CELLULAR AND CIRCUIT LEVEL RESPONSES TO NEURAL STEM CELL TRANSPLANTATION IN THE RODENT CORTEX

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ABSTRACT

CELLULAR AND CIRCUIT LEVEL RESPONSES TO NEURAL STEM CELL TRANSPLANTATION IN THE RODENT CORTEX

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John H. Wolfe

Neural stem cell (NSC) transplantation is a promising strategy for the treatment of neurological disease and injury. NSC transplants have been documented to exert both neurotrophic and immunomodulatory effects in pathological contexts, but grafted cells frequently remain undifferentiated. The specific interactions between undifferentiated NSCs and the normal host microenvironment are not well understood. To investigate the functional impact of undifferentiated NSCs on host activity, a clonal NSC line (C17.2) was utilized. Network dynamics were monitored post-transplant in acute slices of somatosensory cortex using voltage sensitive dye imaging. Single and repetitive callosal stimuli elicited activity that originated in deep layers, propagated vertically along cortical columns, and spread horizontally across superficial layers. Very high levels of C17.2 engraftment (>25%) interfered with parameters of cortical function, including the amplitude, spatial extent, velocity, and integration of evoked potentials. These levels also raised the current threshold required to activate cortical microcircuitry by ten-fold. Conversely, moderate levels of engraftment (<15%) preserved network properties and induced only subtle changes in facilitation during repetitive stimulation. A binning analysis of cortical activity showed that deep cortical layers were more susceptible to the presence of ectopic NSCs than superficial layers. Pharmacological blockade of $GABA_A$ signaling indicated that inhibition was not the predominant cause of circuit dampening in these layers. Instead, highly engrafted cortices showed a marked depletion in host neurons and associated neuronal metabolites. Microglial activation preceded neuronal loss in the transplanted brain and deactivation with doxycycline exerted a neuroprotective effect. Analysis of C17.2-conditioned supernatants showed they secrete a number of proinflammatory cytokines and chemokines. However, these factors did not induce

direct toxicity, but rather enhanced microglial-mediated neuronal apoptosis *in vitro* via tumor necrosis factor alpha (TNFα)-dependent signaling. Primary NSCs from the postnatal subventricular zone showed similar effects on microglial-mediated cytotoxicity. Together, these results suggest that undifferentiated NSCs possess an inherent capacity to modulate microglial functions which can affect neuronal survivability and activity in the host brain.

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General Introduction

I. Neural Stem Cells

For over 100 years, the adult mammalian brain was thought to remain structurally constant after birth, with no further addition of neurons (Gross, 2000). This central dogma stemmed from the histological and anatomic studies of Koelliker, His, and Cajal. In his seminal work (Ramón y Cajal, 1913), Cajal commented "*Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.*" Up to the mid1980s, autoradiographic analysis of S-phase nuclei suggested that neurogenesis was limited to prenatal and early postnatal stages (Rakic, 1985; Eckenhoff and Rakic, 1988). Several developments eventually led to the general acceptance of adult-generated neurons. *In vivo* fate-mapping using BrdU (5-bromo-3'-deoxyuridine) revealed that neurons constitute a large proportion of adult-born cells (Gratzner, 1982; Kuhn et al., 1996). The trophic and mitogenic actions of growth factors, including fibroblast growth factors (FGF) and epidermal growth factor (EGF) family proteins, further permitted the long-term maintenance and subsequent study of neural cells *in vitro* (Morrison et al., 1987; Nurcombe et al., 1993). In the early 1990s, neural cells were isolated from the adult brain that exhibited colony-forming activity, self-renewal, and multipotency (Reynolds and Weiss, 1992, 1996). These *bona fide* neural stem cells (NSCs) were also identified in other regions of the mammalian nervous system (Turner and Cepko, 1987; Morshead et al., 1994; Palmer et al., 1995, 1997; Weiss et al., 1996; Gage, 2000). Advances in the derivation and culture of NSCs led to the idea that these cells could be used to restore normal function in the diseased or damaged brain. Numerous transplantation experiments have been undertaken to test the therapeutic potential of NSCs. The beneficial effects of transplanted NSCs were first demonstrated in a mouse model of lysosomal storage disease (Snyder et al., 1995). Upon neonatal injection, clonally-derived progenitors exhibited widespread engraftment and ameliorated neuropathological lesions. Different sources of NSCs have since been identified for therapeutic use, which are elaborated upon in this section.

Identification of NSCs in the embryonic VZ

Multipotent NSCs reside in the germinal neuroepithelium or ventricular zone (VZ) of the embryonic brain (Frederiksen and McKay, 1988; Anderson, 1989; Temple, 1989; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Davis and Temple, 1994). Early in development, neuroepithelial cells in the VZ undergo expansion through symmetric division. At the onset of cortical neurogenesis, they elongate and convert into radial glial (RG) cells (Noctor et al., 2001). RG cells divide asymmetrically to generate neurons directly or indirectly through intermediate progenitors cells (IPCs) (Noctor et al., 2004). Newly generated neurons migrate along radial glial fibers past earlier-generated cells to reside in successively more superficial positions (Kriegstein et al., 2006). Postmitotic progeny ultimately coalesce into six distinct cortical layers that have specific projections patterns. Sister excitatory neurons are also radially aligned in ontogenic columns (Price and Thurlow, 1988; Kornack and Rakic, 1995) . Subsequent studies have demonstrated that clonally related neurons within a column electrically couple (Yu et al., 2012) and preferentially form synaptic connections (Yu et al., 2009).

The fate of germinal cells in the developing cortex is temporally regulated. Heterochronic transplantation studies have examined the laminar fate of early and late cortical progenitor cells (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Early cortical progenitors, which give rise to deep layer neurons, can retain this fate even after being transplanted into an older host (McConnell and Kaznowski, 1991). Late cortical progenitors grafted into the cerebral cortex of a younger host environment do not acquire an earlier fate. Instead, these cells are restricted to producing upper layer neurons (Frantz and McConnell, 1996). Even in culture, individual progenitor cells sequentially generate distinct neuronal subtypes in the same order as they occur *in vivo*, suggesting that this phenomenon is cell-autonomous (Shen et al., 2006). The separate timing of neurogenesis and gliogenesis in the developing cortex is also well established (Takahashi et al., 1995). Isolated multipotent NSCs *in vitro* recapitulates the normal order of cell generation: neurons followed by glia (Qian et al., 2000). Gliogenic cues, transcription factor sequences, DNA methylation changes, and chromatin modifications may underlie this switch from neurons to glia (Barnabé-Heider et al., 2005; Hirabayashi et al., 2009; Pereira et al., 2010).

Despite their intrinsic capacity to generate multiple neural lineages, embryonic or fetal NSCs raise ethical concerns that might preclude their use in therapies.

Immortalized cell lines

Immortalized lines offer a number of advantages for transplantation studies (Martínez-Serrano, 1997; Jandial et al., 2008). They are self-renewing and can be expanded to large numbers *in vitro*. Immortalized cells are also genetically homogenous because they are isolated from single clone. Due to easy handling of these lines, stable expression of therapeutic genes can be achieved readily.

Although spontaneously occurring NSC lines exist, specifically those isolated from neuronal and glial brain tumors, the vast majority have been generated through genetic manipulation. The introduction of viral oncogenes is an effective approach, presumably because viruses have evolved to enhance host cell proliferation. The SV40 large T antigen has been used extensively for the immortalization of neural progenitors. It maintains host cell replication by inhibiting tumor-suppressor genes p53 and retinoblastoma protein. The mutated form of the large T antigen, tsA58, is particularly favorable because it is inactivated at physiologic temperature (Ray et al., 1992). Thus, NSCs expressing tsA58 can be propagated *in vitro*, but their proliferative drive is removed following transplantation. Two well-characterized, tsA58-carrying lines, HiB5 and RN33B, derive from the embryonic rat hippocampus and medullary raphe, respectively (Frederiksen et al., 1988; Renfranz et al., 1991; Whittemore and White, 1993). Retroviral transfer of *v-myc* is another strategy for generating NSC lines. *V-myc* was identified as the transforming component of the retrovirus MC29 (Alitalo et al., 1983). Retroviruses encoding this oncogene were introduced into primary cultures of dissociated postnatal mouse cerebellum to produce the C17.2 line (Ryder et al., 1990; Snyder et al., 1992). Progenitors from the adult rat hippocampus have also been rendered immortal using *v-myc* (Hoshimaru et al., 1996). The adenoviral early region (E1A) is another viral oncogene capable of inducing sustained replication in neural cells. The Cb-E1A line derives from the cerebellum of the postnatal rat (Seigel et al., 1996).

Genetic perpetuation of human progenitors has been achieved using the retroviral transfer of *v-myc*. Flax et al. (1998) isolated primary cells from the periventricular region of a fifteen week human embryo and subsequently generated an immortalized line that retained multipotency *in vitro*. Additional studies have demonstrated that human lines can be generated from the diencephalic and telencephalic tissue (Villa et al., 2000). Immortalized human NSC lines have also been generated with telomerase. Telomeres are TTAGGG repeats flanking the ends of chromosomes that protect against degradation, fusion, and recombination. Reduction of telomere length after replication contributes to cellular senescence. Telomerase is a cellular ribonucleoprotein reverse transcriptase that increases telomere length and in effect, prolongs cell cycling. Telomerase activity has been conferred upon fetal SVZ cells via introduction of the human TERT gene. Transduced cells could be expanded indefinitely and were found to maintain a normal diploid karyotype (Bai et al., 2004).

One major drawback of immortalized lines is the possibility of cell transformation (Martínez-Serrano, 1997; Jandial et al., 2008). Transformed cells show independence from mitogenic factors, unresponsiveness to antigrowth signals, resistance to apoptosis, and independence from substrate anchorage. In addition, it is unclear whether immortalization itself alters the developmental potential of transplanted NSCs. However, this remains primarily a hypothetical case as no *bona fide* transformation events have been fully documented in the literature after immortalized NSC transplantation.

Adult NSCs

Altman and Das provided the first evidence of adult neurogenesis in the subventricular zone and hippocampal dentate gyrus (DG) of rodents (Altman, 1962; Altman and Das, 1965). Bromodeoxyuridene (BrdU) labeling of dividing cells in the adult rodent forebrain confirmed these early reports (Kuhn et al., 1996, 1997; Kempermann et al., 1997) and showed that adult hippocampal neurogenesis generalized to the human brain (Eriksson et al., 1998). Fate-mapping approaches revealed that a subpopulation of astrocytes gave rise to all adult-born neurons (Doetsch et al., 1999; Seri et al., 2001; Garcia et al., 2004). Similar to their embryonic

counterparts, these neurons displayed spiking activity and were functionally active (Song et al., 2002; van Praag et al., 2002).Although these newborn cells have been shown to synaptically integrate into existing circuits, their contribution to higher order brain functions remains elusive. Several studies have suggested that newborn neurons in the DG may be essential for making fine discriminations between highly similar environments (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011; Gu et al., 2012; Nakashiba et al., 2012).

Much attention has been focused on methods to harvest and propagate NSCs from the adult brain for therapeutic use. Cells have been successfully isolated from the striatum as well as the subgranular zone (SGZ) of the hippocampus of the adult mouse brain and expanded *in vitro* using mitogenic factors (Reynolds and Weiss, 1992; Palmer et al., 1995, 1997). Advanced sorting techniques have been applied to further purify adult progenitors from the SGZ and SVZ (Roy et al., 2000; Pastrana et al., 2009). Progenitor proliferation also occurs in nonneurogenic regions of the adult brain (i.e. cortex and optic nerve), but these cells maintain properties of precursors or become glia. However, progenitors isolated from these nonneurogenic regions can be directed towards a neuronal fate in the presence of retinoic acid and forskolin (Palmer et al., 1999). These studies demonstrate that cell-extrinsic factors are critical for NSC differentiation.

Transplantation experiments further support the importance of a neurogenic niche. For example, DG precursors deposited into the SVZ follow the developmental trajectory of endogenous SVZ precursors and differentiate into olfactory neurons (Suhonen et al., 1996). Moreover, adult NSCs ectopically grafted in nonneurogenic regions generate predominantly oligodendrocytes and astrocytes (Seidenfaden et al., 2006). Interestingly, NSCs from a nonneurogenic region, such as the spinal cord, adopt mature neuronal phenotypes when transplanted into the DG (Shihabuddin et al., 2000). These data support the idea that external cues from the local microenvironment promote neuronal specification of NSCs. More specifically, resident astrocytes (Song et al., 2002), endothelial cells (Shen et al., 2004), microglia (Sierra et al., 2010), and vasculature (Palmer et al., 2000) in the SGZ and SVZ regulate site-specific NSC behavior and contribute to neurogenesis. It is not well understood how these cellular constituents interface with transplanted cells in the diseased or injured brain.

Embryonic and induced pluripotent stem cells

Two major hurdles limit the use of primary adult NSCs in cell-based therapies (Elkabetz et al., 2008). First, adult NSCs show an increased gliogenic bias after long-term culturing. Second, *in vitro* expanded NSCs cannot recapitulate the endogenous diversity of neuronal subtypes. Embryonic stem cell (ESC) lines derived from human blastocysts represent an alternative to primary adult NSCs (Thomson et al., 1998). One notable advancement is the isolation of neural rosettes (Tropepe et al., 2001). ESC-derived neural rosettes are radially organized columnar epithelial cells capable of differentiating into region-specific glial and neuronal cell types (Perrier et al., 2004; Li et al., 2005). They are comparable to *in vivo* neural precursors which emerge at the neural plate after tube closure (Jessell, 2000) and provide improved access to therapeutically relevant neuronal types upon transplantation (Koch et al., 2009). However, human embryonic stem research is ethically and politically controversial because it involves the destruction of human embryos.

