP-positive processes originating from transplanted NPCs residing the parietal cortex crossed over into the contralateral hemisphere via the corpus callosum. GFP-positive projections were observed in all transplanted animals irrespective of injury or transduction profile. Arrow indicates NPC processes in subcortical regions. Representative image from a Sham/MNTS1 animal. Scale bar, 200 µm.

Figure 2.12. GFP-positive processes infiltrating area of pericontusional neuronal fallout in parietal cortex. Box demarcates region of NeuN loss 6 weeks after TBI. Scale bar, 500 µm.
An additional qualitative observation showed that several transplanted NPCs appeared quite proximal to host neurons (GFP-negative; NeuN-positive), which again may demonstrate donor cell interactions with host tissues (Figure 2.14).

Figure 2.13. Spine-like formations observed on GFP-positive process extending from NPC transplant. Scale bar, 10 µm.

Figure 2.14. Representative image of Sham/MNTS1 animal showing close proximity between GFP-positive transplanted NPCs and host neurons. Arrows indicate transplanted cell bodies that appear directly adjacent to host neurons. Box demarcates area of possible interaction. Scale bar, 10 µm.
NPC transplantation significantly improved histopathological outcomes after TBI

Consistent with this rodent model of moderate fluid percussion TBI, we observed well-demarcated lesions along the ipsilateral grey/white matter interface between parietal cortex and external capsule, as well as selective fallout of vulnerable parietal neurons in the cortical region overlying the sliding contusion. Strikingly, although exogenous NPC survival and neuronal differentiation was significantly greater in MNTS1 animals, we found that all groups receiving cells, regardless of transduction profile, demonstrated significant histological improvement 6 weeks after TBI.

Volumetric analysis of brain contusions is a reliable measure of injury severity and corresponding neurological impairment. Six weeks after TBI, we observed a significant reduction in total average contusion volume in both MNTS1- and GFP-control transplanted groups compared to their TBI vehicle counterparts (Figure 2.15).

Quantification of mature neurons at parietal contusions revealed similar findings. NPC transplantation, with or without MNTS1 secretion, was associated with significantly greater levels of NeuN-positive cell survival at the cortical contusion relative to vehicle-injected animals 6 weeks post TBI (Figure 2.16).

Taken together, we observed that both transplanted groups, receiving either GFP-control or MNTS1-transduced NPCs, exhibited significant neuroprotection and preservation of surrounding cytoarchitecture 6 weeks after injury. These findings indicate that transplanted NPCs may have sufficient inherent capabilities to effectively contribute to the protection of vulnerable parenchyma after traumatic insult, without the need for multineurotrophin expression.
Figure 2.15. A) Representative images of lesions (boxes) from a A) TBI vehicle and B) TBI MNTSI animal. C) Volumetric analyses demonstrated significantly reduced contusion volumes in TBI animals receiving cells compared to vehicle-injected TBI animals; *p < 0.05; **p < 0.01. Scale bar, 500µm.
NPC transplantation increased endogenous dentate gyrus neurogenesis

To determine levels of newly-generated neurons in the hippocampal dentate gyrus, a subset of animals was injected with BrdU, a thymidine analog marker for dividing cells that is incorporated into newly replicated DNA during the S-phase of the cell cycle. Quantification of the number of BrdU/NeuN-positive cells in the ipsilateral DG across groups demonstrated considerable differences between NPC-receiving animals and vehicle-injected control groups 6 weeks post TBI (Figure 2.17). Sham/GFP animals had significantly more BrdU/NeuN-positive cells in the ipsilateral DG compared to their sham vehicle counterparts. After TBI, MNTS1 animals had significantly greater levels of endogenous hippocampal neurogenesis compared to both injured and uninjured vehicle groups 6 weeks post TBI. These results were therefore indicative of increased levels of endogenous hippocampal neurogenesis with NPC transplantation.
Figure 2.17. Representative images of BrdU/NeuN double-labeled cells in the ipsilateral DG from a TBI Vehicle (A) and TBI MNTS1 (B) animal 6 weeks post TBI. Scale bar, 200 µm. C) Quantification of double-labeled cells showed that transplantation with GFP-alone transduced NPCs increased levels of BrdU/NeuN-positive cells in the sham group relative to sham/vehicle animals. Injured animals transplanted with MNTS1-expressing NPCs exhibited a greater magnitude of hippocampal neurogenesis compared to injured and uninjured vehicle-injected control animals.
Cell transplantation improved hippocampal-dependent cognitive outcome

Whether these histological changes resulted in any functional differences was evaluated next. Hippocampal-dependent spatial memory capacity was evaluated using the MWM paradigm 5 weeks after surgery. We did not detect any significant differences between sham/vehicle and sham-transplanted groups, so they were collapsed into a single sham group for data analysis. After 12 trials over a period of 3 days, TBI-vehicle control animals exhibited significant spatial memory deficits, consistent with this injury model. In contrast, both NPC-transplanted TBI groups performed significantly better than their TBI vehicle control counterparts in several parameters used to evaluate hippocampal-dependent memory (Figure 2.18). These parameters included escape latency (Figure 2.18, A) and percentage of time spent in the target quadrant (where platform was submerged; Figure 2.18, C). Total path length (the distance traveled to reach submerged platform), another measure of cognitive performance, was only statistically significant between sham- and TBI-vehicle groups, but shorter path lengths were also evident in both NPC-transplanted TBI animals compared to injured vehicle controls (Figure 2.18, B; Supplementary Figure 2.3). Swim speed was not significantly different among groups, indicating that the observed differences were not a result of an inability to execute the swim task (Figure 2.18, D). Improvement in cognitive capacity as assessed by MWM performance in both MNTS1-NPC and GFP-NPC transplanted animals is consistent with the observation that both transplanted groups had histological improvement and greater levels of endogenous hippocampal neurogenesis relative to TBI vehicle control animals.
Figure 2.18. 5 weeks post surgery, all groups underwent the MWM hidden platform task. A) After 3 days of testing, post-hoc analysis revealed that both NPC-TBI transplanted groups and sham animals had significantly reduced escape latencies on the hidden platform task, relative to TBI-vehicle controls (TBI-Vehicle vs. Sham, ##p < 0.001; TBI-Vehicle vs. TBI-GFP, *p < 0.05; TBI-Vehicle vs. TBI-MNTS1, +p < 0.05). Two-way repeated measures ANOVA was significant for trial day (p < 0.001), group (p < 0.05), but not for trial day x group interaction (p = 0.08). B) On Day 3, TBI-Vehicle animals took a significantly longer path length to reach the submerged platform compared to sham animals (**p < 0.001). C) On Day 3, sham and TBI-GFP groups spent a great percentage of time in the target quadrant compared to vehicle control animals (*p < 0.05). D) There were no significant differences in swim speeds among groups suggesting that the injury did not affect swimming skills.
2.4 Major Findings

The experiments conducted in the present study sought to determine whether the inherent benefits of NPCs, including growth factor secretion, immunomodulation, trophic evocation from host tissues, stimulation of endogenous hippocampal neurogenesis, and the ability to integrate within compromised circuitry, would provide sufficient protection and restoration to reverse cognitive outcomes after TBI. We further investigated if enhanced reparative effects would be observed with transplantation of genetically-modified NPCs that constitutively secreted a prosurvival multineurotrophin. Our findings suggested that NPC transplantation, with or without multineurotrophin release, resulted in significant rescue of vulnerable cortical neurons and of the perilesional cytoarchitecture after neurotrauma. Furthermore, NPC transplantation resulted in considerable increases in endogenous hippocampal neurogenesis relative to saline vehicle-treated animals. Importantly, transplanted NPC-mediated changes manifested in significant improvement in hippocampal-dependent spatial memory capacity, exhibiting MWM performances comparable to sham levels, and significantly better than injured vehicle controls.

Six weeks after transplantation, GFP-positive NPCs were easily identifiable and TBI-induced migration was prevalent. Multineurotrophin transduction resulted in robust NPC survival and neuronal differentiation after TBI; however, these MNTS1-specific properties did not seem to have a significant effect on injury-induced outcome measures. Of interest, both MNTS1- and GFP-alone-transduced NPCs displayed long-distance projections with spine-like morphologies throughout the length of the process. Future investigations as to the nature and functional-relevance of these long-distance neurites and spine-like formations would be exciting and may reveal additional important
properties of NPCs to target and optimize the therapeutic potential of cell transplantation strategies for the treatment of TBI.