Takahashi and Yamanaka devised a method to dedifferentiate mouse somatic cells into pluripotent cells that avoids the problems specific to ESCs (Takahashi and Yamanaka, 2006). They were able to circumvent the rigid bounds of cell fate and lineage commitment using four transcription factors, Oct 3/4, Sox2, c-Myc, and Klf4. Several groups replicated and refined these methods to generate induced pluripotent stem cells (iPSCs) of human origin (Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007). IPSCs can be guided towards an NSC fate using a number of different culturing protocols (Chambers et al., 2009). This technology promises patient-derived tissue for future transplantation, thereby minimizing the possibility of immune rejection. However, there are several practical concerns introduced by the selective pressures inherent to the reprogramming procedure. These issues include chromosomal aberrations (Mayshar et al., 2010), somatic mutations (Gore et al., 2011), abnormal DNA methylation (Lister et al., 2011), and copy number variations (Hussein et al., 2011). The pluripotency of these cell types and their extended derivation times increase their potential for tumorigenicity (Ben-David and Benvenisty, 2011). There is also concern that iPSC-derived NSCs may generate tumors due to contaminating iPSCs or incomplete reprogramming. Direct reprogramming of somatic cells to

multipotent, lineage-restricted cells could lower the potential risk of teratoma formation. More recently, human fibroblasts have been directly converted into induced neural stem cells (iNSCs), bypassing a pluripotent state (Han et al., 2012; Ring et al., 2012; Thier et al., 2012).

Another outstanding issue is the generation of mature neural progeny from iPSC-derived transplants. Over the past decade, researchers have demonstrated that the morphogenic gradients present in neurodevelopment may be applied *in vitro* to drive the differentiation of specific neuronal subtypes from iPSCs (Ma et al., 2011; Shi et al., 2012; Liu et al., 2013; Qu et al., 2014). However, these precisely regulated developmental processes do not tolerate significant *in vivo* deviation, making the replacement of adult neurons by ectopic NSCs a considerable challenge. For example, in the absence of developmental guidance cues, deriving spinal motor neurons that can receive the appropriate regulatory input and are able to extend their axons long distances to reinnervate muscles is a significant experimental hurdle. Apart from this issue, both transplanted ESC and iPSC-derived cells may have an intrinsic specification that may limit the cell types that can be produced *in vivo* (Gaspard et al., 2008; Espuny-Camacho et al., 2013). ESC and iPSC lines may also vary in their neurogenic capacity as revealed by genomic and functional analyses (Wu et al., 2007; Hu et al., 2010; Kim et al., 2010, 2011)

II. NSC Transplantation in CNS Disorders

The feasibility of different NSC-based strategies has been tested in a number of preclinical models of human disease. Many of these therapeutic platforms have raised issues related to the restricted differentiation potential of transplanted precursors.

Lysosomal storage disease

Lysosomal storage diseases (LSDs) represent a group of more than fifty metabolic disorders typically inherited in an autosomal recessive manner (Shihabuddin and Cheng, 2011). LSDs are caused by mutations in one or more catabolic enzymes involved in the degradation of macromolecules within the lysosome. Impaired lysosomal activity leads to progressive accumulation of substrates, resulting in cellular and organ dysfunction. Systemic enzyme

replacement therapy has been effective at correcting visceral disease for some LSDs, such as Gaucher, Fabry, Pompe, mucopolysaccharidosis (MPS) type I, II, and VII. Treating CNS pathology associated with many LSDs remains a major technical challenge due to limited blood-brain barrier permeability. Moreover, the disseminated lesions characteristic of LSDs necessitate widespread delivery of therapeutic products.

An effective stem cell-based therapy for LSDs requires cells to migrate throughout brain parenchyma and secrete appropriate levels of lysosomal enzyme (Desnick and Schuchman, 2002). Transplanted NSCs can produce deficient enzyme for uptake by adjacent diseased host cells, contributing to a larger area of clearance and neuroprotection. This phenomenon of cross-correction is mediated by mannose 6-phosphate receptor-mediated endocytosis. NSCs can be further engineered *ex vivo* to maximize expression and secretion of lysosomal enzymes that are deficient in the host brain. Preclinical studies must establish the levels of enzyme activity required for therapeutic effect.

Several mouse models of LSDs with neuropathic disease have been treated using NSC transplants. The first successful demonstration came from studies in MPS VII rodents using the NSC line C17.2 (Snyder et al., 1995) . Neonatal intraventricular transplantation resulted in the widespread engraftment of donor cells, β-glucuronidase secretion, and cross-correction of neighboring neurons and glia. Similar findings were reported using a human NSC line (HB1.F3) in this mouse model (Meng et al., 2003). Cell-type specific markers and morphological features suggested that some grafted cells in this study differentiated into astrocytes, however, no evidence of mature neuronal differentiation was reported. In a Tay-Sachs rodent model, C17.2-mediated gene transfer resulted in broad distribution of β-hexosaminidase, at levels potentially sufficient to correct pathology (Lacorazza et al., 1996). Benefits of cell-based therapies have also been reported in rodent models of Krabbe disease (Taylor et al., 2006), metachromatic leukodystrophy (Klein et al., 2006), and Niemann-Pick type A (Shihabuddin et al., 2004). In the majority of these studies, neonatal transplants result in the arrest of disease progression. However, most affected patients are not diagnosed until their symptomology is clinically evident. In a proof-of-principle study, intracranial transplantation of C17.2 cells into adult symptomatic

Sandhoff disease mice restored β-hexosaminidase activity, reduced storage levels, and decreased inflammation (Jeyakumar et al., 2009). Although both neurons and glia were present in C17.2 grafts, the vast majority of grafted cells persisted as undifferentiated cells.

Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder associated with a progressive loss of dopaminergic neurons in the substantia nigra (SN) (Gaillard and Jaber, 2011). Symptoms include rigidity, hypokinesia, tremor, and postural instability. Current treatments for PD such as L-dihydroxyphenylalanine (L-dopa), dopamine agonists, enzyme inhibitors, and deep brain stimulation fail to counteract disease progression. Cell-based approaches aim to provide long-term clinical benefit by restoring physiological levels of dopamine in the striatum.

Open-label clinical trials have demonstrated that intrastriatal grafting of human fetal mesencephalic tissue, rich in dopaminergic neuroblasts, can provide some relief (Lindvall et al., 1992; Kordower et al., 1995; Piccini et al., 1999). The resulting neuronal progeny can reinnervate the denervated striatum and restore striatal dopamine release. Contradictory data obtained from two double-blind placebo trials reported no significant transplant effects (Freed et al., 2001; Olanow et al., 2003). A considerable issue in these particular studies was the occurrence of post-operative graft-induced dyskinesia, or involuntary muscle movements. This adverse event was thought to arise from unregulated dopamine release by grafted cells. In fact, fetal tissue obtained from mice lacking the dopamine active transporter (DAT), which regulates dopamine transmission via reuptake, exacerbates L-dopa-induced dyskinesias in grafted rodents (Vinuela et al., 2008).

Recent work has addressed the long-term survival of fetal-derived grafts in PD patients (Kordower et al., 2008; Li et al., 2008; Mendez et al., 2008). In one study, dopaminergic neurons derived from transplants survived without signs of degeneration for up to 14 years(Mendez et al., 2008). Conflicting reports cite that a small proportion of transplanted cells acquired host pathology (Kordower et al., 2008; Li et al., 2008). However, the progression of pathology in graft-derived neurons was found to be slow and patients still showed long-term clinical benefit.

The substantial variability in patient outcomes may partly stem from the poor standardization of transplanted material. Post-mortem analysis of PD patients reveals that transplanted cells yielded a proportion of serotonergic neurons (Mendez et al., 2008). Grafts of fetal ventral mesencephalon also contain a mixture of SN and ventral tegmental area (VTA) dopaminergic neuronal subtypes. Only the SN subtype retains the capacity to innervate the striatum and induce substantial benefit in PD (Mendez et al., 2005; Grealish et al., 2010). In fact, when fetal neurons from the olfactory bulb are transplanted into the SN, few fibers extend to the striatum, despite a significant number of dopaminergic neurons being present in the transplant (Gaillard et al., 2009). These issues and the limited fetal mesencephalic tissue available for transplantation may be resolved by generating dopaminergic neuroblasts from stem cells.

Dopaminergic neuroblasts have been derived from human ESCs using a variety of methods, including stromal feeder layers, growth factors, morphogens, and transcription factors (Kawasaki et al., 2000; Bjorklund et al., 2002; Kim et al., 2002; Takagi et al., 2005; Roy et al., 2006; Rodríguez-Gómez et al., 2007; Cho et al., 2008; Sanchez-Pernaute et al., 2008; Kriks et al., 2011). ESC-derived neurons can survive in rodent models of PD and mediate functional recovery (Roy et al., 2006; Cho et al., 2008). IPSCs from PD patients have also been differentiated into dopaminergic neurons (Hargus et al., 2010). IPSC-derived grafts in the adult rat striatum did not show signs of neurodegeneration. However, few dopaminergic neurons within the graft projected to appropriate subcortical targets. A significant therapeutic concern related to the use of ESC and iPSC-derived transplants is the presence of undifferentiated tumor-forming cells (Roy et al., 2006). Cell purification with FACS prior to transplantation may alleviate these concerns (Ganat et al., 2012). There is a current need for strategies that will promote the proper differentiation of grafted cells into SN dopaminergic neurons.

Epilepsy

Epileptic seizures may develop as result of brain insult, developmental malformation, and genetic mutation, or may be idiopathic in nature (Sebe and Baraban, 2011). These surges of aberrant hyperactivity reflect an imbalance between excitatory and inhibitory networks in the

brain. Approximately one third of affected individuals are unresponsive to existing medications, which primarily target the GABAergic system. Although surgical resection may be option for a minority of these patients, it can lead to further neurological impairment. Thus, refractory epilepsy remains a large clinical problem. Cell-based approaches are currently being considered as an alternative treatment (Alvarez Dolado and Broccoli, 2011; Sebe and Baraban, 2011; Tyson and Anderson, 2014). GABAergic grafts may directly replace dysfunctional or lost interneurons or indirectly modulate a hyperactive excitatory network.

Several studies over the past decade have elucidated the origin of cortical GABAergic interneurons (Lavdas et al., 1999; Sussel et al., 1999; Xu et al., 2004; Butt et al., 2005) . Located in a restricted region of the ventral telencephalon known as the medial ganglionic eminence (MGE), these precursors migrate tangentially over long distances to populate the cortex (Marín and Rubenstein, 2001). Grafting studies have further demonstrated the remarkable migratory capacity of MGE cells in an adult host (Wichterle et al., 1999). Accordingly, MGE-derived interneuron precursors may be especially suited for use in the epileptic brain.

In a proof-of-principle study, MGE precursors ectopically grafted into the normal postnatal brain (P3-P4) gave rise to neurons that migrated extensively throughout the cortex, striatum, and hippocampus (Alvarez-Dolado et al., 2006). Over seventy percent of grafted cells adopted a GABAergic interneuron phenotype, based on electrophysiological measures, and a subset expressed subtype specific markers (i.e. somatostatin, neuropeptide Y, parvalbumin, and calretinin). More importantly, patch-clamp recordings of IPSCs in host pyramidal neurons revealed that GABAergic-mediated inhibition was elevated in engrafted regions. Thus, MGE-derived grafts could increase the inhibitory tone of local circuits via a synaptic mechanism. MGE transplants also show intrinsic anti-epileptogenic activity. Cells transplanted into the cortex of neonatal Kv1.1 mutant mice, which lack the voltage-gated K+ channel, Kv1.1, significantly reduced the frequency and duration of spontaneous electrographic seizures (Baraban et al., 2009). This approach can also be used to ameliorate seizure activity and attenuate behavioral phenotypes in an adult model. MGE progenitors were transplanted into the hippocampus or

amygdala of pilocaprine-treated rodents (Hunt et al., 2013). Nearly sixty percent of grafted cells exhibited electrophysiological phenotypes consistent with those of mature interneurons.

The limited number of cells that can be harvested from the MGE for therapeutic use is a potential concern. MGE cells can be expanded *in vitro* as neurospheres, but the interaction of MGE cells with mitogens in culture may alter their neurogenic potential. Consequently, MGE neurospheres injected into a kainic acid model of epilepsy generated largely astrocytes and a small proportion of GABAergic cells after transplantation into the adult hippocampus (Waldau et al., 2010). Interestingly, grafted cells still restrained spontaneous recurrent motor seizures, suggesting that these cells acted through a non-synaptic mechanism. Another strategy to obtain sufficient cell numbers for transplantation is the directed differentiation of ESC and iPSC cells into MGE like progenitors. Several studies have demonstrated that such cell types, which mimic endogenous human neural development, can be derived *in vitro* (Maisano et al., 2012; Maroof et al., 2013; Nicholas et al., 2013).

Stroke

Cell death following ischemic stroke can contribute to sensory, motor, and cognitive impairments (Kokaia and Lindvall, 2012). Though endogenous, surviving neurons may contribute to the restoration of disrupted circuitries, the plasticity of these mature cells is limited, particularly in the aged brain. Cell-based approaches are of particular interest as a means to restore function via neuronal replacement.

Human fetal-derived NSCs have been transplanted into the stroke-damaged rodent cortex (Kelly et al., 2004) and striatum (Darsalia et al., 2007). Intracortically transplanted neurospheres survived robustly in ischemic brains for at least four weeks post-transplant. Human cells exhibited directed, long-distance migration towards the lesion in ischemic animals. Migrating cells expressed the neuroblast marker doublecortin (DCX). Cells at the lesion border predominantly expressed the immature neuronal marker βIII-tubulin and a small percentage expressed glial fibrillary acidic protein (GFAP). Human NSCs in the lesioned striatum also exhibited robust survival and extensive migration (Darsalia et al., 2007). A small number of

grafted cells displayed mature neuronal morphology and expressed subtype-specific markers, such as calbindin and parvalbumin. However, the majority of cells remained undifferentiated and expressed nestin, a marker of neural stem cells. Immunohistochemistry also revealed a very low percentage of proliferating cells within the graft core. Altering the timing of transplantation and number of input cells did not significantly increase the extent of neuronal differentiation (Darsalia et al., 2011). Despite their lack of complete neuronal maturation, fetal grafts may promote dendritic plasticity in stroke-lesioned and contralateral cortices (Andres et al., 2011). Transplanted rats exhibited enhanced corticocortical, corticostriatal, corticothalamic, and corticospinal axonal rewiring from the intact hemisphere, which correlated with functional recovery.

Fetal-derived NSCs are currently being used in clinical trial in stroke patients. Clinical use is limited to the human NSC line CTX0E03, which was derived from fetal cortex following conditional immortalization (Pollock et al., 2006). Previously, intrastriatal transplantation of CTX0E03 in a rat model of stroke significantly improved sensorimotor function and gross motor asymmetry. Grafted cells expressed the neuron-specific cytoskeletal protein neurofilament (NF). None of the queried NF-reactive cells had elaborated dendritic arborizations characteristic of striatal neurons. Thus, it is unlikely these cells act by neuronal replacement.

NSCs for neuronal replacement may also be obtained from human ESCs. Human ESC-derived NSCs migrated towards the ischemic lesion following intrastriatal delivery in a rat stroke model (Daadi et al., 2008). Treated rats improved in the independent use of their impaired forelimb two months post-grafting. Nearly sixty percent of grafted cells differentiated into βIII-tubulin-positive neurons, half of which were GABAergic and only two percent of which were glutamatergic. Further analysis revealed the presence of voltage-gated sodium currents and excitatory postsynaptic currents in a subset of grafted cells, suggesting that they were functionally active (Daadi et al., 2009). In another study, seven percent of mouse ES-derived NSCs differentiated into mature glial cells and thirty percent differentiated into mature neurons that were capable of firing action potentials (Bühnemann et al., 2006). While ES-derived NSCs can give rise to functional neurons, it is unclear whether these cells integrate into host circuits to restore function.

More recently, human iPSC-derived NSCs were transplanted into the stroke-injured rat brain (Oki et al., 2012; Tornero et al., 2013). Long-term expandable neuroepithelial-like stem cells (lt-NES) generated from human iPS cells promoted the recovery of forepaw movements at one week post-transplant (Oki et al., 2012). The rapid recovery period suggests that improvement was most likely not due to neuronal replacement. However, a proportion of lt-NES cells were capable of differentiating into morphologically mature neurons that received synaptic inputs and sent projections to the proper target area. Nearly twenty-five percent of iPSC derived progenitors grafted into the ischemic cortex generated electrophysiologically mature neurons, a subset of which adopted cortical phenotypes (Tornero et al., 2013). Although transplanted groups exhibited bilateral recovery in a motor task, this improvement occurred at an early time point and was likely not due to the reconstruction of circuitry.

III. Modulation of NSC Fate by Immune Cells

The limited survival and differentiation of grafted NSCs may be a consequence of innate and adaptive immune mechanisms that accompany virtually every injury or disease state.

Immune signaling in the CNS

Innate immunity in the CNS is stimulated by tissue injury, infection, and degenerative disease (Lampron et al., 2013). Molecular hallmarks of these pathological processes, coined pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), are recognized by pattern recognition receptors (PRRs). PRRs are subdivided into three major receptor families: Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG1-like receptors (RLRs). Although PRR functions are best characterized in resident microglia, the innate immune cells of the CNS, astrocytes also express a subset of these receptors. PRR signaling in glia converges on the nuclear factor κB (NFκB) and p38 mitogen-activated protein kinase (p38 MAPK) pathways to induce the production of cytokines (IL-1β, IL-6, TNFα, IL-12, IFNγ, TGFβ, etc.), chemokines (MIP-1α, MCP-1, RANTES, etc.), reactive oxygen species, and free radicals. The impact of activated microglia and the effectors they release is complex because

immune signaling has time- and context-dependent proinflammatory and anti-inflammatory components.

Cytokine release also triggers the adaptive immune response, which involves the activation and recruitment of peripheral immune cells to sites of injury or infection (Ransohoff and Brown, 2012). Cytokines act locally to activate vasculature and in circulation to activate lymphocytes. Circulating lymphocytes bind to activated endothelium and extravasate into brain parenchyma. Once present, lymphocytes can eliminate pathogens through antigen recognition, T-cell mediated cytolysis and B-cell mediated antibody production. The brain has mechanisms in place to resist an adaptive immune response. Relative to other tissues of the body, peripheral lymphocyte recruitment is attenuated by the astrocytic blood-brain barrier (BBB). The brain also tightly regulates T-cell mediated adaptive immune recognition. This is achieved in part by constitutive expression of Fas ligand (FasL) by astrocytes and neurons, which triggers FasL-mediated apoptosis of activated T-cells (Niederkorn, 2006). The CNS parenchymal environment is also anti-inflammatory, containing elevated concentrations of anti-inflammatory cytokines (i.e.TGF-β and IL-10) and gangliosides that are toxic to T-cells. Still, a sufficiently large immune stimulus can degrade BBB function and permit a full-scale adaptive response (Ransohoff and Brown, 2012) .

Immunogenicity of grafted NSCs

Neural stem cells, surprisingly, express numerous immune receptors and ligands, including TLRs, cytokine and chemokine receptors, major histocompatibility complex type I and type II (MHCI and II) molecules, and several cell adhesion molecules utilized for extravasation by lymphocytes (Pluchino and Cossetti, 2013). Consequently, signaling mechanisms thought to pertain only to cells of the immune system can potentially influence the survival and differentiation of transplanted NSCs. These mechanisms may be significantly altered or even enhanced by pathologic processes. Allogeneic NSCs in the healthy brain can evade immune surveillance through the downregulation of MHC antigens (Hori et al., 2003). However, proinflammatory cytokines present in the diseased brain can increase the immunogenicity of allografts. In

particular, exposure to IFNγ or TNFα can upregulate surface expression of MHC and costimulatory markers that subsequently trigger an adaptive immune response (Imitola et al., 2004; Mammolenti, 2004). IFNγ or TNFα can also enhance the expression of toll-like receptors (TLR2, TLR4) in NSCs and affect how these cells respond to TLR2 and TLR4 agonists (Covacu, 2009). The immunogenicity of NSCs may also be elevated by long-term culturing or cycling *in vivo* (Fainstein et al., 2013). Considerable efforts are underway to improve graft survival through suppression of the adaptive response (Pearl et al., 2011). However, innate immune signaling also plays a prominent role in graft survival (Chen et al., 2011). In some circumstances, the actions of the innate immune system may not be sufficient to eliminate allografts. Instead, innate responses may suppress the neuronal differentiation of transplanted progenitors (Ideguchi et al., 2008; Gomi et al., 2011). Consistent with these data, numerous studies have reported the detrimental effect of inflammation on hippocampal neurogenesis (Carpentier and Palmer, 2009).

IV. Effects of NSCs on Host Microenvironment

The limited terminal differentiation of transplanted NSCs within host tissue suggests that they may exert therapeutic effects that are alternative to cell replacement.

Neurotrophic support

Grafted NSCs can enhance endogenous repair and protective mechanisms via neurotrophin signaling (Martino and Pluchino, 2006). Neurotrophins are a family of proteins central to a number of CNS functions, including differentiation, neuronal survival, synaptogenesis, and activity-dependent synaptic plasticity (Lu et al., 2005). Four neurotrophins have been identified in the mammalian brain: nerve growth factor (NGF) (Levi-Montalcini, 1987), brain derived neurotrophic factor (BDNF) (Barde et al., 1982) , neurotrophin 3 (NT3) (Maisonpierre et al., 1990), and neurotrophin 4/5 (NT4/5) (Hallböök et al., 1991) . These molecules act by binding two distinct categories of transmembrane receptors: the low-affinity p75 neurotrophin receptor ($p75^{NTR}$) and the high-affinity Trk family of receptor tyrosine kinases (TrkA, TrkB, TrkC) (Meakin and Shooter, 1992; Chao and Hempstead, 1995). In addition to neurotrophins, other secreted

factors similarly possess neurotrophic activities. These include ciliary neurotrophic factor (CNTF)(Ip and Yancopoulos, 1996) , glial cell line–derived neurotrophic factor (GDNF)(Lindsay and Yancopoulos, 1996), insulin-like growth factor (IGF)(Doré et al., 1997), and basic fibroblast growth factor (bFGF)(Walicke et al., 1986).

The increased bioavailability of neurotrophic factors correlates with functional recovery in several disease models. NSC-secreted factors, such as CNTF and leukemia inhibitory factor (LIF), are known to enhance mature oligodendrocyte survival in demyelinating CNS diseases (Butzkueven et al., 2002; Linker et al., 2002). Transplanted cells releasing platelet-derived growth factor (PDGF)-AA and FGF-2 induce oligodendrocyte precursor cell (OPC) proliferation and remyelination in a rodent model of multiple sclerosis (MS) (Einstein et al., 2009). Similarly, transplantation of iPSC-derived neural precursors promotes the survival, differentiation, and remyelination capacity of endogenous precursors via LIF secretion (Laterza et al., 2013). Grafted NSCs also influence the injury environment in the spinal cord; GDNF, NGF, and BDNF released by an immortalized NSC line (C17.2) promote substantial outgrowth of host axons after spinal cord injury (SCI) (Lu et al., 2003). Human iPSC-derived neurospheres similarly secrete BDNF and NGF and support improved motor function post-SCI (Nori et al., 2011).

NSCs also confer neuroprotection via growth factors in neurodegenerative diseases. Transplanted C17.2 cells intrinsically express GDNF and rescue dysfunctional host neurons in Parkinsonian mice (Ourednik et al., 2002). Moreover, human progenitor cells engineered to constitutively secrete GDNF can preserve the viability of neurons in experimental models of Parkinson's disease (Behrstock et al., 2005) and amyotrophic lateral sclerosis (ALS) (Suzuki et al., 2007). Transplantation of NSCs into the lumbar spinal cord of ALS rodents further postpones disease onset and progression, an effect that is correlated to increased levels of VEGF and IGF-1 (Corti et al., 2007). In models of stroke, VEGF also appears to be required for NSC-induced recovery (Horie et al., 2011). In particular, human fetal-derived neurospheres secreting VEGF enhanced peri-infarct vascular regeneration, an effect that was abolished following selective neutralization of human VEGF with Avastatin. NSC-secreted VEGF has also been implicated in

dendritic sprouting, axonal plasticity, and axonal transport *in vitro* and may contribute to cortical rewiring in stroke (Andres et al., 2011).

Transplanted NSCs may also exert neuroprotective antioxidant effects towards surrounding host neurons. Accumulating evidence suggests that oxidative stress, which results from the upregulated production of reactive oxygen species (ROS), may contribute to the pathogenesis and progression of several neurodegenerative diseases (Andersen, 2004; Barnham et al., 2004). Although mammalian cells have evolved repair and resistance mechanisms to counteract oxidative stress, the activities of several antioxidant molecules (i.e. SOD2, CAT, and GPx) are reduced in the brain. NSCs appear to be more resistant to oxidative insult than their postmitotic progeny. At steady state, primary and immortalized NSCs have a lower content of intracellular ROS and higher basal levels of key antioxidant enzymes UCP2 and GPx (Madhavan et al., 2006). Following exposure to the mitochondrial toxin 3-nitropropionic acid, NSCs can upregulate UCP2, GPx, and SOD2 to recover from initial deterioration (Madhavan et al., 2006). Furthermore, NSCs can promote an effective antioxidant response in surrounding neuronal cells when co-cultured with primary neurons or grafted into the striatum (Madhavan et al., 2008). In particular, NSCs interfere with the production of free radicals by upregulating neuronal SOD2 expression, which is mediated by upstream VEGF and CNTF signaling.

Although future therapies may take advantage of the neuroprotective properties of undifferentiated NSCs, such strategies will require an in-depth understanding of how trophic factors signal and how these specific mechanisms are regulated. The biological actions of neurotrophins depend on the form of the neurotrophin (i.e. pro- versus mature) and the class of receptor that is activated. Pro-neurotrophins are functionally active and preferentially bind $p75^{NTR}$ to mediate apoptosis whereas mature neurotrophins preferentially bind Trk to initiate survival (Lee et al., 2001; Lu et al., 2005). One could imagine that pro- and mature neurotrophins exert diametrically opposing effects on neuronal survival through their preferred cognate receptors. However, evidence suggests each receptor can engage in both survival-promoting and death-promoting signaling pathways (Kalb, 2005). Therapeutic manipulation of neurotrophin signaling requires a better understanding of the cellular context within which signaling occurs.

Immune modulation

Undifferentiated NSCs can also modify the inflammatory environment. NSCs isolated from the neonatal rat SVZ can attenuate pathology in acute experimental allergic encephalomyelitis (EAE) (Einstein et al., 2003). Acute EAE is a model for disseminated brain inflammation associated with CNS injury and insult (i.e. traumatic, ischemic, and immune-mediated). Neurological symptoms in acute EAE result from the breakdown of the blood-brain barrier. The extravasation of T cells and peripheral monocytes/macrophages into the CNS gives rise to widespread perivascular lesions. Transplanted NSCs downregulate the expression of intercellular adhesion molecule-1 (ICAM-1) and the leukocyte integrin LFA-1, limiting the transendothelial migration of leukocytes (Einstein et al., 2003). NSCs in this model can further inhibit the proliferation of infiltrating T-lymphocytes.