2.5 Supplemental Figures

**Supplemental Figure 2.1 Experimental timeline**

<table>
<thead>
<tr>
<th>Day 0 Surgery</th>
<th>Day 7 NPC transplantation or vehicle injection</th>
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<tr>
<td>6 animal groups</td>
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<tr>
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Days 5 - 21 Immunosuppression
Days 7 - 14 BrdU injections (1μg/100g, i.p.)
Days 35 - 38 Water maze testing

**Supplemental Figure 2.2. Ultrascope image of cleared MNTS1-transplanted brain 6 weeks post TBI.** Arrows indicate extensive GFP-positive processes with spine-like morphologies projecting from transplanted NPCs. Box demarcates cells that have migrated away from transplant graft site towards contused regions. Scale bar, 300 μm
Supplemental Figure 2.3. Two representative swim tracks from a A) TBI GFP and B) TBI vehicle animal on Day 3 of the hidden platform MWM task. Platform (yellow circle) was submerged in northeast quadrant of pool. Animals were released from the West release point. The TBI GFP (A) animal exhibited a much shorter path length to reach the submerged platform compared to the TBI vehicle control animal, which is indicative of intact spatial memory.
2.6 Supplemental Table

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*Supplemental Table 2.1. All physiological variables were normal during surgical procedures. Data are expressed as mean ± SEM*
Chapter 3

Neuroprotective Efficacy of a Novel Proneurogenic Compound after Traumatic Brain Injury

3.1 Introduction to the Experiment

As discussed in Chapter 1, TBI is a major cause of death and disability in the United States (Langlois and Rutland-Brown, 2005), Focal contusive injury, diffuse axonal damage, and selective patterns of neuronal loss contribute to long-term functional impairments (Bramlett and Dietrich, 2007). The hippocampus is highly vulnerable to TBI (Kotapka et al., 1991; Lowenstein et al., 1992) and subsequent circuit disruption leads to hippocampal-dependent memory dysfunction (Bramlett et al., 1997; Witgen et al., 2005). Thus, identification of novel therapeutic approaches that include both neuroprotective and reparative strategies is critically important.

New neurons are continuously generated throughout life in the SGZ of the hippocampal dentate gyrus, where NPCs undergo distinct developmental stages of proliferation, fate specification, migration, integration, and maturation (Altman, 1969; Gage, 2000; Gross, 2000; van Praag et al., 2002; Abrous et al., 2005; Emery et al., 2005; Zhao et al., 2006; Toni et al., 2008). Newly-generated DGCs integrate within hippocampal circuitry to participate in hippocampal-dependent memory (Zhao et al., 2008; Deng et al., 2010). Stimuli that augment hippocampal neurogenesis are associated with memory improvement (Gould et al., 1999; van Praag et al., 1999; Gaulke et al., 2005), while specific exposures that decrease hippocampal neurogenesis correlate with impairment of hippocampal-dependent learning and memory (Kuhn et al., 1996, Mcdonald and Wojtowicz, 2005; Schmidt and Duman, 2007).
Several studies have reported that NPC proliferation is significantly enhanced after TBI (Dash et al., 2001; Chirumamilla et al., 2002; Urrea et al., 2007; Gao et al., 2009). This response is transient however as levels of DCX-positive cells decrease to below baseline levels and remain persistently depressed, indicating a chronic depletion of immature neurons and hippocampal neurogenesis (Rola et al., 2006; Gao et al., 2008; Potts et al., 2009; Atkins et al., 2010) Given that hippocampal neurogenesis is closely correlated with hippocampal-dependent memory (Snyder et al., 2005; Zhao et al., 2008; Deng, et al., 2010), therapeutic interventions to augment the survival of newly-generated hippocampal neurons and enhance neurogenesis may improve hippocampal-dependent functional outcomes after brain trauma.

Using a target-agnostic in vivo screen of 1000 compounds to identify small drug-like molecules with neurogenic efficacy in the hippocampus of living mice, Pieper and colleagues (2010) discovered and characterized P7C3, an aminopropyl carbazole that elicited robust neurogenic responses. Both potency and efficacy, as well as toxicity profile, were further improved through synthesis of an analog, P7C3-A20, which differs from P7C3 chiefly by substitution of a fluorine for the hydroxyl group within the linker region of the molecule (Pieper et al., 2010; MacMillain et al., 2011) (Figure 3.1).

![Figure 3.1 Chemical structure of P7C3-A20](image-url)
The P7C3-class of molecules, including P7C3-A20, enhances hippocampal neurogenesis by blocking apoptosis of newly-generated hippocampal neurons, thus resulting in a greater survival of new neurons, and is thought to operate through stabilization of mitochondria under otherwise toxic conditions (Pieper et al., 2010).

As discussed in Chapter 1, the potent proneurogenic effects of P7C3-A20 (and its parent compound, P7C3) were demonstrated using two different rodent models of impaired adult neurogenesis (Pieper et al., 2005; 2010). After prolonged administration of P7C3 and P7C3-A20 (hereafter referred to as A20), Pieper and colleagues showed that the pathologically high levels of apoptosis was significantly attenuated, restoring hippocampal structure and function. Furthermore, P7C3 and A20 administration enhanced endogenous neurogenesis, prevented neuronal death, and preserved hippocampal-dependent learning (Pieper et al., 2010).

Given the vulnerability of newly-born dentate granular neurons to brain injury and their important role in hippocampal-dependent memory, we hypothesized that administration of the A20 proneurogenic compound after TBI would enhance hippocampal neurogenesis and protect against hippocampal-dependent spatial memory deficits. We also hypothesized that A20 would protect mature cortical neurons from degeneration after TBI and attenuate sensorimotor impairment. To these hypotheses, we investigated whether administration of A20 would 1) reduce contusion volume, 2) protect vulnerable cortical neurons, 3) enhance SGZ NPC proliferation, 4) promote and prolong enhanced SGZ neurogenesis, and 5) protect against sensorimotor and hippocampal-dependent cognitive deficits after TBI.
3.2 Materials and Methods

Experimental Design

When performing fluid percussion injury studies, there is a considerable amount of physiological and metabolic variations. Therefore sufficient numbers of animals are required to obtain proper statistical significance. In accordance with this, we performed a sample size estimate based on our historical data from over the past 15 years using SigmaStat (Systat Software, Inc., Chicago, IL) with a power of 0.80, indicating that there is an 80% chance of detecting a difference between groups. Based on this analysis, we determined that 8-12 rats are needed per group for each proposed experiment.

Animals

80 adult male Sprague Dawley rats were randomly assigned to a 1-week, 3-week, or 5-week group (1-week group: TBI-vehicle, n = 10; TBI-A20, n = 10; 3-week group: sham-vehicle, n = 5; sham-A20, n = 5; TBI-vehicle, n = 10; TBI-A20, n = 10; 5-week group: sham-vehicle, n = 5; sham-A20, n = 5; TBI-vehicle, n = 10; TBI-A20, n = 10). Animal care was in accordance with the guidelines set forth by the University of Miami Animal Care and Use Committee and the NIH Guide for the Care and Use of Laboratory Animals. Animals were housed in a temperature-controlled room (22°C) with a 12-h light/dark cycle. They had at least 7 days of acclimation time before undergoing any experimentation. All animals had access to food and water ad libitum, except for a 24 h fast before the surgical procedure in order to maintain consistent glucose levels.

Traumatic brain injury

Rats underwent moderate parasagittal fluid percussion injury or sham surgery as previously described in Chapter 2. Thirty min post-TBI, animals were administered 10
mg/kg of A20 or vehicle IP using a randomized selection process. This dosage was selected based on previous study (Pieper et al., 2010) and administered every day 2x per day for 7 days post surgery. Animals also received injections of a concentrated aqueous solution of BrdU and FdUrd labeling reagent (10:1; 1 mL/100g of rat bodyweight administered IP; Invitrogen Life Technologies, Grand Island, NY) 24 h post TBI and daily thereafter for 7 days.

**Physiology**

All physiological variables including pH, pO2, pCO2 and MABP were within normal ranges before and after the fluid percussion insult (Supplemental Table 3.1). Animals displayed normal activity within 24 h after recovery from anesthesia as detected by visual inspection of grooming behavior, posture, and locomotion. There was no significant weight loss between experimental groups at the time of injury or at the time of perfusion-fixation.

**Behavior analyses**

*Spontaneous forelimb task:* Sensorimotor performance was evaluated by an investigator blinded to the experimental groups using the spontaneous forelimb task. A transparent Plexiglas cylinder (20 cm diameter, 30 cm high) encourages use of the forelimbs for vertical wall exploration. Animals were placed in the cylinder for 5 min per test and videotaped. The videotape was played in slow motion (1/5th real time speed) and blinded observers quantified the number of times the right forelimb, the left forelimb, or both forelimbs simultaneously made contact with the cylinder wall during a rearing movement. The asymmetry index was calculated by dividing the number of contralateral placements by the total placements of both paws (Bramlett et al., 1995; Baskin et al.,
2003). Animals were tested 1 day prior to surgery (baseline) and 7 days after. Identical experimental procedures were carried out for both 3-week and 5-week groups. Data presented are from 3-wk animals. Similar outcomes were observed in the 5-week group (data not shown).

**Spatial memory:** One week before sacrifice (2 or 4 weeks after TBI or sham surgery), spatial memory was evaluated via the MWM as previously described in Chapter 2, Section 2.2). Swim patterns were recorded and analyzed via Ethovision (Noldus Information Technology, Leesburg, VA). Parameters evaluated included escape latency (time to locate platform), swim speed, path length to platform (distance traveled), and percentage of total time spent in each quadrant.

Animals were tested for 4 consecutive days, each day consisting of 4 trials at 5-min intervals. For each trial, animals were released from the edge of the pool into a randomly selected quadrant. Release point order was identical for all animal groups. During each trial, the animals were allowed 60 sec to find the hidden platform. If they failed to locate it, they were guided to the platform. Animals were required to remain on the platform for an additional 30 sec for memory acquisition training.