The immunomodulatory effect of NSCs has also been reported in a mouse model of chronic CNS inflammation, relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE), which mimics aspects of human MS (Pluchino et al., 2005). Unlike acute EAE, this model exhibits significant immune-mediated demyelination, leading to chronic neurologic disability in affected mice. NSCs harvested from the adult mouse SVZ and intravenously injected into R-EAE mice can home to and persist in perivascular regions of the CNS. Grafted NSCs induce the programmed death of infiltrating pro-inflammatory T-cells, reducing the extent of demyelination and axonal loss. This effect has been attributed to binding of extrinsic death receptor and intrinsic mitochondrial-mediated pathways. In fact, inhibition of death receptor ligands (i.e. FasL, Apo3L or TRAIL) significantly reduces the NSC-mediated pro-apoptotic effect *in vitro* (Pluchino et al., 2005).

Intravenous delivery of adult NSCs acutely after ischemic stroke can protect the brain from delayed injury by altering the immune landscape. In particular, NSCs downregulate the expression of proinflammatory cytokines (i.e.TNFα, IFNγ, IL-1β, and IL-6) and significantly reduce numbers of activated microglia and infiltrating leukocytes in peri-ischemic regions (Bacigaluppi et al., 2009).

NSC transplantation in the subacute phase of spinal cord injury also induces an immunomodulatory effect (Cusimano et al., 2012). NSCs occupying perivascular regions establish contacts via junctional coupling with macrophages. These NSCs reduce the proportion of infiltrating proinflammatory macrophages, and have profound effects on their cytokine profile. Specifically, transplanted NSCs enhance levels of neuroprotective *Nos2* in macrophages invading the lesioned cord environment.

V. Rationale for Dissertation Studies

The *in vivo* replacement of degenerating neurons in the diseased brain with exogenous NSCs remains an elusive goal. The adult brain largely lacks the extrinsic factors that regulate differentiation developmentally. NSC transplants in this non-permissive environment generate few cells that appropriately mature and integrate into host tissue. Accumulating evidence suggests that ectopic NSCs exert remarkable trophic and immunomodulatory effects in a number of CNS diseases while retaining predominantly undifferentiated features. In addition, NSCs express a number of costimulatory and possibly antigenic components that may elicit an immune response even in the absence of host pathology. The therapeutic potential of undifferentiated NSCs relies on an improved understanding of their effects on the normal host brain. To address this issue, research was undertaken with the following discrete goals. First, to identify alterations to neural network activity induced by different densities of NSC engraftment in the mature rodent cortex. Second, to investigate the interactions of undifferentiated NSCs with resident microglia and neurons *in vivo* as well as in a well-controlled co-culture system. Completion of these goals will provide progress towards the clinical application of NSC-based strategies.

CHAPTER 2

Undifferentiated Neural Stem Cells Reduce Cortical Network Excitability in a Dose-Dependent Manner

(This chapter is adapted from Weerakkody TN, Patel TP, Yue C, Takano H, Anderson HC, Meaney DF, Coulter DA, and Wolfe JH, Engraftment of Nonintegrating Neural Stem Cells Differentially Perturbs Cortical Activity in a Dose-Dependent Manner, Mol Ther., 21(12):2258-67, Copyright 2013)

I. Introduction

Neural stem cells (NSCs) are promising candidates to treat a number of neurodegenerative diseases, as reviewed in (Lindvall and Kokaia, 2010). Such neurological disorders have been refractory to therapy due to their ubiquitous pathology. NSCs possess an inherent ability to self-renew and migrate to multifocal lesions, circumventing limitations of other gene delivery vehicles (Müller et al., 2006). However, primary NSC transplants, as well as NSCs derived from embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) generate a high proportion of cells that do not show evidence of neuronal differentiation or synaptic integration (Roy et al., 2006; Wernig et al., 2008; Koch et al., 2009; Miura et al., 2009; Jeon et al., 2012; Chaubey and Wolfe, 2013). It is important to understand whether undifferentiated or non-integrating donor cells influence host circuit activity and if these cells cause unintended neurological impairment.

Neurophysiological data from previous transplantation studies exclusively characterized single-cell dynamics and did not assess the emergent properties of neuronal ensembles (Englund et al., 2002; Wernig et al., 2004; Alvarez-Dolado et al., 2006; Koch et al., 2009; Oki et al., 2012). The neocortex, which largely mediates cognitive processes, is composed of interacting laminar and columnar circuits (Petersen, 2007). Due to its stereotypic connectivity, the cortex is an amenable system to define host circuit properties and identify abnormalities induced by exogenous cells. Voltage sensitive dye (VSD) imaging directly measures the spatiotemporal dynamics of neural networks, including the functional connectivity of the neurons involved, with high temporal resolution (Cohen and Salzberg, 1978; Contreras and Llinas, 2001; Carlson and Coulter, 2008). Furthermore, since VSD signals reflect membrane depolarization, subthreshold synaptic connections between functionally related areas that are difficult to detect with conventional electrophysiology can be visualized.

In this study, we used VSD imaging to test the functional impact of physiologically immature, non-integrating donor cells in the cerebral cortex. For donor NSCs, we selected the well established clonal line C17.2 (Ryder et al., 1990) that is refractory to differentiation in the cortex (Watson et al., 2006). In contrast to primary (Magnitsky et al., 2008; Chaubey and Wolfe,

2013) and immortalized NSC transplants(Lundberg et al., 2002; Demeter et al., 2004) that show limited distribution, C17.2 cells yield high density, titratable levels of engraftment. This system provides an ideal, testable model to evaluate the limits of physiological tolerance of host circuits to donor cells, without confounding contributions from ectopic neurons and glia. Here, we provide the first direct evidence that exogenous NSCs can disrupt neural network activity. While moderate NSC levels largely preserved physiological function, high levels severely dampened cortical activity through a mechanism not requiring GABAergic neurotransmission. Furthermore, our study revealed that there was a significant dose-dependent depletion of host cells within engrafted regions. We demonstrate that non-integrating NSCs can induce differential network alterations as a function of engraftment level, which puts a premium on methods used to derive donor cells as well as appropriate controls for engraftment effects.

II. Results

Distribution and Differentiation of Grafted NSCs

To evaluate the functional impact of exogenous NSCs on host cortical networks *in vivo*, we used the immortalized NSC line C17.2 in an established murine transplantation model (Snyder et al., 1995) (**Fig. 2.1a**). C17.2 cells are amenable to expansion and genetic manipulation *in vitro*, and able to migrate and survive long term *in vivo* (Snyder et al., 1995; Flax et al., 1998; Park et al., 2002; Riess et al., 2002; Teng et al., 2002) compared to primary-derived cells (Magnitsky et al., 2008; Chaubey and Wolfe, 2013). The NSCs were modified to constitutively express GFP and injected intraventricularly into the neonatal (P0-P2) mouse brain (Snyder et al., 1995). At 1 day post-injection (DPI), donor NSCs occupied periventricular regions (**Fig. 2.1b**), at 3 DPI we observed chains of migrating NSCs, and by 14 DPI *in vivo* expansion resulted in robust cortical engraftment throughout the neuroaxis (**Fig. 2.1c**). To phenotype donor cells, we performed immunofluorescence analysis 2 months post-transplant (**Fig. 2.2**), which showed that engrafted NSCs remained in a largely non-proliferative, undifferentiated state.

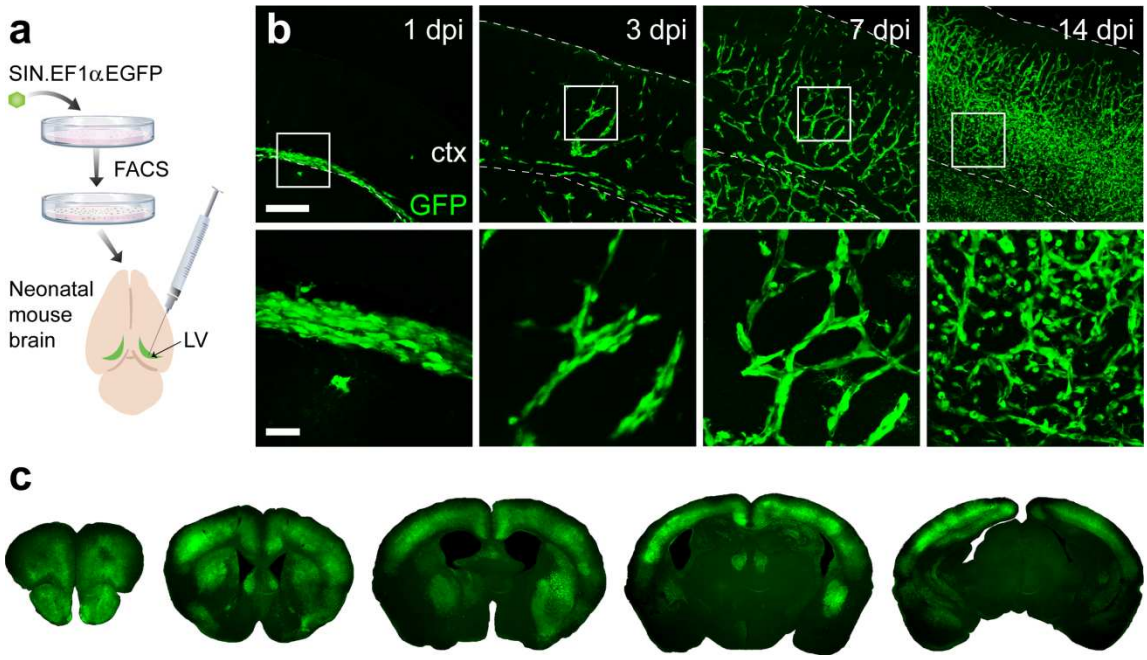

Figure 2.1. Engrafted NSCs migrate and proliferate extensively during first two postnatal weeks. (**a**) Schematic illustration of intraventricular NSC transplantation in neonatal rodent brain. (**b**) Trajectory of transplanted NSCs during first two postnatal weeks. Lower panels are magnified view (4x) of boxed region in upper panels. (**c**) Representative coronal sections along rostrocaudal axis feature stable cortical grafts at 8 wks post-transplant. (Scale bars in **b**: 250 μm, *Upper*; 50 μm, *Lower*)

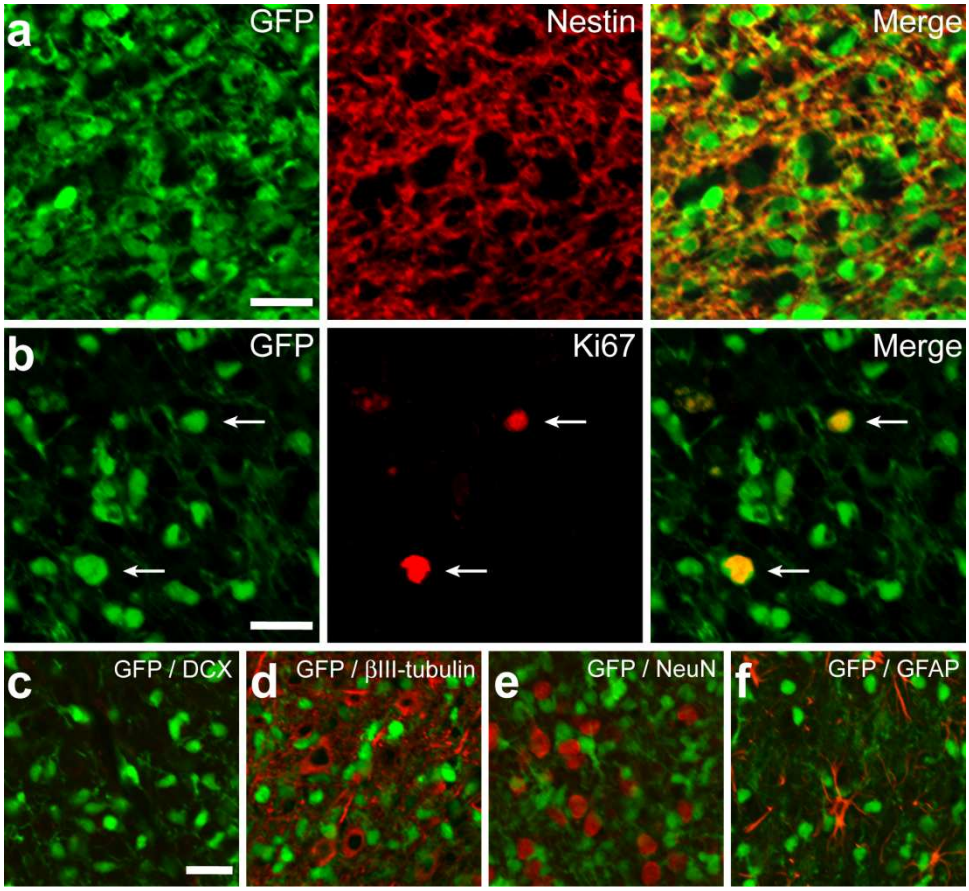

Figure 2.2. Exogenous NSCs show limited differentiation potential *in vivo*. (**a**) Cortical grafts are immunopositive for nestin, a marker of undifferentiated NSCs, at 8 wks post-transplant. (**b**) GFP-labeled cells were largely quiescent, with only a small percentage continuing to proliferate, as indicated by Ki67 immunonoreactivity. (**c-f**) Exogenous NSCs show no evidence of differentiation into mature neural lineages, as suggested by absence of DCX, βIII-tubulin, NeuN, and GFAP colabeling. (Scale bars: 25 μm)

Dose-Dependent Effects on Amplitude of Cortical Activation

We previously found that stable engraftment of ectopic NSCs caused no gross behavioral abnormalities (Snyder et al., 1995). However, it is unclear whether high density of engraftment in some areas could disrupt existing neural networks. To investigate whether cortical dynamics were influenced by engraftment density, NSC levels were titrated *in vivo* using three different input doses (80,000, 40,000, and 8,000 cells/ventricle). We quantified engraftment using two-dimensional confocal projections of each slice and expressed values as percent GFP-positive area normalized to total cortical area (**Fig. 2.3a**). Automated cell counts on an independent set of slices validated this measurement method. Graft area measurements strongly correlated to cell counts (Pearson's correlation $r=0.99$ $p<0.0001$), and thus served as a metric for NSC engraftment level (**Figs. 2.3b-e**).