**Histopathology and immunohistochemistry**

At 1 or 5 weeks post TBI or sham procedures, animals were anesthetized with 3% isoflurane in 30% O₂/70% N₂O and transcardially perfused with saline (80 mL for 2 min) followed by 4% paraformaldehyde (4°C, 350 mL at a pressure of 100–120 mmHg for 28 min). Brains were immediately removed, placed in 4% paraformaldehyde for 48 h, and then cryoprotected in 20% sucrose in phosphate-buffered saline. Brains sections (45 µm thickness) were cut with a frozen sliding microtome (LEICA SM200R, Leica
Microsystems, Inc., Buffalo Grove, IL). Serial sections at 270 µm intervals were immunostained with H&E, goat anti-DCX (1:200), rat anti-BrdU (1:100), mouse anti-BrdU (1:200), or mouse anti-NeuN (1:500) as previously described (Atkins et al., 2010; Bregy et al., 2012). Development of immunostaining was conducted with goat anti-rat IgG (1:200), horse anti-mouse (1:200), goat anti-rat Alexa Fluor 488 (1:200), or goat anti-mouse Alexa Fluor 594 (1:200).

**Volumetric and stereological analyses:**

Antibody penetration was verified in all sections using 100x magnification. Cortical contusion volumes were determined by tracing the contused areas in serial H&E sections with a 5x objective on an Axiophot 200 M microscope (Zeiss Microscopy, LLC, Thornwood, NY) using Neurolucida software (MicroBrightfield, Inc., Williston, VT, USA). Cortical contusion boundaries were well demarcated by hemorrhage and shearing at the gray/white matter interface between the cortex and external capsule on the ipsilateral hemisphere. Contusion areas were calculated for 6 coronal levels proximal and distal to the epicenter of injury at -3.8 bregma (-0.8, -1.8, -3.3, -4.3, -5.8, and -6.3 mm posterior to bregma). Separate serial sections from -3.0 to -5.8 mm posterior to bregma were chosen to determine neuron survival within vulnerable cortical regions. NeuN-immunoreactive cells were quantified by a blinded observer in an unbiased manner using Stereoinvestigator software (MicroBrightfield, Inc.; Gundersen et al., 1988). The parietal cortex overlying the contusion area was contoured at 5x and a counting grid of 150 x 150 µm was placed over the contoured parietal cortex. Using a 35 × 35 µm counting frame, NeuN-positive cells were counted in 30-40 randomly-placed sampling sites with a 63x 1.4 NA objective. Stereoinvestigator software was also used to conduct quantitative
assessment of BrdU-, DCX-, and BrdU/NeuN-immunoreactive cells (Gundersen et al., 1988). The DG cell layer was contoured using a BX51TRF Olympus microscope (Olympus America, Center Valley, PA) at 4x magnification. A blinded observer analyzed 5 sections between bregma levels -3.0 mm and -5.0 mm posterior to bregma using a 70 x 70 μm counting frame in 40-55 randomly-placed sampling sites and a PlanApo N 60X/1.42 oil objective.

Statistical Analysis

Data are expressed as mean ± SEM. Data were analyzed using Student’s t-test, one-way ANOVA, or two-way repeated measures ANOVA depending on the outcome measure. Post hoc analysis was performed using Bonferroni corrections. Significance level was p < 0.05 using two-tailed testing.

3.3 Results

A20 decreased contusion volume and blocked mature cortical neuron death after traumatic brain injury

Consistent with our previous experience with the rat model of fluid percussion-induced moderate TBI, we observed well-demarcated contusions along the gray/white matter interface between the cortex and external capsule on the ipsilateral, traumatized hemisphere (Figure 3.2 A, B). Volumetric analyses of contusion areas at 1 week post TBI demonstrated that animals receiving A20 had significantly reduced contusions at multiple bregma levels surrounding the site of injury (Figure 3.2, D). A20 administration reduced average contusion volume 2-fold compared to vehicle-treated control animals (Figure 3.2, C).
In addition to a subcortical contusion after TBI, we observed selective neuronal dropout within a reproducibly-located pericontusional cortical region (Figure 3.3 A, B). NeuN- (a marker of mature neurons) immunoreactive cell survival in this susceptible
cortical region was quantified. At 1 week post injury, A20 administration resulted in significantly greater numbers of NeuN-positive cells in ipsilateral parietal cortical regions compared to vehicle-treated TBI animals (Figure 3.3 C). Furthermore, TBI control animals exhibited expansion of the lateral ventricles and greater external capsule loss (Figure 3.3 A).

Figure 3.3. A20 administration was neuroprotective in vulnerable ipsilateral cortical regions 1 week post TBI. (A, B) Representative NeuN-stained images of vehicle- (A) and A20- (B) treated animals. Selective neuronal loss was observed in the cortical region overlying the contusion site. Treatment with A20 significantly reduced NeuN-positive cell loss. Boxes in A, B enclose the areas of neuronal loss. Arrows indicate subcortical lesion. C) Quantification of NeuN-positive cells between treatment groups revealed a significant neuroprotective effect of A20 1 week post injury; *p < 0.05. Scale bar, 500 μm.


**A20 increased generation of new hippocampal neurons in the DG after TBI**

To determine whether A20 would significantly increase hippocampal neurogenesis in the DG, animal groups were injected with BrdU, a thymidine analog marker for dividing cells that is incorporated into newly-replicated DNA during the s-phase of the cell cycle. After TBI, A20 administration resulted in greater numbers of BrdU-positive cells observed throughout the full extent of the blades of the DG. In contrast, TBI-vehicle animals had less BrdU-positive cells, which were localized predominately to the apex of the DG (Figure 3.4 A, B, C, D, I).

After TBI, BrdU-positive cells have been shown to differentiate into astrocytes and microglia as well as neurons (Chirumamilla et al., 2002). Thus, to determine whether A20 enhanced the generation of new neurons, we performed immunohistochemical staining with DCX, a marker of immature neurons at an early stage of neurogenesis. At 1 week post TBI, there was a significant increase in DCX-positive cells in animals administered A20 compared to vehicle controls (Figure 3.4 J). As with BrdU-staining, DCX-labeled cells in A20 animals were more widely dispersed throughout the DG relative to what was observed in vehicle-treated animals after TBI (Figure 3.4 E, F, G, H).
Figure 3.4. Administration of A20 increased progenitor cell and immature neuron proliferation 1 week post TBI. Representative images showing BrdU- (A-D) and DCX- (E-H) immunoreactive cells in TBI vehicle- (left) and A20- (right) treated groups. Images in C, D and G, H are magnified regions of boxed areas in A, B, E, F. (I, J) Quantification of BrdU- and DCX- positive cells. A20 administration significantly increased both BrdU- and DCX-positive cells, indicative of early stage neurogenesis; *p < 0.05; ***p < 0.001. Scale bars, 200 μm.
Under normal conditions, newly-born DG neurons in rats take approximately 4-6 weeks to mature and integrate within the hippocampal circuitry (Ming and Song, 2005). In order to determine whether proliferating immature neurons observed at 1 week also survived to this stage, we double-stained cells in the SGZ for BrdU and NeuN 5 weeks after TBI. In these chronic experiments, animals were assigned to a sham-vehicle, sham-A20, TBI-vehicle, or TBI-A20 group. No differences were observed between sham-vehicle and sham-A20 groups, so they were collapsed into a single sham group for data analysis. Five weeks after TBI or sham surgery, TBI-vehicle and TBI-A20 groups both had BrdU/NeuN-positive cells in the ipsilateral DG (Figure 3.5 A, B). In sham animals, confocal micrographs revealed that BrdU/NeuN-positive cells were sparse and confined to the SGZ (Figure 3.5 A). Quantification of double-labeled cells supported these qualitative observations (Figure 3.6). The TBI-vehicle group had a significantly greater number of double-labeled cells relative to sham animals, which is consistent with acute proliferative responses induced by TBI. However, A20 administration after TBI further augmented hippocampal neurogenesis, as demonstrated by more robust levels of BrdU/NeuN-positive cells in the ipsilateral DG. Cells coexpressing BrdU/NeuN in TBI-A20 animals appeared to migrate outside of the SGZ into the granular layer of the DG (Figure 3.5 B) consistent with maturation profiles of newborn neurons.
Figure 3.5 Confocal micrographs showing BrdU- and NeuN-immunoreactive cells within the DG 5 weeks after TBI. A) Representative fluorescent images showing cells that colabeled for BrdU and NeuN in the ipsilateral DG (scale bars, 200 μm. Boxed regions outline the areas of higher magnification shown in bottom row of A. B) Representative micrographs from a TBI-A20 animal showing several cells coexpressing markers for BrdU and NeuN (arrows) in the ipsilateral DG. Double-labeled cells in A20 animals appeared to migrate out of the SGZ into more superficial layers of the DG, consistent with neuronal maturation profiles (GCL, granular cell layer; SGZ, subgranular zone). Scale bar, 10 μm.
A significant functional deficit commonly observed in this model of moderate TBI is a contralateral sensorimotor deficit, in which a right hemisphere traumatic insult results in a persistent left forelimb deficit that can be quantified using the spontaneous forelimb task (Bramlett et al., 1995). This task encourages use of the forelimbs during vertical wall exploration, and animals with deficits do not use forelimbs equally. We evaluated baseline sensorimotor ability 1 day before TBI, and then measured again 1 week after TBI (Figure 3.7). Prior to surgery, all animal groups used both forelimbs equally. At 1 week post TBI however, TBI-vehicle control animals demonstrated a significant contralateral forelimb deficit. By contrast, TBI animals receiving A20
exhibited no contralateral forelimb sensorimotor deficit, performing equally well to sham-operated animals and significantly better than their TBI-control counterparts.

We next investigated whether A20 administration could improve the spatial memory deficits that are frequently observed in this model of TBI (Bramlett et al., 1995, 1997b). Hippocampal-dependent cognitive capacity was evaluated using the MWM

Figure 3.7 A20 protected from contralateral forelimb deficits 1 week post TBI. An asymmetry index of less than 50% indicates a contralateral deficit. All animal groups performed similarly pre-surgery (baseline). One week after TBI, vehicle-treated controls exhibited a significant contralateral forelimb deficit compared to their baseline time point. Contralateral forelimb usage in the TBI-vehicle control group 1 week post injury was significantly decreased compared to sham-uninjured animals, consistent with this injury model. In contrast, TBI animals treated with A20 showed no signs of a contralateral deficit and performed similar to sham-operated animals and significantly better than TBI-vehicle controls; sham vs. TBI-vehicle: *p < 0.05; TBI-vehicle vs. TBI-A20: **p < 0.01.
hidden platform task 4 weeks post TBI. This interval was selected based on the temporal maturation and electrophysiological profiles of newly-generated hippocampal neurons. After 16 trials over a period of 4 days, TBI control animals exhibited significant spatial memory deficits. Injured animals receiving A20, however, performed significantly better than the TBI-vehicle control group in several parameters used to evaluate hippocampal-dependent spatial memory. These parameters included escape latency (Figure 3.8 A), path length (Figure 3.8 B), and time spent in the target quadrant (where the platform was submerged; Figure 3.8 C). TBI-vehicle animals did not exhibit a monotonic swim pattern during the hidden platform task (Figure 3.8 A, B). Swim speed was not significantly different among groups, indicating that the observed differences were not due to an inability to execute the swim task (Figure 3.8 D).

3.4 Major Findings

The experiments conducted in this study showed that treatment with the proneurogenic/neuroprotective compound A20 after TBI reduced overall contusion volume and improved pericontusional neuronal survival in the parietal cortex. A20 treatment also increased acute proliferative responses at 1 week and the magnitude of hippocampal neurogenesis in the SGZ 5 weeks post surgery. Importantly, the morphological changes correlated with improved sensorimotor capacity and hippocampal-dependent spatial memory outcomes relative to TBI-vehicle control animals. This study is the first to show both neuroprotective and neurogenic effects of A20, one of the new P7C3-class of neuroprotective agents, in an established model of TBI.
Figure 3.8 A20 administration significantly improved MWM performance 4 weeks after TBI. (A) Post-hoc analysis revealed that TBI-A20 and sham animals had significantly reduced escape latencies on the hidden platform task relative to TBI-vehicle controls on the 4th day of testing (Bonferroni’s Multiple Comparison Test: TBI-A20 vs. TBI-vehicle, **p < 0.01; sham vs. TBI-vehicle, ###p < 0.001). Two-way repeated measures ANOVA was significant for trial day (p < 0.0001), treatment (p = 0.007), and treatment x trial day interaction (p = 0.0005). (B) By day 4, sham and TBI-A20 animals exhibited significantly shorter path lengths to reach the platform compared to TBI-vehicle controls (Bonferroni’s Multiple Comparison Test: **p < 0.01). Two-way repeated measures ANOVA was significant for trial day (p < 0.0001), treatment (p = 0.0013), and treatment x trial day interaction (p < 0.016). (C) On day 4, sham and TBI-A20 animals spent a greater percentage of time in the target quadrant compared to TBI-vehicle control animals (Bonferroni’s Multiple Comparison Test: *p < 0.05; **p < 0.01). Two-way repeated measures ANOVA showed significance for trial day (p < 0.0001) and treatment (0.0168), but not for treatment x trial day interaction (p = 0.131). (D) There were no significant differences in swim speeds among groups, suggesting that the injury did not affect swimming skills (one-way ANOVA: p = 0.129).
3.5 Supplemental Table

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<td>39 ± 0.98</td>
<td>37.96 ± 0.31</td>
<td>38.45 ± 0.63</td>
<td>38.22 ± 0.62</td>
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<tr>
<td>pO₂</td>
<td>141.2 ± 13.09</td>
<td>154.8 ± 10.93</td>
<td>148.50 ± 9.81</td>
<td>139.8 ± 5.49</td>
</tr>
<tr>
<td>MABP</td>
<td>130.4 ± 2.69</td>
<td>126.6 ± 2.36</td>
<td>107.15 ± 2.17</td>
<td>115.8 ± 3.72</td>
</tr>
<tr>
<td>Brain temperature</td>
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<td>36.86 ± 0.10</td>
<td>36.89 ± 0.09</td>
<td>36.76 ± 0.12</td>
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<tr>
<td>Rectal temperature</td>
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<td>37.02 ± 0.08</td>
<td>36.87 ± 0.07</td>
<td>36.83 ± 0.07</td>
</tr>
<tr>
<td><strong>30 min posttrauma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
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<td>7.44 ± 0.01</td>
<td>7.44 ± 0.01</td>
<td>7.43 ± 0.01</td>
</tr>
<tr>
<td>pCO₂</td>
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<td>39.38 ± 0.38</td>
<td>38.7 ± 0.80</td>
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</tr>
<tr>
<td>pO₂</td>
<td>141.2 ± 9.89</td>
<td>152.2 ± 14.87</td>
<td>138.3 ± 7.70</td>
<td>139.8 ± 6.81</td>
</tr>
<tr>
<td>MABP</td>
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<td>123.52 ± 3.88</td>
<td>104.14 ± 3.77</td>
<td>118.9 ± 4.04</td>
</tr>
<tr>
<td>Brain temperature</td>
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</tr>
<tr>
<td>Rectal temperature</td>
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<td>36.88 ± 0.07</td>
<td>36.97 ± 0.09</td>
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Supplemental Table 3.1. All physiological variables were within normal ranges before and after surgical procedures. Data are expressed as mean ± SEM.
Chapter 4

Discussion

The studies conducted in this dissertation project sought to explore the effects of promoting endogenous regenerative processes after TBI by utilizing different treatment strategies. It has been established that the endogenous upregulation of specific neurorestorative programs may be insufficient to confer significant functional repair after neurotrauma. Thus, we hypothesized that supplementary exogenous therapies by providing a proneurogenic molecule or by transplanting transduced NPCs would augment and promote endogenous programs to facilitate recovery after TBI.

4.1 Neural Progenitor Cell Transplantation

This study was conducted to determine whether transplantation of syngeneic neural progenitor cells, with or without genetic modification to secrete a human multineurotrophin, would improve histological and functional outcomes after moderate TBI. We found that transplantation of NPCs, irrespective of transduction profile, resulted in protection of vulnerable parietal neurons, preservation of the pericontusional cytoarchitecture, increased endogenous hippocampal neurogenesis, and cognitive improvement on hippocampal-dependent spatial memory tests. MNTS1 expression in particular had significant effects on the survival and neuronal differentiation capacity of transplanted NPCs, while the injured brain milieu influenced NPC targeted migration profiles.

Our findings were consistent with published reports demonstrating that NPC transplantation is a viable treatment option for traumatic brain injury. Furthermore, ex vivo genetic manipulation of these therapeutic cells prior to transplantation may provide
additional support in terms of long-term survival and neuronal differentiation. As discussed in Chapter 2 (Figures 2.1 – 2.4), work in our lab has previously shown the prosurvival capacity of MNTS1 *in vitro*, including experiments illustrating extensive DRG survival and neurite outgrowth with application of MNTS1-rich medium. MNTS1 recapitulated the biological and prosurvival activity as effectively as a “cocktail” of neurotrophins containing NGF, BDNF, and NT-3. Additional *in vitro* experiments established that NPCs transduced with MNTS1 remained indistinguishable from control NPCs with multipotentiality properties intact and unaffected by transduction.

*Survival and Neuronal Differentiation of MNTS1-NPCs*

One of our early qualitative observations was the robust survival and neuronal differentiation of MNTS1-transduced NPCs. Subsequent quantitative assessments confirmed these observations. As discussed in Chapters 1 and 2, MNTS1 is a mature neurotrophin with multifunctional, multitargeting trophic activity. Mature neurotrophin-Trk binding interactions initiate several prosurvival signaling pathways that regulate neuronal viability and survival, differentiation, neurite outgrowth, and synaptic plasticity (Thoenen, 1995; Bibel and Barde, 2000; Huang and Reichardt, 2003). In addition to secreting trophic factors, NPCs also express Trk receptors. Thus, neurotrophin-Trk signaling can affect cells in both a paracrine and an autocrine manner. We hypothesized that the greater transplant survival rates observed in MNTS1-receiving animals resulted from multineurotrophin secretion engaging neighboring NPCs (paracrine), as well as the secreting cells themselves (autocrine), in prosurvival signaling pathways.