Optical recordings were made in *acute slices of somatosensory cortex* at 2 months post-transplant in response to a single callosal stimulation (**Fig. 2.4a,b**). We observed a progressive reduction in peak signal amplitude ($\Delta F/F_0$) with increased cortical engraftment, suggesting that exogenous NSCs can modulate network excitability (**Fig. 2.4c**). To determine the locus of dampened cortical activity, we generated color-coded maps depicting maximum $\Delta F/F_0$ for individual pixels across all movie frames (**Fig. 2.4d**). We observed a strong negative correlation between engraftment level and corresponding peak $\Delta F/F_0$ values (Pearson's correlation $r=-0.82$ $p<0.0001$) (**Fig. 2.4e**). K-means clustering of maximum $\Delta F/F_0$ values partitioned the slices into three engraftment densities: control, moderate, and high (**Fig. 2.4f**). We expressed engraftment as percent GFP-positive area normalized to total cortical area. Whereas high levels (> 25%) caused marked reductions in the amplitude of activation ($0.10\pm0.01\%$ vs.$0.22\pm0.01\%$, $p<0.0001$), moderate levels (<15%) did not alter this network property ($0.19\pm0.01\%$ vs. $0.22\pm0.01\%$, $p>0.05$). In accordance with these results, we found that highly engrafted slices had elevated thresholds of cortical activation, as determined by local field recordings (**Fig. 2.5**). Furthermore, the injection procedure itself did not significantly perturb host physiology (**Figs. 2.6a,b**). Collectively, these data indicate that network alterations induced by exogenous NSCs are dose-dependent.

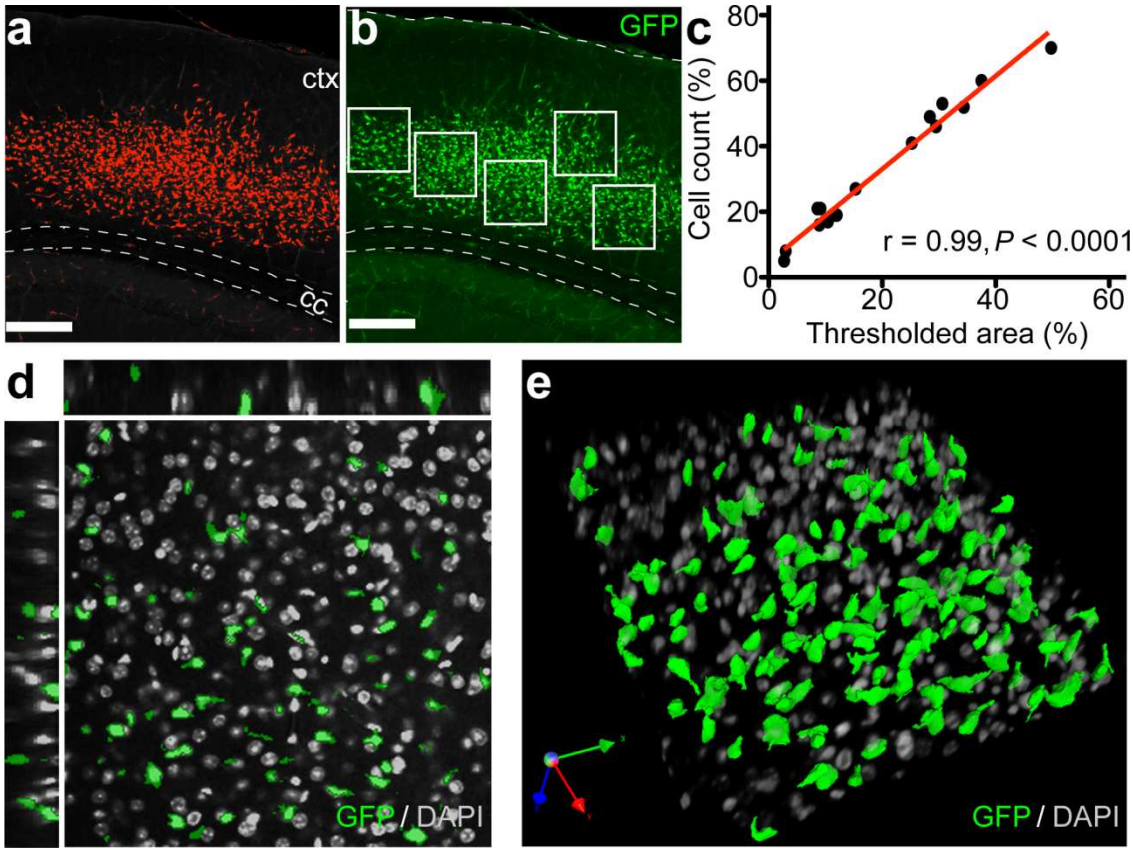

Figure 2.3. Exogenous NSCs exhibit robust levels of engraftment in cortex. (**a**) Maximum intensity projection showing thresholded GFP+ graft at 8 wks (red mask represents all pixel intensities ≥ 2 SD above mean background intensity). (**b**) Automated counts performed on 5 randomly selected cortical ROIs (white boxes) validate graft area measurements. (**c**) Correlation plot with linear fit comparing quantitation methods from **a** and **b** (n=16 slices). (**d**) Representative optical plane from engrafted ROI in E showing colocalization of GFP and DAPI fluorescence. (**e**) 3-D reconstruction of engrafted ROI in E rendered from confocal z-stack. (Scale bars in **a** and **b**: 250 μm)

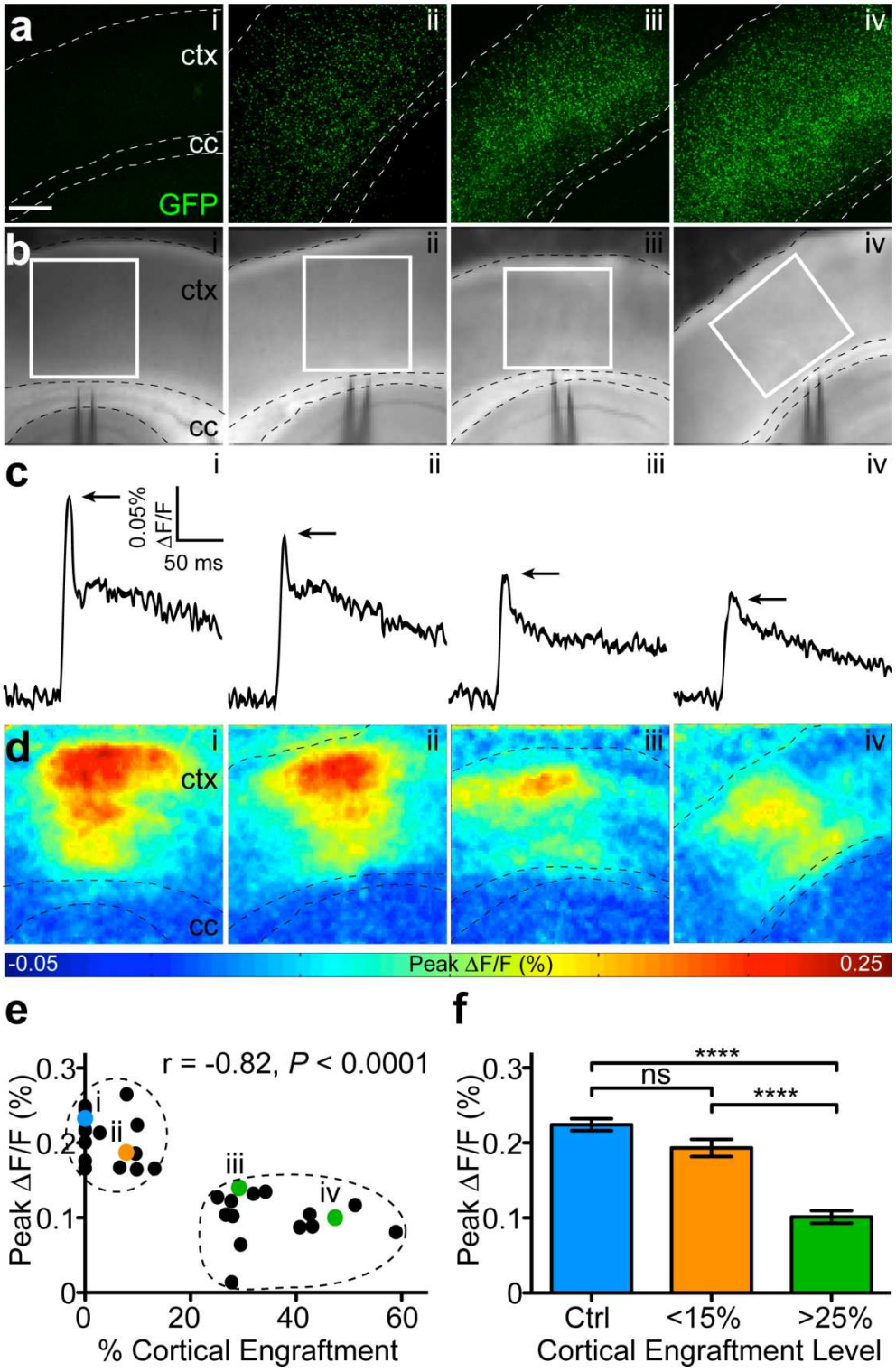

Figure 2.4. NSC engraftment reduces the amplitude of cortical activation in a dose-dependent manner.

Figure 2.4. NSC engraftment reduces the amplitude of cortical activation in a dose-dependent manner. a) Confocal images of representative cortical grafts (i-iv) at 8 wks. (**b**) Bright-field images showing cortical slice preparation and electrode placement for VSD imaging. (**c**) VSD traces of time resolved mean fluorescence intensity change ($\Delta F/F_0$) within defined cortical ROI (white boxes in **b**). (**d**) Color-coded maps of cortical activation, depicting maximum F/F_0 for individual pixels within a 1024 ms recording interval. (**e**) Correlation plot of maximum signal amplitude versus cortical engraftment level (n=33 slices). (**f**) Histogram showing differential effects of engraftment on evoked VSD signal (control, n=9; <15%, n=9; >25%, n=15). All imaged slices grouped into engraftment densities based on K-means clustering of maximum $\Delta F/F_0$ values (dotted circles in **e**). Data are means ± SEM (****P<0.0001). (Scale bar in **a**: 250 μm)

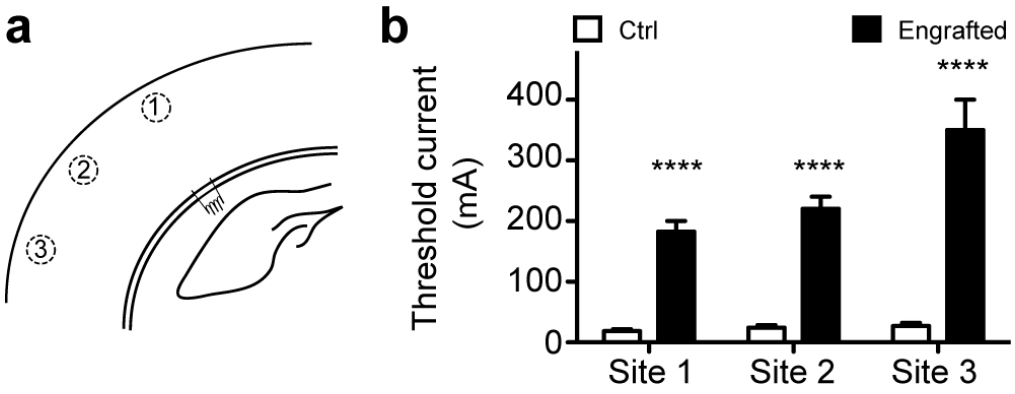

Figure 2.5. High loads of ectopic cells elevate the current threshold required to activate cortical microcircuitry. (**a**) Schematic illustration of cortical slice preparation showing recording sites (dotted circles) in layer 2/3 and stimulation electrode placement in corpus callosum. (**b**) Histogram showing threshold current for local field potentials across sites for control (n=7) and highly engrafted slices (n=8).