After injury, the brain rapidly undergoes a series of neurochemical and metabolic alterations, including excess excitatory amino acid release, free radical formation, and
activation of deleterious inflammatory cascades (Bullock et al., 1995; Bramlett and Dietrich, 2004). All of these secondary injury mechanisms can significantly affect the survival and function of transplanted cells. Thus, one would anticipate that NPC survival would be reduced in TBI groups compared to sham animals. However, TBI/MNTS1-transplanted animals had greater internalized cell body survival compared to the sham/MNTS1-transplanted group. Thus, in addition to potentially hostile posttraumatic events, there appears to be an injury-induced upregulation of factors that may have beneficial effects on transplanted cell survival after TBI. These include the upregulation of VEGF, BDNF, NGF, FGF2, acute hippocampal neurogenesis, angiogenesis, and release of anti-inflammatory cytokines such as IL-6, -8, and -10 (Morganti-Kossman et al., 1997; Yoshimura et al., 2001; Sköld et al., 2005). Injury-induced endogenous reparative effects are thought to be an attempt by the brain to repair itself, albeit they are insufficient in many pathological conditions. Several studies have elucidated the proneurogenic and prosurvival effects of angiogenesis and VEGF on endogenous and exogenous NPCs after TBI (Lee and Agoston, 2010; Xiong et al., 2010). In our experiments, we observed several GFP-positive NPCs surrounding blood vessels, which may have contributed to transplant survival.

Furthermore, after neurotrauma there is a phenotypic switch of microglia/macrophage populations resulting in a disproportionate number of M1 (activated) relative to M2 (resting) microglia/macrophages (Loane and Byrnes, 2010). M1 phagocytes are toxic to neurons and OPCs. They release proteases, proinflammatory cytokines, and free radicals, which contribute to the hostile posttraumatic environment. (Loane and Byrnes, 2010; David and Kroner, 2011). In contrast, M2 resting
microglia/macrophages promote remyelination and axon regeneration, clear inhibitory myelin debris, release neurotrophic factors, such as BDNF, and block the production of ROS and proinflammatory factors (Rapalino et al., 1998; Dougherty et al., 2000). Wang et al. (2013) showed that after TBI, the number of M1 microglia/macrophages increased in the cortex, striatum, and corpus callosum, and remained elevated for at least 2 weeks. Conversely, M2 resting phagocytes were decreased at chronic time points post TBI (Wang et al., 2013).

After exogenous cell transplantation however, studies have reported that multipotent NPCs modulate the resident microglia/macrophage population, resulting in a greater M2 phenotype relative to M1 (Walker et al., 2012). Recent work showed that M2 resting microglia co-cultured with NPCs exerted reciprocal, symbiotic effects. Both NPCs and M2 phagocytes exhibited increased survival and proliferation compared to mono cultures (Liu et al., 2013). Thus, the presence of NPCs appears to promote the M2 population, which in turn supports NPC survival. A recent in vivo study reported that the NPC-mediated increase in M2 microglia/macrophages was associated with greater levels of VEGF expression (Mosher et al., 2012). The greater magnitude of NPC transplant survival in TBI/MNTS1 animals compared to sham/MNTS1-transplanted groups in our studies may therefore be attributed to endogenous protective modifications that allow the inflammatory environment to be more permissive for supplementary neurorestorative strategies, such as NPC transplantation, which in turn confer reciprocal remedial effects.

While we did observe greater MNTS1-expressing cell survival in sham animals compared to GFP-control groups, it appeared that the injury profile together with MNTS secretion greatly increased transplant survival. Our findings support the prosurvival
capacity of MNTS1-transduced NPCs, which may be acting in a paracrine and/or autocrine manner to engage multiple survival pathways for transplant survival. Because the injury profile was a significant factor in determining graft cell survival, we surmise that endogenous neuroreparative events initiated post trauma, such as greater angiogenesis, upregulation of VEGF and other neurotrophic factors, were also conducive to NPC survival. Furthermore, there may be reciprocal benefits occurring concomitantly; the presence of NPC grafts promotes the switch from a trauma-induced activated M1 microglia/macrophage phenotype to a resting M2 remedial phenotype, which in turn supports the survival of transplanted NPCs in the injured CNS.

In addition to increased MNTS1-NPC survival, we also found a greater proportion of NeuN-positive NPCs after MNTS1 transduction compared to GFP-control NPCs, indicative of differentiation into a mature neuronal phenotype. While significantly increased levels of neuronal differentiation in transplants were observed only in the TBI/MNTS1 group, sham/MNTS1 animals also exhibited higher levels of NeuN-positive NPCs that trended towards significance relative to GFP-control NPCs in injured animals. We conclude that neuronal differentiation occurred as a result of multineurotrophin-induced enhanced Trk signaling. As discussed in previous sections, differentiation into a mature neuronal phenotype is associated with Trk-mediated signaling cascades, including PI3K/AKT and MAPK/ERK activation. Whether MNTS1 was acting in an autocrine/paracrine manner to stimulate a neuronal phenotype in transplanted cells or if the presence of MNTS1 created a favorable host milieu conducive to neuronal differentiation has yet to be clarified.
Transplanted NPC Migration in the Injured Brain

We found that after TBI, transplanted NPCs, with or without MNTS1 transduction, exhibited significant tropism and considerable targeted migration towards lesions and areas of histopathology. This observation is consistent with published findings in both experimental animal models and TBI patients (Belmadani et al., 2006; Zhu et al., 2006; Darkazalli and Levenson, 2012; Wu et al., 2012). After TBI, there is a significant upregulation of various cytokines and chemokines, such as stromal-derived factor-1α (SDF-1α), and monocyte chemoattractant protein-1 (MCP-1), that are secreted by reactive astrocytes at neuroinflammatory lesions. Their upregulation creates a chemoattractant gradient to which tropic stem cells are recruited (Babcock et al., 2003; Xu et al., 2007; Wu et al., 2012). NPCs constitutively express cell adhesion molecules (CAMs; such as CD44), integrins (such as α4), and several chemokine receptors, including CCR1, CCR2, and CXCR4. These cell surface molecules are important for stem cell homing towards the injured CNS as demonstrated by migration of NPCs in response to CCL5 (RANTES) and SDF-1α chemokines in a dose-dependent manner (Pluchino et al., 2005; Martino and Pluchino, 2006). Although the phenomenon of chemoattractant-induced NPC migration has been demonstrated in several models of CNS injury, the precise mechanisms are still not fully understood. However, in a rodent model of chronic neuroinflammation, work by Pluchino et al. (2005) showed inflamed endothelial cells expressing specific ligands such as hyaluronic acid and vascular cell adhesion molecule 1 (VCAM1) that interacted with the NPC cell surface molecules, CAMs and integrins, respectively. NPCs firmly adhered and migrated towards chemoattractant gradients through the activation of their chemokine receptors, which
induced the activation of α4 and β1 integrins. These events subsequently induce G-coupled protein receptor (GPCR)-dependent cell-cell interactions that underlie NPC migration (Jacques et al., 1998; Pluchino et al., 2005; Martino and Pluchino, 2006). Thus, our observation of greater NPC tropism in the inflamed brain post trauma is consistent with the permissibility of the injured host milieu for migration. We surmised that trauma-induced upregulation of chemokines generated chemoattractant gradients and inflamed endothelial cell-mediated “molecular tethering” of NPCs towards neuroinflammatory lesions. Consistent with this notion, targeted migration was shown to be negligible in sham, uninjured animals.

There was more robust migration in MNTS1-receiving animals compared to TBI/GFP animals. This may be due to enhanced neurotrophin-mediated migratory mechanisms conferred by MNTS1 secretion. As mentioned previously, neurotrophin binding initiates several specific pathways. Activation of TrkB receptors leads to direct phosphorylation of PLCγ, which in turn activates diacylglycerol (DAG). DAG activates PKC cascades, which influence intracellular signaling mechanisms that promote persistent cellular responses including neurite outgrowth and migration. Furthermore, Chiaramello and colleagues (2007) found that BDNF-TrkB binding modulates neuroblast migration through both PI3K/AKT and MAPK/ERK signaling cascades.

*Long-distance neurite outgrowth*

In all transplanted groups, we observed significant long-distance neurite outgrowth from transplanted NPCs. GFP-positive processes were extensive and infiltrated several cortical and subcortical structures, including the contralateral hemisphere. Of note, the majority of these processes exhibited spine-like formations,
which may be indicative of host-donor cell interactions. Although both groups displayed long-distance processes, qualitative observations suggested that process formation and infiltration was greater in MNTS1-transduced NPCs. Elevated neurotrophin activity associated with MNTS1 expression was most likely a contributing factor. PI3K/AKT, MAPK/ERK, and PLCγ activation via neurotrophin-Trk interactions have been shown to be associated with neuritogenesis. Several groups have reported on the role of BDNF and TrkB signaling in dendritic arborization and spine formation, particularly in the SGZ of the hippocampus (Bergami et al., 2008). Dendritic spinogenesis is frequently correlated with learning and strengthening of synapses (Alonso et al., 2004; Zhou et al., 2004). The present studies did not determine whether the spine-like formations were forming functional synaptic contacts with neighboring cells. If GFP-positive processes from transplanted NPCs were indeed participating in the local circuitry, this may have increased their propensity to survive in an activity-dependent manner, mediated in part by BDNF-TrkB interactions.

Similar to progenitor cell body migration, we observed a considerable number of GFP-positive processes specifically targeting the contused area and infiltrating within damaged tissue. Mature NGF-p75NTR interactions can reduce the magnitude of the glial scar by decreasing reactive astrocyte proliferation (Cragnolini et al., 2009). NPC neuritogenesis in the traumatized hemisphere may have occurred due to greater permissibility for neurite outgrowth attributed to trophic factor secretion.

*Protection of pericontusional parenchyma and cortical neurons*

After brain injury, there is significant cell death and dysfunction as a result of enhanced excitotoxicity, free radical generation, diffuse axonal injury, disruption of
circuits, and initiation of proapoptotic inflammatory cascades. Within 1 h after moderate TBI, cell death caspases are upregulated in the pericontusional cortex (Keane et al., 2001). Consistent with this animal model of parasagittal fluid percussion injury, we observed sliding contusions within the external capsule at the gray/white matter interface, as well as significant neuronal fallout in the ipsilateral parietal cortex due to the selective vulnerability of these brain regions. In experimental models, the severity of these contusions and disruption of perilesional parenchyma predicts levels of functional impairment on specific behavioral assessments. After NPC transplantation, we found that both transplanted groups had significantly reduced contusion volumes and greater cortical neuron survival compared to TBI/vehicle-control counterparts 6 weeks post TBI. Because both MNTS1- and GFP-alone-transduced NPCs displayed significant cytoprotective effects after TBI, we hypothesized that tissue preservation was attributed to the inherent remedial properties of transplanted NPCs. In addition to prosurvival and neuroprotective growth factor secretion, NPCs can also modulate neuroinflammatory responses in the traumatized brain, either through direct induction of apoptotic programs in encephalitogenic Th1 helper cells (Plucino et al., 2005) or by promoting M2-like microglia/macrophage phenotypes (Walker et al., 2012), thus creating a more permissive milieu for endogenous reparative processes to transpire.

The conventional rationale for cell transplantation therapy is the physical replacement of dying or dysfunctional host cells by transplant grafts, and subsequent integration within the existing circuitry to mitigate functional deficits in neurodegenerative or traumatic conditions. Additionally, several studies have provided compelling reports of “bystander” mechanisms conferred by NPCs (Martino and
Pluchino, 2006; Baraniak and McDevitt, 2010; De Feo et al., 2012). There is complementary evidence demonstrating that functional integration and interaction between donor and host cells contribute to the attenuation of trauma-induced impairment (Flax et al., 1998; De Feo et al., 2012). Thus, NPC-mediated neuroprotection after TBI may be due in part to compensatory cellular replacement. NPC participation in compromised host circuitry may protect vulnerable cells from undergoing cell death, due to activity-dependent survival mechanisms. This speculation is further corroborated by the presence of numerous spine-like formations throughout the length of most GFP-positive processes and direct adjacency of grafted cells to host neurons, two observations that may indicate functional interactions with the host environment.

Endogenous Hippocampal Neurogenesis after NPC Transplantation

Several studies have reported significant increases in SGZ progenitor cell proliferation and maturation in the traumatized hemisphere after experimental TBI (Dash et al., 2001; Kernie et al., 2001; Chirumamilla et al., 2002; Urrea et al., 2007; Gao et al., 2009). However whether the acute upregulation of hippocampal neurogenesis post injury is sustained and functionally-relevant remains unclear. Previous work has demonstrated a persistent depression of DCX-positive cells at chronic time points after TBI, resulting in a prolonged depletion of immature neurons and impaired hippocampal neurogenesis (Rola et al., 2006; Gao et al., 2008; Potts et al., 2009; Atkins et al., 2010). In the present study, at 6 weeks post injury, we observed no significant differences in DG neurogenesis between sham-vehicle and TBI-vehicle groups. This finding may be suggestive of trauma-enhanced hippocampal neurogenesis returning to – and perhaps subsequently below – baseline levels after the acute phase post trauma.
In contrast, we found that both MNTS1 and GFP-control NPCs exerted robust endogenous neurogenic effects in the hippocampal DG 6 weeks post surgery compared to vehicle-treated groups. We suspect that trophic factor secretion may have contributed in part to this proneurogenic effect. Although NPCs were transplanted into the right parietal cortex, we observed numerous GFP-positive processes infiltrating the hippocampal formation, including CA1 and stratum radiatum regions. Thus, endogenous hippocampal neurogenic responses may have been elicited by the release of trophic factors from the distal neurites of transplanted NPCs or due to transplanted NPCs evoking trophic support from neighboring hippocampal host cells. These two NPC-mediated events could therefore result in sufficient trophic signaling for significant endogenous hippocampal neurogenesis to take place. Another possible explanation for increased SGZ neurogenesis with cell transplantation is NPC-mediated immune modulation, which would allow for a more permissive environment conducive to greater SGZ neurogenesis after TBI.

Immature neurons in the SGZ are highly vulnerable and the mechanisms responsible for the persistent loss of immature neurons are not fully understood. Several studies have reported a unique vulnerability of the posttraumatic hippocampal progenitor pool to necrotic and apoptotic cell death processes. Gao and colleagues (2008) reported significant numbers of degenerating cells in the DG, with 80% being immature neurons after moderate TBI. Given that hippocampal neurogenesis is closely correlated with hippocampal-dependent memory (Gould et al., 1999; Zhao et al., 2008; Snyder et al., 2005; Deng, et al., 2010), therapeutic interventions, such as neurotrophic factor support, to augment the survival of newly-generated hippocampal neurons may be critical.
**Improved cognitive outcomes with NPC transplantation**

Consistent with our immunohistochemical findings, we observed improved hippocampal-dependent cognitive function on the spatial memory-dependent MWM task in both GFP- and MNTS1-NPC transplanted animals relative to vehicle-injected control groups. Learning and memory impairment is one of the most lingering and persistent deficits after brain injury (McAllister, 2011). Thus, novel therapies targeting these functional outcomes are critical. We cannot definitively conclude whether the spatial memory rescue observed in our transplanted animals was attributed to cytoprotective protective effects and/or elevated endogenous hippocampal neurogenesis, or to some other parameter that was not fully evaluated, such as long-distance processes and spine-like morphologies. In agreement with our histological findings, we observed no significant cognitive differences between GFP-NPC and MNTS1-NPC transplanted groups. In fact, GFP-alone-NPC transplanted animals spent significantly greater periods of time in the target quadrant on the third day of testing 5 weeks post injury. In this paradigm, TBI/MNTS1 animals were no different from TBI-Vehicle controls. Thus, sustained NPC transplant survival and increased neuronal differentiation with MNTS1 secretion may not be necessary for functional improvement.

**Limitations**

We did not assess transplanted NPC survival and differentiation at time points greater than 5 weeks post transplantation (6 weeks post surgery). Although we did not visually detect any behavioral evidence of seizure activity or increased neuropathic pain in any animal groups, excessive exogenous cell survival and neuritogenesis, as well as constitutive MNTS1 secretion, may potentially result in adverse effects. This is especially
pertinent in cases of TBI, in which PTE is a common comorbidity (Garga and Lowenstein, 2006). It is also important to ascertain whether cell transplantation leads to prolonged immune responses or tumorigenicity. Studies conducted to assess long-term effects and temporal profiles of cell transplants are required to fully interpret the range of implications of these findings. Encouragingly, Lepore et al. (2006) conducted a longitudinal study in vivo investigating the long-term effects of NPC transplantation into the injured adult CNS. They found that at 15 months, transplanted NPC survival was perceptible and grafts did not result in tumor formation or elicit pronounced immunoreactivity (Lepore et al., 2006).

The therapeutic window for cellular transplantation will also have to be critically assessed to further clarify translational relevance. It is possible that if transplantation is delayed until more chronic phases of TBI, the NPC-mediated cytoprotection and neurogenic responses may be highly dependent on multineurotrophin treatment. Future studies are required to assess critical factors including cellular dose-response studies, treatment windows for greatest therapeutic value, and any long term risk factors associated with this experimental treatment approach.

While we observed significant preservation of the pericontusional cytoarchitecture with multineurotrophin-expressing NPCs, it is important to consider that endogenous neurotrophins are differentially regulated and direct distinct actions on neuronal viability and development. In layers IV and VI of the cerebral cortex, BDNF and NT-3 have opposing effects on neuronal dendritic growth (McAllister et al., 1997). McAllister and colleagues (1997) reported that in layer IV, BDNF stimulated dendritic growth and NT-3 inhibited it, while the reverse was true for layer VI. Thus, because of
the “push-pull” activity of endogenous neurotrophin signaling and the delicate balance required to maintain proper neuronal growth and differentiation, the consequences of the constitutive expression of a pleotropic neurotrophin may be complicated. This underscores the need for meticulous pharmacokinetic assessment and thorough investigation.

In the NPC transplantation experiments executed in this dissertation project, we did not observe any negative effects of multineurotrophin treatment in our animal models. That being said, there were no significant differences between MNTS1-transduced NPC and GFP-alone transduced NPC transplanted groups. Thus, perhaps greater NPC graft survival and neuronal differentiation with MNTS1 secretion is superfluous and may pose possible complications at longer survival time points after neurotrauma.