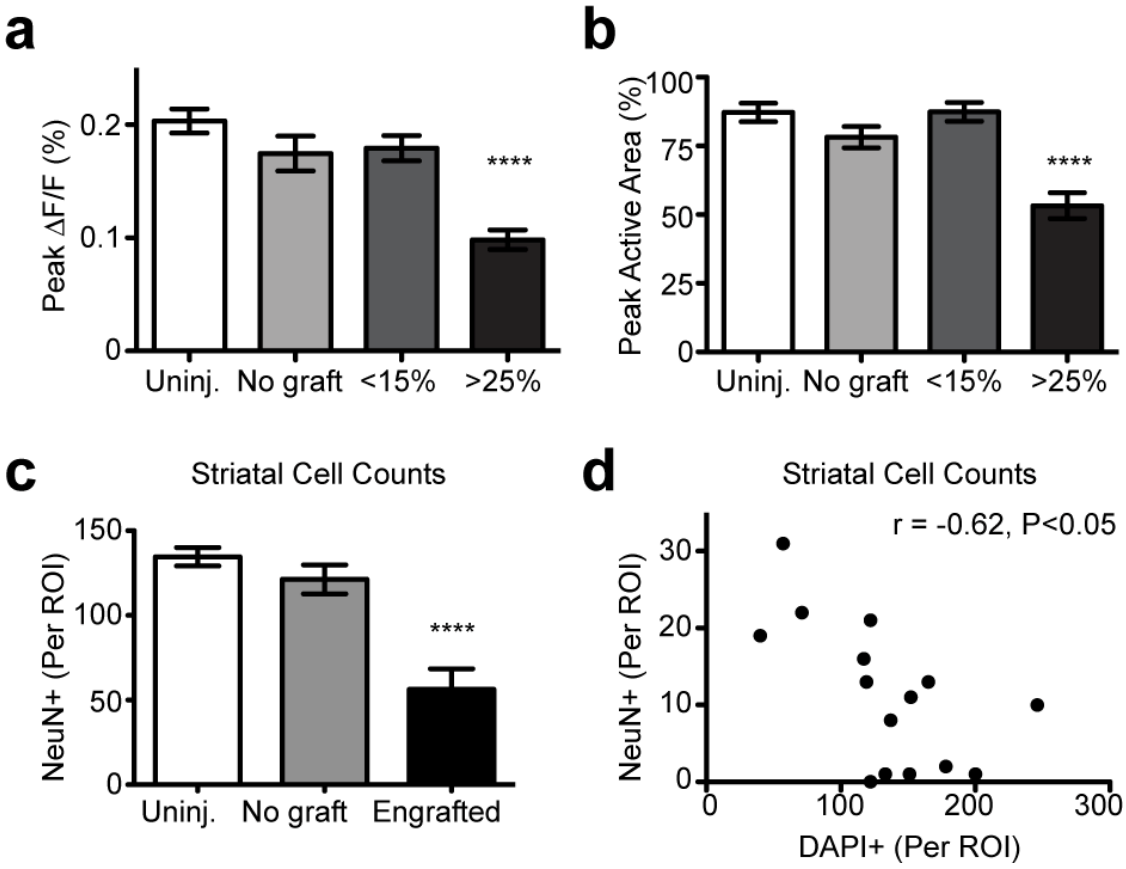

Figure 2.6. Functional outcomes are not directly influenced by injection route or region of engraftment. (a) Histogram showing differential effects of striatal engraftment on evoked VSD signal (uninjected, n=10; no graft, n=8; <15%, n=12; >25%, n=15). **(b)** Histogram showing differential effects of engraftment on peak active area (uninjected, n=10; no graft, n=8; <15%, n=12; >25%, n=15). **(c)** Histogram showing neuronal count by ROI across engraftment conditions (uninjected, n=24; no graft, n=12; engrafted, n=15). **(d)** Correlation plot showing neuronal (NeuN+) versus total (DAPI+) cell count across engrafted striatal regions (n=15) at 8 wks. (Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Spatiotemporal Patterns of Cortical Excitation

We next investigated the spatiotemporal patterns of excitation across engraftment densities (**Figs. 2.7a,b**). Consistent with earlier work (Contreras and Llinas, 2001; Wester and Contreras, 2012), a single stimulus activated deep layers (L5/6) in control slices (see frames at 3 and 6 ms), followed by columnar activation to L1 with simultaneous horizontal spread in L5/6 (see frames at 8 and 10 ms). Within superficial layers (L2/3), excitation propagated laterally (see frames at 14 and 18 ms). Moderately engrafted slices showed activity patterns similar to control, whereas highly engrafted slices exhibited columnar activity with minimal lateral spread. To quantify the global extent of activation, we determined the number of pixels that exhibited significant depolarization after callosal stimulation. The activated pixel number in a defined cortical region was normalized against the total pixel number, generating an activation measure, and plotted against time. Time of peak activation, rise time, and fall time extrapolated from these plots were not significantly altered across engraftment densities, suggesting that aspects of cortical function were preserved in this transplantation model. However, the maximum activated area negatively correlated to engraftment level (Pearson's correlation $r= -0.78$, $p<0.0001$) (**Fig. 2.7c**). Furthermore, whereas high levels of ectopic cells spatially constrained activity (0.81 ± 0.12 mm^2 vs. 1.94 ± 0.12 mm^2, $p<0.0001$), moderate levels maintained excitatory spread across lamina (1.52 ± 0.10 mm^2 vs. 1.94 ± 0.12, $p>0.05$) (**Fig. 2.7d**). These results suggest that undifferentiated NSCs, at high levels, block the horizontal propagation of excitatory potentials, while preserving columnar connectivity in the somatosensory cortex.

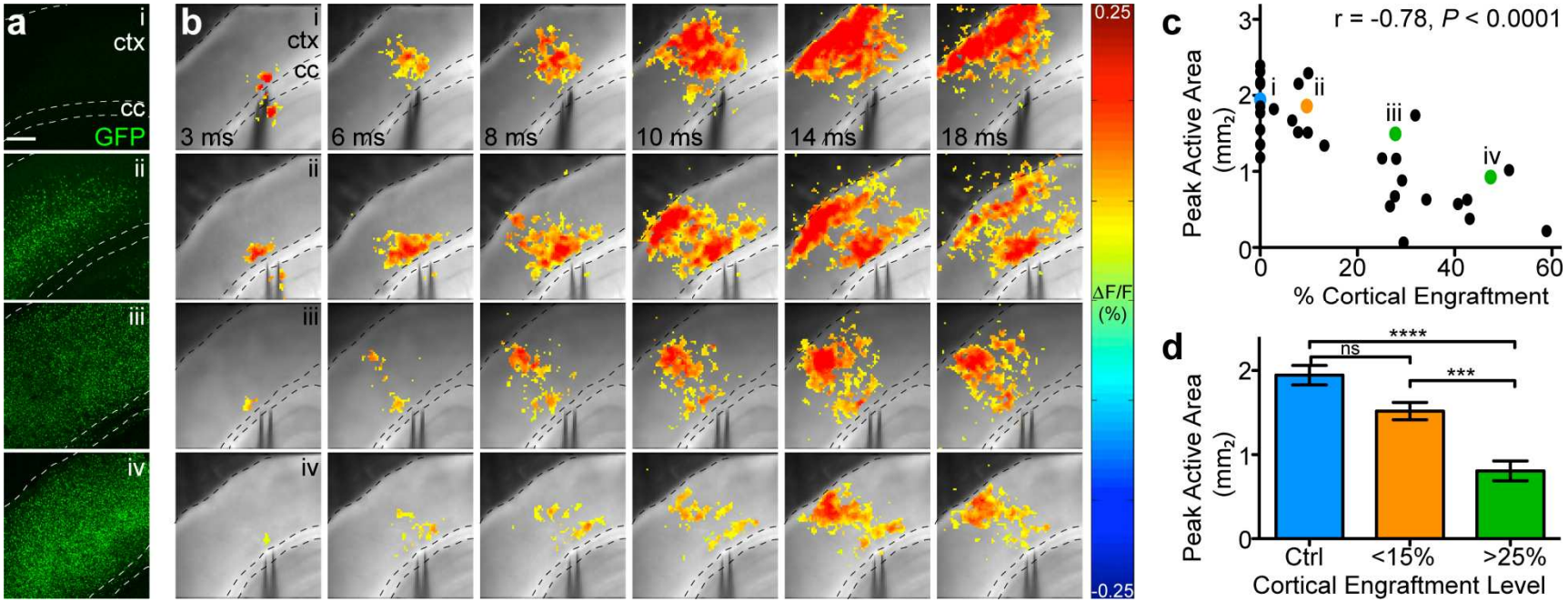

Figure 2.7. Stereotypic pattern of cortical excitation is conserved but spatially restricted at high engraftment levels. (**a**) Confocal images of representative cortical grafts (i-iv) at 8 wks. (**b**) Spatiotemporal maps of cortical activity following callosal stimulation. For each representative slice, corresponding series of frames shows pattern of VSD signal propagation. (**c**) Correlation plot of maximum activated area versus cortical engraftment level (n=33 slices). (**d**) Peak area of cortical activation is smaller in highly engrafted (green bars) but not significantly altered moderately engrafted slices (red bars), (control, n=9; <15%, n=9; >25%, n=15). Data are means ± SEM (***P<0.001, ****P<0.0001). (Scale bars in **a**: 250μm)

Defects within Laminar Circuits

Spatiotemporal properties of cortical activity are determined by interactions between local laminar and columnar circuits (Petersen, 2007; Lefort et al., 2009). Therefore, we examined the effect of exogenous NSCs on cortical layers (L2-L6), approximated by horizontally aligned bins that were perpendicular to the axis of columnar activity (**Fig. 2.8a**). Bin 1 and 2 corresponded to the supragranular layers (L2/3), bin 3 aligned with layer 4; and bins 4 and 5 largely represented infragranular layers (L5/6). In each binned response (**Fig. 2.8b**), we examined several indices of circuit function: peak amplitude, peak active area, peak displacement, and peak velocity of propagating potentials. Consistent with the global measures (**Figs. 2.4e,f**), binned responses demonstrated a progressive reduction in peak $\Delta F/F_0$ (**Fig. 2.8c**), peak active area (**Fig. 2.8d**), and peak horizontal displacement (**Fig. 2.8e**) with increased engraftment density. The peak propagation velocity was calculated as the maximal difference in active area between any two consecutive movie frames over the imaging interval. While layer-specific velocity was reduced in all bins of highly engrafted slices, moderately engrafted slices showed pronounced defects in deep layers exclusively (0.70±0.04 mm/ms vs. 0.99±0.06 mm/ms, $p<0.05$) (**Fig. 2.8f**).

The temporal and spatial integration of afferent inputs is critical to the formation of complex representations during wake states (Civillico and Contreras, 2006). Repetitive callosal stimuli were applied at two frequencies, 10 and 40 Hz, to mimic prevailing rhythms present *in vivo* during slow wave sleep (SWS) and activated states, respectively. Both stimulation trains are known to produce facilitating responses in the rodent somatosensory cortex (Contreras and Llinas, 2001). High levels of exogenous NSCs blocked the enhancement of peak $\Delta F/F_0$ in all cortical bins (**Figs2.8g,h**). Facilitation was differentially impaired in bin 4 (1.63±09 vs. 2.08±0.13 mm^2, $p<0.05$) and bin 5 (1.46±0.07 mm^2 vs. 1.95±0.13, $p<0.01$) of moderately engrafted slices. This data indicates that moderate NSC levels cause measureable defects in cortical computations within infragranular circuits.

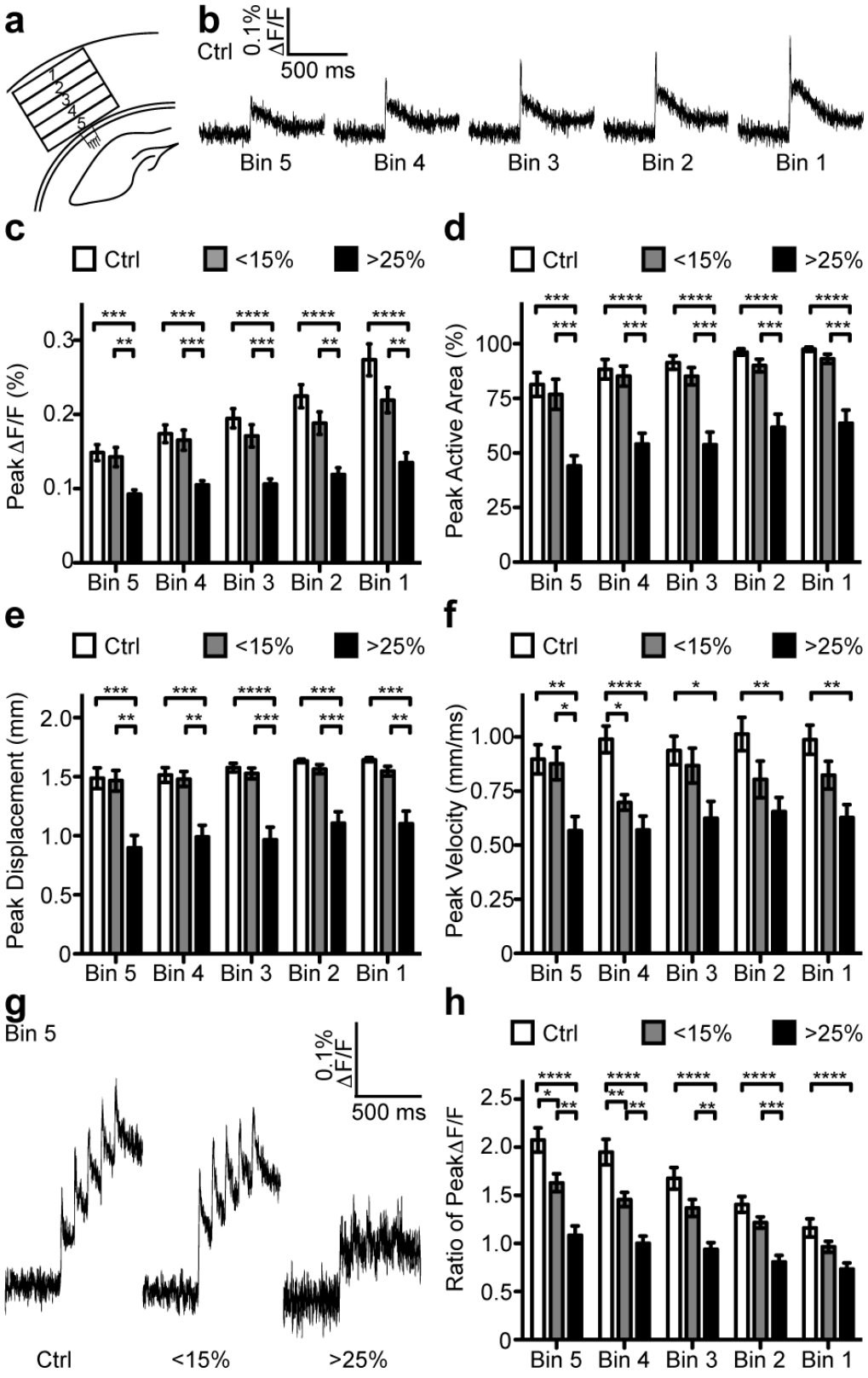

Figure 2.8. High levels of cortical engraftment lead to layer-specific disruptions in network function.