### 4.2 Proneurogenic Compound Administration

In the original paper by Pieper et al. (2010), the aminopropyl carbazoles exerted significant proneurogenic effects in several animal models of impaired neurogenesis, an outcome they attributed to the protection of newborn neurons from apoptosis. Because brain trauma initiates widespread apoptosis and chronically impairs hippocampal neurogenesis, the studies described in Chapter 3 were conducted to evaluate whether A20 would be neuroprotective, rescue adult neurogenesis, and subsequently improve functional outcomes in an experimental model of moderate TBI. The results of these studies demonstrated that treatment with the proneurogenic/neuroprotective compound reduced overall contusion volume and improved pericontusional neuronal survival. A20
treatment also increased acute proliferative responses and the magnitude of hippocampal neurogenesis in the SGZ 5 weeks post injury. These morphological changes correlated with improved sensorimotor and cognitive function, which are significant clinical problems experienced by patients after TBI.

*Preservation of Parenchyma, Mature and Immature Neuronal Survival, and Improved Functional Outcomes with A20 Administration after TBI*

Animals administered A20 had significant preservation of vulnerable NeuN-positive mature cortical neurons and protection of the surrounding cytoarchitecture of the traumatized hemisphere. Using the spontaneous forelimb task to evaluate sensorimotor capacity, we observed that A20-treated animals performed at levels similar to sham-operated animals, and significantly better than vehicle controls 1 week after TBI. The beneficial effects of early posttraumatic A20 treatment on significantly reducing contusion volume and protecting against cortical neuron vulnerability may participate in this clinically-relevant sensorimotor improvement.

As discussed previously, the vulnerability of SGZ immature neurons has been identified as a prominent feature of damage after TBI (Rola et al., 2006; Gao et al., 2008; Atkins et al., 2010; Blaiss et al., 2011). Hippocampal proliferation is acutely increased after TBI with a traumatic insult, however this cellular response appears to be transient and a chronic loss of immature neurons has been reported to persist up to 12 weeks (Rola et al., 2006; Potts et al., 2009; Atkins et al., 2010; Gao et al., 2013). Here we report that A20 administration increased acute proliferative responses as well as the magnitude of hippocampal neurogenesis in the ipsilateral DG compared to vehicle-treated animals 5 weeks post TBI. The ability of A20 to enhance the survival of newborn hippocampal
neurons after brain injury may be a significant and new therapeutic treatment strategy targeting endogenous reparative processes.

In rodents, newly-generated hippocampal neurons take approximately 4-6 weeks to functionally integrate within the hippocampal circuitry (Li et al., 2009). Animals receiving A20 performed better overall than TBI vehicle controls on the MWM at 4 weeks post injury. Several specific measureable parameters, including escape latency, total distance traveled, and percentage of time spent in the target quadrant, supported the conclusion that administration of A20 in the early posttraumatic period improved cognitive outcome.

Consistent with the notion of time-dependent neuronal maturation profiles, we also tested a separate group of animals on spatial memory capacity 2 weeks post TBI, prior to when the majority of newly-generated neurons are functionally incorporated into hippocampal circuitry. Interestingly, at this earlier time point, A20-treated animals performed no better than TBI-vehicle controls (Figure 4.1).
Thus, based on the established time-dependent progression of neurogenesis and the integration of new neurons into the hippocampal circuitry, our immunohistochemical and behavioral findings support the view that augmentation of hippocampal neurogenesis with A20 administration was a significant factor underlying improved cognitive function at 4 weeks post TBI.

In terms of the clinical relevance of this discovery, this therapeutic compound has advantageous pharmacokinetic properties, including desirable toxicity profiles, favorable blood/brain distribution, sufficient oral availability, and excellent metabolic stability in rodents (MacMillian et al., 2011). Furthermore, the beneficial effects of P7C3-compounds have been demonstrated in other animal models of CNS pathology, including...
ALS, AD, and models of impaired adult hippocampal neurogenesis (Pieper et al., 2010; de Jesús-Cortéz et al., 2012; Tesla et al., 2012). Additional experiments evaluating the efficacy of A20 treatment in other animal models of acute or more progressive neuropathology would be warranted for future clinical consideration. In addition, the assessment of A20 on cytoprotection, neurogenesis, and functional outcomes when treatment is delayed after TBI needs to be determined. Cause and effect relationships between A20-induced hippocampal neurogenesis and improvements in cognitive function after TBI also need to be evaluated using available approaches including antimitotic treatment, irradiation, or genetic ablation models. Nevertheless, findings to date suggest that the newly discovered P7C3-class of neuroprotective agents might promote clinically-relevant functional recovery in patients after TBI by preserving mature brain structures and helping to promote endogenous reparative processes within the hippocampus.

*Potential Mechanisms Underlying the Therapeutic Effects of the P7C3-class of Aminopropyl Carbazoles*

The precise mechanism(s) by which A20 exerts its effects has yet to be fully clarified. Pieper and colleagues (2010) proposed that the proneurogenic capacity of A20 was due to protection of newly-generated hippocampal neurons from apoptotic cell death through preservation of mitochondrial membrane integrity following a calcium challenge. As discussed in Chapter 1, apoptosis is one of the most pervasive and deleterious secondary injuries after TBI. Mitochondrial dysfunction contributes to the pathogenesis of cell death in both a caspase-dependent and caspase-independent manner (Zhang et al., 2005). Under death-inducing conditions, such as mechanical stress or excitotoxicity, mitochondria sequester excessive amounts of calcium, which is a primary mediator of
mitochondrial membrane depolarization, formation of the permeability transition pore (PTP), and subsequent release of cytochrome c into the cytosol to form the apoptosome complex (Zhang *et al*., 2005). The preliminary studies conducted by Pieper and colleagues demonstrated that A20 and its derivatives decreased the prevalence of apoptogenic cleaved caspase-3 and protected mitochondria from membrane dissolution in a dose-dependent manner (Pieper *et al*., 2010). Mitochondria-associated cell death involves numerous molecular mediators, ionic fluxes, and multiprotein interactions (Fiskum, 2000). Thus, it is difficult to delineate which specific apoptotic event(s) A20 is affecting.

Through SAR assays and the generation of several P7C3-derivatives, it has been established that the unique chemical scaffold of A20 is essential for its proneurogenic, antiapoptotic activity. The P7C3-class of compounds was discovered (Pieper *et al*., 2010) and characterized (Macmillan *et al*., 2011) relatively recently. Thus, there has not yet been ample time to fully characterize the molecular mechanisms underlying its therapeutic activity. As discussed in Chapter 1, Dimebon (latrepirdine) shares structural similarities with the P7C3-class of aminopropyl carbazoles, including a three-ring heterocycle. Dimebon was approved for commercial use in 1983 and has since been the subject of several experimental and clinical trials assessing its therapeutic efficacy in neurodegenerative diseases such as AD and HD. Side-by-side comparisons of Dimebon and P7C3-compounds revealed greater neuroprotective/neurogenic efficacy and potency of A20 in several neurodegenerative conditions (Pieper *et al*., 2010; de Jesús-Cortéz *et al*., 2012; Tesla *et al*., 2012). A recently published meta-analysis of all previous clinical reports and trials with Dimebon asserts that there is a dearth of preliminary mechanistic
and target studies delineating the molecular interactions that underlie Dimebon activity (Cano-Cuenca et al., 2014). However, another recent report reviewed the experimental literature on Dimebon and proposed several molecular mechanisms underlying its potential therapeutic activity (Bharadwaj et al., 2013). Thus, given the scaffold similarities between A20 and Dimebon, several hypotheses may be proposed as to how A20 is conferring antiapoptotic actions based on previous molecular studies with Dimebon. Dimebon has been reported to target ion channels and receptor activity (Schambra et al., 2005), to inhibit protein aggregation and autophagy (Klionsky and Emr, 2000; Tampellini et al., 2009), to modulate calcium metabolism and neurotransmitters (Bachurin et al., 2001; Okun et al., 2010), as well as to protect mitochondria function under stress conditions (Bachurin et al., 2003; Zhang et al., 2010). The etiology of TBI-induced neuropathology likely involves several of these pathological events. Because preliminary A20 experiments suggested an antiapoptotic effect mediated through mitochondria, we will primarily discuss Dimebon and mitochondrial protection; however there may be additional protective effects conferred by A20 occurring concomitantly.

Studies have shown that Dimebon modulated the opening of the mitochondrial PTP. Under normal conditions, membrane permeability allows the flux of calcium and other small compounds in and out of the mitochondria. However, pathological PTP opening, such as what occurs after TBI, results in membrane destabilization, swelling, and expulsion of neurotoxic apoptogenic proteins, including cytochrome c (Rasola and Bernardi, 2007). Dimebon suppressed PTP opening induced by several toxic agents (Bachurin et al., 2003) and significantly enhanced mitochondrial cerebral glucose utilization (Day et al., 2011). Additional research showed that Dimebon inhibited
mitochondria swelling triggered with excessive calcium toxicity (Naga and Geddes, 2011). In their review, Bharadwaj et al. (2013) elucidated two separate studies demonstrating Dimebon-induced stabilization of mitochondria membrane potential, rescue of ATP synthesis, and protection from Aβ-induced respiratory chain complex perturbations (Zhang et al., 2010; Eckert et al., 2012). These preclinical data illustrating the antiapoptotic effects of the P7C3-related compound Dimebon may reveal new molecular targets to delineate the therapeutic mechanisms underlying the P7C3-class of aminopropyl carbazoles.

Limitations

One of the disadvantages of using pharmacotherapies for the treatment of progressive disorders such as brain injury is the need for readministration and possible dosage adjustment, potentially for extended periods of time. In the present experiments, animal groups were treated with 10 mg/kg of A20 for only 1 week after injury. Upon evaluation 4 weeks later, endogenous neurogenic responses remained sustained and cognitive improvement was evident. Future studies will be required to determine the therapeutic window and optimal duration of treatment necessary to promote chronic protection and long-term improvement in cognitive function after TBI.

The mechanisms and molecular targets of A20 need to be more thoroughly delineated before it can be considered for clinical use. TBI often occurs in the presence of other injuries (polytrauma) and patients may be taking additional medications. A20 is delivered systemically. Adverse drug interactions and/or cross-tolerance with A20 administration may be significant concerns that require thorough evaluation. Furthermore, if systemic A20 administration is elevating endogenous neurogenesis by
protecting immature neurons from undergoing apoptosis, one must assess whether undesired, non-target antiapoptotic effects may be occurring simultaneously. In the posttraumatic brain, there may be some benefits to apoptosis, including signaling for the clearing of cellular debris, pruning aberrant trauma-induced connections, as well as inducing cell death to make room for new neurite growth.

4.3 Neuroprotection and Neurogenesis for the Treatment of TBI: Future Directions

Collectively, the experiments carried out in this dissertation project demonstrate that therapeutic interventions targeting and enhancing endogenous neuroprotective and neurogenic processes are effective in mitigating some of the deleterious histological and functional impairments after neurotrauma. However, based on the available data, we cannot yet ascertain whether functional improvement was directly correlated to either neuroprotection or neurogenesis. We hypothesize that both likely played a crucial role in behavioral outcomes. In the proneurogenic A20 experiments, we observed significant improvement in sensorimotor faculties as early as 1 week post TBI, prior to the maturation and functional integration of newborn DG neurons. This reversal of sensorimotor deficits coincided with the neuro- and cytoprotection effects observed in A20 groups 1 week post trauma. Interestingly, A20-induced cognitive improvement was not observed until 4 weeks post TBI. Spatial memory evaluation at 2 weeks revealed no differences between TBI-Vehicle and TBI-A20 groups. This finding is consistent with established neuronal maturation patterns and functionality during normal development stages. Thus, we propose that protection of mature parietal neurons in the cortical lesion and reduction of contusion volumes contributed to sensorimotor improvement, whereas
enhanced SGZ neurogenesis contributed to hippocampal-dependent spatial memory performance on relevant behavioral tasks. The latter notion cannot be drawn definitively, although there is compelling evidence connecting SGZ neurogenesis levels to hippocampal-dependent learning and memory capacity. Although we did not evaluate sensorimotor ability in the NPC transplantation experiments, we did observe significantly improved hippocampal-dependent spatial memory on the MWM task 5 weeks post TBI. This is an appropriate time point to assess functional participation of newly-generated DG neurons in hippocampal circuitry, as neuronal maturation in the rodent occurs over 4-6 weeks.

Adult hippocampal neurogenesis is determined by the interplay between intrinsic genetic mechanisms and extrinsic cues such as growth factors and specific environmental stimuli. In traumatic conditions, there is an imbalance between immature neuron death and survival. Treatments developed to tip the balance in favor of cell survival, either through NPC grafts to deliver and evoke trophic support or via pharmacotherapies to preclude pathological apoptosis, are critical for restoring functional outcomes. A recent paper demonstrated that there are two distinct Sox2-positive, Notch-dependent NSC populations in the adult hippocampus, and each responds selectively to aging, physiological, and pathological stimuli (Lungert et al., 2010). Quiescent radial NSCs are activated by exercise, while epileptic seizures induced the expansion of the neurogenically-active horizontal NSC pool. Furthermore, the reduction of adult neurogenesis in aging mammals results from a loss of active horizontal NSCs rather than a total loss of the stem cell pool (Lugert et al., 2010). It would be worthwhile to investigate which stem cell pool is affected by TBI and responsible for the acute
increases and chronic depression of adult hippocampal neurogenesis. Given the complex interplay of signaling mechanisms involved in all aspects of neuronal maturation, these investigations may unveil novel molecular targets to restore DG neurogenesis to uninjured-baseline levels after neurotrauma. There is still much to be clarified about these relationships and the mechanisms involved.

To further delineate the mechanisms underlying the therapeutic effects of the two treatment strategies employed in this dissertation project, it is crucial to understand the molecular interactions and events that contribute to the pathogenesis of secondary injuries after TBI. This knowledge can identify possible targets that may be influenced by the protective and neurogenic effects of our therapeutic approaches. To determine if the elevated DG neurogenesis levels conferred by NPC grafts and A20 contributed to improved cognitive outcomes, it would be necessary to conditionally ablate or block adult hippocampal neurogenesis in the presence of treatment. If our hypotheses are correct, we would expect to see a reversal of spatial memory improvement and a return to TBI-control levels. Negative manipulation of adult neurogenesis, through the use of dominant-negative Wnt agents, cranial irradiation, antimitotic drugs, conditional transgenic models, or by some other means, would provide critical insight on the therapeutic effects of A20- and NPC-induced hippocampal neurogenesis in cognitive recovery after TBI.

Additional experiments to elucidate therapeutic mechanisms of behavioral recovery with the treatments investigated in this dissertation project include those to determine if functional and true integration of transplanted NPCs and/or newly-generated DG neurons indeed occurred. Electrophysiological evaluation, assessment of LTP
induction and maintenance, expression of immediate early genes, as well as pre- and postsynaptic stains, would highlight possible important changes that may have occurred with our experimental strategies after TBI.

After brain injury, NPC transplantation and P7C3-A20 administration mitigated some of the deleterious histopathological and functional outcomes observed in both experimental and clinic settings. Furthermore, these treatments enhanced hippocampal neurogenesis, which is compromised after neurotrauma. Because of the diverse nature of these two therapies, we surmise they act on separate targets and through divergent pathways. There may also be some cross talk and shared actions due to the redundancy of various signaling cascades and multi-action molecular mediators. Corroborating this notion, we observed shared neuro- and cytoprotective effects, elevated hippocampal neurogenic responses, and cognitive improvement with both NPC transplantation and A20 therapy (Proposed Schematic, Figure 4.2). We hypothesize that a combinatorial treatment strategy, engaging both interventions, may have more clinical-relevance and exhibit augmented effects as a result of synergistic actions, and hence be more efficacious given the heterogeneous, complex, and progressive nature of traumatic brain injury.
Figure 4.2. Proposed schematic of therapeutic actions conferred by NPC transplantation and A20 administration in the traumatized brain.

The inherent protective and neuroreparative effects of transplanted NPCs may include direct cellular replacement and integration within compromised host tissues, immunomodulatory mechanisms, and/or trophic factor secretion, which was enhanced in some groups with genetic modification to secrete the prosurvival multineurotrophin, MNTS1. Neurotrophin-activated neurite outgrowth and synaptic plasticity may in turn facilitate and strengthen functional integration of transplanted cells into host circuitry, possibly resulting in greater neuroprotection and survival of newborn neurons due to activity-dependent mechanisms. Neurotrophin signaling also mediates cell survival and neuronal differentiation, and possibly endogenous adult hippocampal neurogenesis, (although a direct association with the latter has not been fully established as neurotrophins may be acting through prototypic survival and differentiation pathways and not necessarily affecting proliferative responses). We found that NPC transplantation protected the perilesional cytoarchitecture, promoted endogenous hippocampal neurogenesis, and mitigated cognitive deficits after brain trauma. The therapeutic effects of A20 administration after TBI are less understood, but have been attributed to the attenuation of apoptosis. Previous mechanistic studies with a structurally-related compound demonstrated antiapoptotic activity via protection of mitochondrial membranes and reduction of excitotoxic events. Treatment with A20 resulted in significant neuroprotection and enhanced hippocampal neurogenesis, and subsequent functional improvement in sensorimotor and spatial memory outcomes after trauma. NPC transplantation and A20 treatment both protected vulnerable parenchyma and restored endogenous neurogenic reparative responses. These structural and cellular changes effectively attenuated some of the deleterious residual consequences of neurotrauma. As illustrated here, we propose that each therapeutic intervention may be acting through distinct pathways and targeting separate TBI-induced neuropathological events. However, there may be some overlap as both strategies resulted in similar histological and functional outcomes. Engaging a combinatorial approach consisting of both progenitor cell therapy and proneurogenic compound administration may be more effective in ameliorating functional deficits after injury (dashed lines represent less established interactions).
References


95. Döbrössy, M.D., Drapeau, E., Aurousseau, C., Le Moal, M., Piazza, P.V., and Abrous, D.N. (2003). Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Mol Psychiatry 8, 974-982.


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