Figure 2.8. High levels of cortical engraftment lead to layer-specific disruptions in network function. (**a**) Schematic illustration of spatial binning within a neocortical ROI. For each imaged slice, five horizontally aligned bins were generated, each perpendicular to the axis of columnar activity. (**b**) Representative traces showing evoked VSD response within cortical bins of a control slice. (**c-f**) Histograms demonstrating effect of engraftment on VSD signal properties in binned cortical regions (control, n=9; <15%, n=9; >25%, n=15). Peak intensity (**c**), activated area (**d**), displacement (**e**), and propagation velocity (**f**) of potentials are significantly altered across cortical bins of highly engrafted slices (green), but not in moderately engrafted slices (red bars). (**g**) Representative traces showing evoked VSD response in bin 5 to repetitive stimuli (5 pulses, 10 Hz) across engraftment conditions. (**h**) Comparison of facilitating responses across cortical bins and engraftment conditions (control, n=9; <15%, n=9; >25%, n=15). Shown are ratios of peak $\Delta F/F_0$ signal (fifth response is normalized to first response) after repetitive callosal stimulation. Data are means ± SEM (* P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).

Early implantation exposes NSCs to endogenous growth signals that promote rapid graft expansion, causing both mild and severe defects to host physiology. To determine whether such deficits are induced following delivery into the mature brain, NSCs were stereotaxically injected into the left cortical hemisphere of adult mice. Vehicle (mock) injections were administered to the right hemisphere to control for effects induced by the injection route. Mock injected responses were indistinguishable from those in uninjected controls. Grafts established 2 months post-transplant did not exceed moderate levels. Consistent with neonatal transplants, this level of engraftment did not perturb gross measures of host function (amplitude, area, displacement, velocity) (**Fig. 2.9a-d**). Interestingly, a more subtle measure of network function (integration of repetitive inputs in deep layers), that was disrupted in neonatal transplant recipients, was not altered in adult recipients (**Fig. 2.9f**). These results suggest that the developmental stage of the host brain can largely influence functional outcome of cell therapies; however, engraftment in adult transplants is limited to the area of injection.

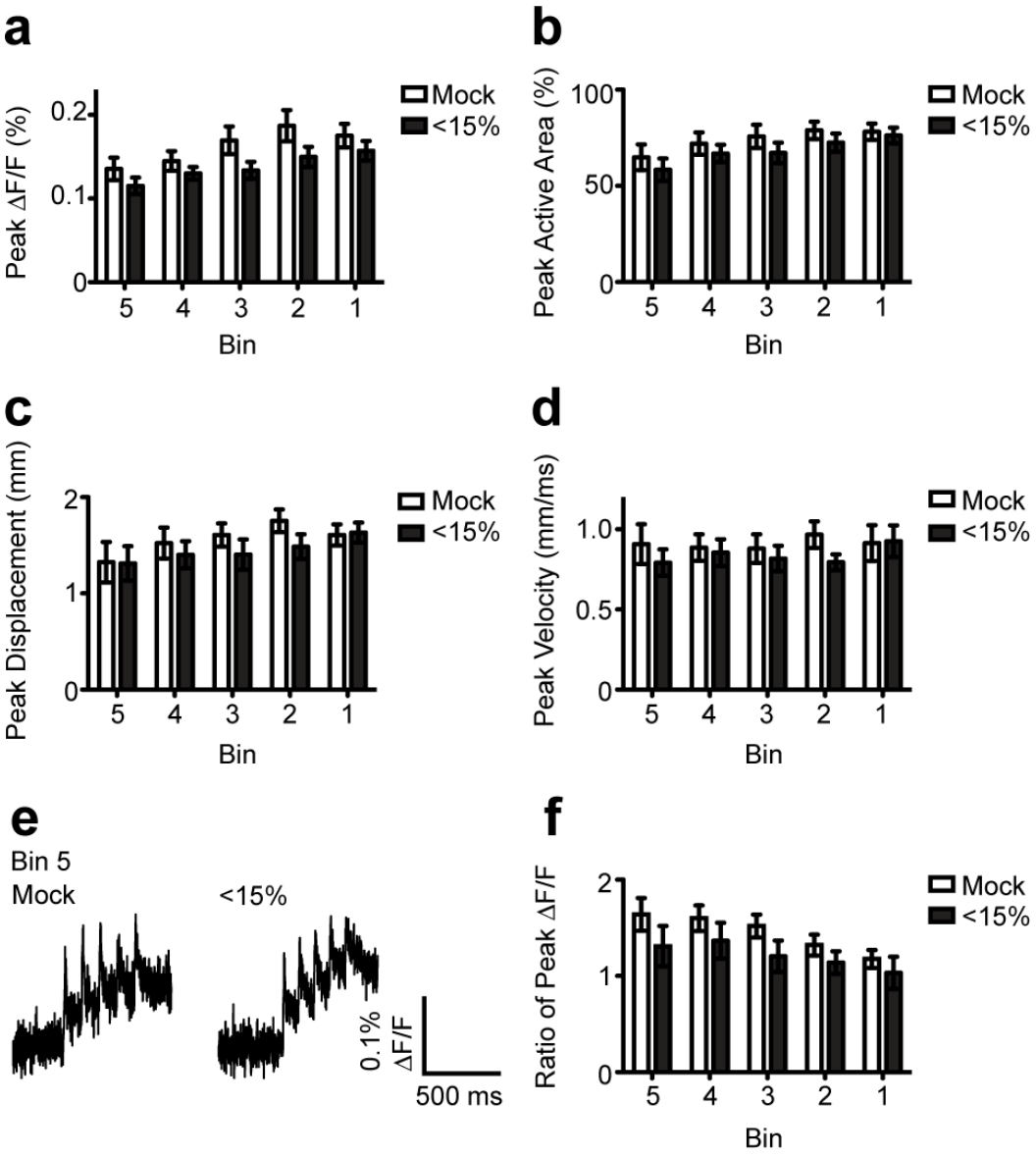

Figure 2.9. Adult transplants yield levels of engraftment that do not alter host circuit function. (**a-d**) Histograms demonstrating effect of engraftment on VSD signal properties in binned cortical regions (mock, n=7; <15%, n=11). Peak intensity (**a**), activated area (**b)**, displacement (**c**), and propagation velocity (**d**) of potentials are unchanged across cortical bins. (**e**) Representative traces showing evoked VSD response in bin 5 to repetitive stimuli (5 pulses, 10 Hz) across engraftment conditions. (**f**) Comparison of facilitating responses across cortical bins and engraftment conditions (mock, n=7; <15%, n=8). Shown are ratios of peak $\Delta F/F_0$ signal (fifth response is normalized to first response) after repetitive callosal stimulation. Data are means ± SEM (* P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).

Alterations to Excitatory and Inhibitory Network Tone

NSC-induced alterations in cortical excitability may be a consequence of either reduced excitatory or enhanced inhibitory network tone (Contreras and Llinas, 2001; Petersen and Sakmann, 2001; Petersen et al., 2003). To distinguish between these two possibilities, we blocked γ-Aminobutyric acid type A receptor ($GABA_AR$)-mediated inhibition with picrotoxin (PTX). Cortical responses to single callosal stimuli were monitored before and 30 min after PTX treatment. Control and highly engrafted slices exhibited PTX-induced hyperexcitability, as shown in color-coded activity maps (**Fig. 2.10a**). Suppression of GABAergic signaling expanded the boundaries of cortical activation (**Fig. 2.10a**) and also, prolonged depolarizing responses in all cortical bins (**Fig. 2.10b**). Differences in the magnitude of excitation pre- and post-PTX application were comparable in all cortical bins of control and highly engrafted groups, except in bin 4 (0.010±0.001 vs. 0.007±0.001, $p<0.05$) (**Fig. 2.10c**). We conclude that host neurons can be recruited, even in the presence of many exogenous NSCs, to increase cortical excitation. However, our results also suggest that ectopic cells differentially impaired infragranular excitatory circuits. Grafted NSCs markedly lowered the absolute level of excitation attained following PTX treatment in bin 3 (0.80±0.10% vs. 1.20±0.07%, $p=0.02$), bin 4 (0.76±0.10% vs. 1.20±0.07%, $p=0.006$), and bin 5 (0.71±0.09% vs.1.00±0.07%, $p=0.02$) (**Fig. 2.10d**). These data indicate that grafted NSCs reduced the excitatory network tone in deep layers. Furthermore, we can conclude that exogenous cells do not require $GABA_AR$ signaling mechanisms to modulate network activity. GABA-A signaling may partially contribute to observed alterations; however, this is coupled with additional changes to either excitatory connections or the intrinsic excitability of host neurons.

Depletion of Host Cells in Engrafted Cortices

We observed a depletion of DAPI+/GFP- host cells in the cortex, which strongly correlated with engraftment level (Pearson's correlation $r= 0.99$ $p<0.0001$) (**Fig. 2.10e**). No concomitant change to cortical thickness was detected. We also performed a microcircuit analysis of lightly and heavily engrafted regions within the same acute slice preparation. Based on this analysis, we found that host cell number is negatively correlated to donor cell number (Pearson's

correlation r= -0.60, p<0.0001, n= 78 regions) **(Fig. 2.10f).** In all cases, total cell number was conserved across control, moderate, and high-density engraftment conditions (p=0.14) as indicated by automated DAPI counts averaged across 5 regions of interest **(Fig. 2.10g).** We also observed marked neuronal depletion in subcortical regions, based on NeuN quantification within engrafted striatal tissue (11.27±2.42 vs. 26.92±1.10, p<0.0001) **(Fig. 2.6c).** In engrafted striatal regions, the number of neurons also varied inversely with total number of cells (Pearson's correlation r= -0.62, p<0.05, n= 15 regions) **(Fig. 2.6d).** Collectively, these results indicate that engraftment of NSCs was gained at the cost of endogenous cells.

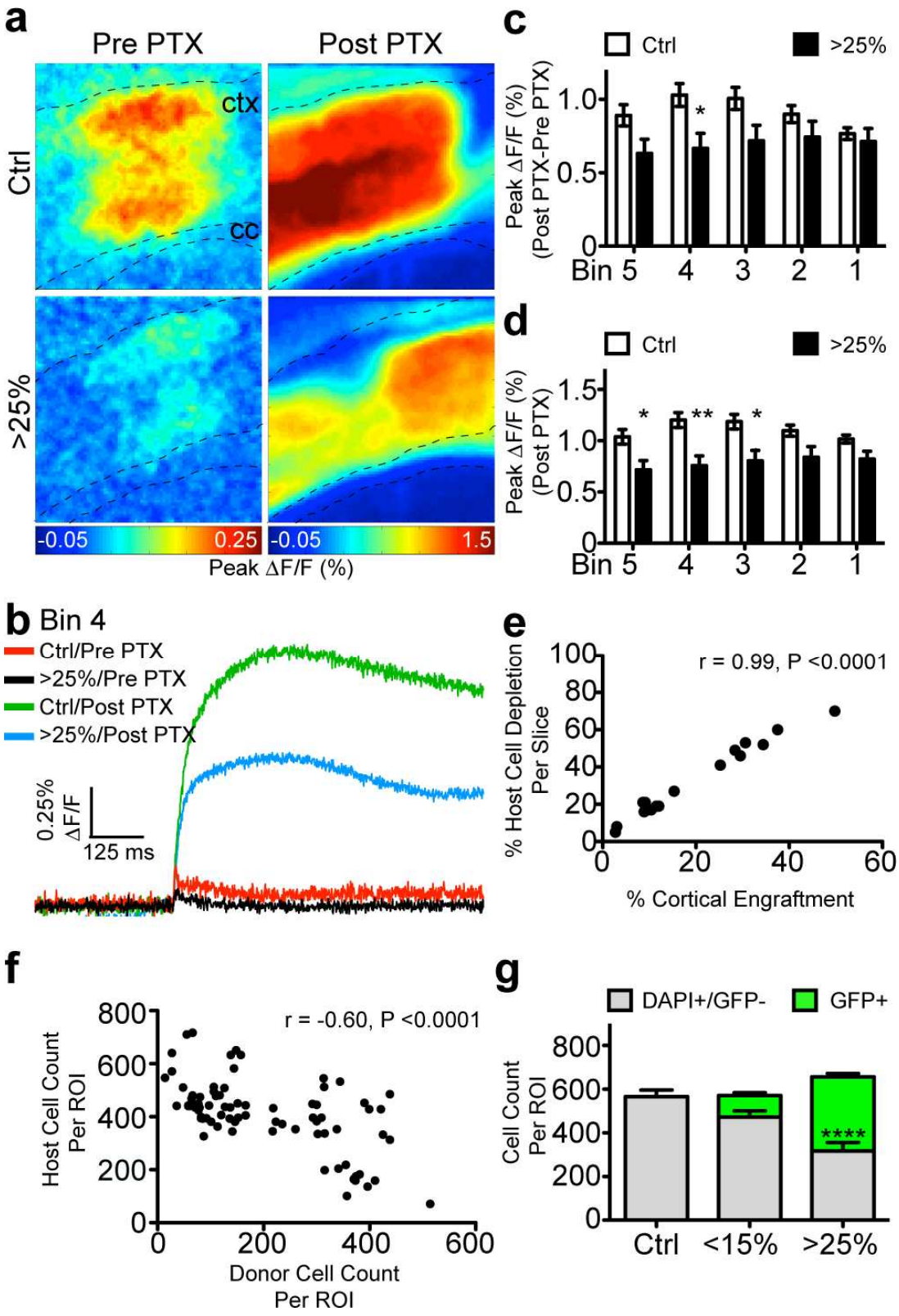

Figure 2.10. Ectopic NSCs impair excitatory network structure in deep layers and induce host cell depletion.

Figure 2.10. Ectopic NSCs impair excitatory network structure in deep layers and induce host cell depletion. (**a**) Color maps of cortical activation, depicting maximum $\Delta F/F_0$ for individual pixels within a 1024 ms interval. Representative maps show effect of $GABA_A$ receptor antagonist, PTX, on cortical activity in control (Upper) and highly engrafted (Lower) slices. Note that both control and engrafted slices exhibit PTX-induced hyperexcitability (left panels, baseline; right panels, 30 min after picrotoxin application). (**b**) Representative optical recordings from control and highly engrafted slices pre and post PTX treatment. (**c**) Histogram showing relative change in excitability (post PTX – pre PTX) following drug application (n=5). (**d**) Histogram displaying peak $\Delta F/F_0$ values during blockade of GABAergic inhibition (n=5). (**e**) Correlation plot showing depletion of DAPI+/GFP- host cells versus cortical engraftment level across imaged slices (n=16) at 8 wks. (**f**) Correlation plot showing host (DAPI+/GFP-) versus donor (DAPI+/GFP+) cell count across imaged ROIs (n=78) at 8 wks. (**g**) Histogram showing endogenous (gray bar) and donor (green bar) cell count by ROI across engraftment conditions (control, n=9; <15%, n=9; >25%, n=7). Total cell count is preserved across conditions (p=0.14) highly slices (n=7) at 8 wks. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

III. Discussion

Transplanted NSCs have the potential to provide therapeutic benefit in a number of disease states through gene or drug delivery, cell replacement, or by exerting trophic or neuroprotective effects. However, there has been considerable difficulty achieving efficient integration of implanted cells, independent of source (Roy et al., 2006; Wernig et al., 2008; Koch et al., 2009; Miura et al., 2009; Jeon et al., 2012) . In the current study, we used a NSC line that remains undifferentiated in the cortex to investigate the physiological effect of non-integrating NSCs across a range of engraftment levels. Based on VSD imaging of network dynamics, we found that the cortex can safely accommodate quantities of immature cells comparable to those currently attained from primary NSCs, ES-NSCs, and iPS-NSCs. At levels of engraftment up to 15%, we observed subtle yet, physiologically relevant disruptions to network function exclusively in deep cortical layers (L5/6). However, at very high levels of engraftment (exceeding 25% engraftment) there were much more extensive and severe alterations to activity, specifically to the amplitude, spatial extent, and velocity of propagating potentials. The results suggest that a threshold of inefficiency in integration may confound analysis of deficits in models of neurological disease and interfere with the therapeutic effect of cell therapy.

These data are consistent with findings that ectopic C17.2 cells can functionally interact with host circuits well before electrophysiological maturation (Jäderstad et al., 2010). In this previous study, grafted NSCs were engineered to overexpress neurotrophin-3, which allowed them to differentiate into neurons and form gap junctions with host neurons. Gap junctions lower the input resistance of coupled cells in the developing cortex (Lo Turco and Kriegstein, 1991), and provide a mechanism by which grafted cells could lower the intrinsic excitability of intact host neurons. However, we found no evidence of gap junction formation between grafted, unengineered NSCs and endogenous cells (data not shown). Thus, the cellular mechanism underlying circuit interference remains unclear.

One possibility is that exogenous cells used GABA-dependent mechanisms to modulate cortical excitability. GABAergic inhibition plays a pivotal role in shaping the spatiotemporal properties of evoked cortical responses *in vitro* (Contreras and Llinas, 2001; Petersen and

Sakmann, 2001) and *in vivo* (Petersen et al., 2003), including the integration of afferent inputs (Borgdorff et al., 2007). Transplanted primary embryonic cerebellar and cortical tissue, rich in GABA, can raise thresholds for seizure initiation in rodent models of epilepsy (Stevens et al., 1988). Furthermore, transplanted neural precursors isolated from the medial ganglionic eminence (MGE) can differentiate into mature cortical interneurons that increase local inhibition (Alvarez-Dolado et al., 2006) or globally suppress seizure activity in the epileptic brain (Baraban et al., 2009). These findings suggest that transplantation of GABAergic progenitor cells is sufficient to markedly dampen host activity. However, donor cells in our study were not GABAergic, as indicated by negative GAD67 immunolabeling (data not shown). We also performed $GABA_AR$ blockade experiments to test whether ectopic NSCs potentiated inhibitory neurotransmission in the host cortex. Application of the GABAergic antagonist picrotoxin to acute slices revealed all latent excitatory connections. Preservation of activity in the presence of drug would suggest that excitatory network structure is intact within engrafted cortices and that functional alterations are mediated exclusively by enhanced GABAergic signaling. However, we found that activity within deep cortical layers was significantly reduced in picrotoxin-treated engrafted slices, suggesting that that donor cells potentially disrupted the number, trajectory, or targeting of excitatory host cells or their pathways.

We did observe a dose-dependent depletion of host cells in engrafted regions, with no concomitant changes in cortical thickness. The total cell number was conserved, suggesting that endogenous cells were lost. Consistent with this finding, neuron numbers in subcortical regions were reduced with increased numbers of exogenous cells. Competition for external trophic signals in the host brain may mediate this loss. Trophic requirements have been shown to tightly regulate total cell number in the cerebral cortex. The size of interneuronal grafts in the normal rodent brain has been restricted in this way, with a number of precursors undergoing apoptosis to maintain a fixed cell number (Southwell et al., 2012). Furthermore, programmed cell death has been found to restrain cell number in the developing cortex (Blaschke et al., 1996). Therefore, it is possible that grafted NSCs compete with resident excitatory neurons for trophic support, leading to increased death of endogenous cells.

The loss of host cells is sufficient to substantially diminish cortical network excitability, as evidenced by previous studies (Pucilowska et al., 2012). We found the physiological effects of host cell depletion to be more nuanced in our model. Whereas moderate engraftment depleted endogenous populations to levels that affected network integration, high engraftment was required to deplete host cells to levels that globally impaired activity. Our results also suggest that grafted NSCs preferentially affect infragranular circuits. Although moderate engraftment largely preserved cortical function, these levels noticeably reduced the velocity of signal propagation and blocked facilitation in L5/6. Moreover, excitatory infragranular circuits, unmasked by PTX application, showed dampened activity in highly engrafted slices. These results are consistent with the cortical distribution of grafted NSCs, which localized to deep cortical layers. We did, however, observe alterations to the peak amplitude, spatial extent, and velocity of potentials in L2/3, suggesting that supragranular circuit function is also disrupted to some degree.

We propose that functional defects in L2/3 of highly engrafted slices may be the result of columnar interactions with underlying L5/6. Recent findings have shown that blocking L5 activity with tetrodotoxin (TTX) significantly reduces peak $\Delta F/F_0$ signal throughout the depth of the cortex, suggesting that L5 amplifies activity in L2/3. (Wester and Contreras, 2012). Thus, high levels of exogenous NSCs in L5/6 may contribute to the reduction of $\Delta F/F_0$ signal in L2/3 that we observed. Additionally, high levels of engraftment blocked the lateral spread of excitation in L2/3. Local inactivation of L5 with TTX produces a similar effect (Wester and Contreras, 2012), suggesting that defects in L2/3 signal propagation may be attributed to reduced L5 input. Layers 2/3 have sparse connectivity, with 10 times as many inhibitory connections as excitatory connections (Holmgren et al., 2003), and more hyperpolarized neurons (Manns et al., 2004; Lefort et al., 2009). Thus, L2/3 requires powerful excitatory drive from L5 to depolarize. Accordingly, when inhibitory input was pharmacologically suppressed with PTX in our study, superficial layers sustained activity without L5 input. Additionally, L2/3 blockade with TTX does not significantly alter $\Delta F/F_0$ in the cortex and only minimally affects activation of surrounding columns (Wester and Contreras, 2012). These results indicate that impairment to L5 function alone is sufficient to influence activity in superficial cortical layers. We propose that exogenous NSCs may directly

interfere with infragranular circuitry by altering the number of host cells, and consequently, the number of functional synapses. Sparse L5/6 connectivity may reduce excitatory input to L2/3, interfering with the initiation and lateral propagation of activity within L2/3, which is consistent with our results.

Overall, our study has a number of implications for cell therapy in the CNS. In particular, these data put a premium on the method used to obtain cells as well as appropriate controls for engraftment. A number of studies have demonstrated that fully differentiated grafts can preserve host function, suggesting that alterations in our study are due to non-integrating cell types. For example, the cortex can accommodate a large number of ectopic, fully differentiated interneurons without significant alteration to activity (Southwell et al., 2012). Transplantation of interneuronal progenitors expanded the cortical interneuronal population by up to 35%, but the frequency of inhibitory synaptic events did not scale up proportionately. Moreover, it has been shown that transplanted ES-derived neurons can adopt and drive activity of endogenous hippocampal networks (Weick et al., 2011; Piña-Crespo et al., 2012). Finally, mature NT2N neurons derived from a clonal human teratocarcinoma line (NT2) have been transplanted in phase I(Nelson et al., 2002) and II(Kondziolka et al., 2005) clinical trials for stroke therapy. These cells, selected for their potent neuronal lineage commitment, did not cause deleterious effects in affected patients. Collectively, these studies suggest that the host brain can safely accommodate fully differentiated cells.

Additionally, our findings establish a safe limit for the number of engrafted progenitors in cortex. Low levels of engraftment have been achieved with primary NSCs(Magnitsky et al., 2008; Chaubey and Wolfe, 2013) and other NSC lines (Lundberg et al., 2002; Demeter et al., 2004), but such levels are likely to be subtherapeutic (Taylor and Wolfe, 1997). Similarly, adult parenchymal transplants resulted in limited cortical engraftment. Although these levels were well tolerated by the adult recipients, they restrict the utility of NSCs for the treatment of widespread pathology of neurogenetic diseases. Optimization of graft survival and migration using genetic engineering may improve therapeutic outcomes through enhanced distribution of the NSCs. However, our data also suggest that such improved engraftment levels may also introduce defects in the

network function, which may interfere with the beneficial effect of the cell therapy. It will be necessary to find a balance between the engrafted cell density that is needed for a therapeutic effect and preserving normal host circuit functions.

IV. Materials and Methods

Cell culture, labeling, and sorting

The C17.2 line was derived after v-myc immortalization of progenitor cells isolated from the postnatal mouse cerebellum (Ryder et al., 1990) . NSCs were maintained as an adherent monolayer on uncoated 10-cm dishes at 37℃ and 5% CO_2 and passaged at a ratio of 1:10 by trypsinization twice per week. Growth medium contained 83% Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5g/L) and 1mM sodium pyruvate, 10% fetal bovine serum, 5% horse serum, 1% L-glutamine, and 1% penicillin-streptomycin-fungizone (all from Gibco, Grand Island, NY). Lentiviral-mediated labeling of C17.2 cells was performed as described previously to enable reliable graft identification (Watson et al., 2006). A self-inactivating (SIN) lentiviral vector driving constitutive EGFP expression from the human elongation factor 1α promoter (EF1α) was generated using standard triple transfection approach (Watson et al., 2006). C17.2 cells were transduced for 12 h in conditioned medium containing the vector (SIN.EF1 α.EGFP) at multiplicities of infection (MOIs) of approximately 10. Labeled populations were sorted for EGFP on the FACSVantage SE cell sorter (BD Biosciences, San Jose, CA) and expanded *in vitro* following recovery for two additional passages prior to transplantation. EGFP has been used as a reporter gene in a number of other transplantation studies without observable alteration to donor cell physiology (Englund et al., 2002; Alvarez-Dolado et al., 2006b; Koch et al., 2009). Additionally, several genetic voltage and calcium indicators, developed to reliably monitor cell physiology, are EGFP fusions (Nakai et al., 2001; Peterka et al., 2011). EGFP toxicity has not been observed using these functional reporters. Thus, it is unlikely that functional outcomes in this study are the result of some unknown impact of EGFP expression.

Neonatal transplantation

Cells were harvested for transplantation as previously described (Watson et al., 2006). Briefly, cells were trypsinized and washed twice in PBS before final resuspension in PBS to yield a final concentration of 40,000 cells/µl. Only viable cells, determined using trypan blue exclusion, were included in cell counts. For the dose-response study, cell preparations were serially diluted in PBS. Mice were divided into four groups according to the number of input cells/ventricle: 80,000 (n=3), 40,000 (n=3), 8,000 (n=3), and uninjected (n=3). During the transplantation procedure, the heads of cryoanesthetized neonatal (P0-2) C57BL/6 mice were transilluminated and approximately 2 µl of cell suspension was slowly injected into each lateral ventricle with a finely drawn glass micropipette. The angle of injection is such that the needle does not penetrate through the somatosensory cortex, but instead enters through caudal aspect of the brain to minimize tissue damage. Injected pups were warmed up and returned to maternal care after recovery. All procedures were approved by the Institutional Care and Use Committee at the Children's Hospital of Philadelphia.

Stereotaxic adult injections

All animals receiving injections were older than 2 months of age at the time of injection. Under sterile conditions, SCID mice (n=4) were anesthetized with isofluorane and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and holes the size of the injection needle were drilled into the skull. Cell injections were done unilaterally with 0.5 µl of suspension (40,000 cells total). An equivalent volume of PBS was injected into the contralateral hemisphere. The injection syringe (Hamilton, Reno, NV) delivered cells or vehicle at a constant volume of 0.1 µl/min using a syringe pump (KD Scientific, Holliston, MA). The needle was left in place for 3 min after each injection to minimize upward flow of viral solution after raising the needle. Coordinates [in millimeters; rostral (+) or caudal (−) to bregma, left of midline, ventral to pial surface] for the cortex were −2.1, 1.25, and 1.1. All procedures were approved by the Institutional Care and Use Committee at the Children's Hospital of Philadelphia.

Cortical slice preparation

Brains were harvested from both neonatal and adult recipients 8-10 weeks post-transplant for live imaging. Mice were anesthetized with isoflurane, decapitated, and the brains removed and blocked in ice-cold artificial cerebral spinal fluid (ACSF) (3 mM KCl, 1.25 mM $NaHPO_4$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose), in which NaCl was replaced with an equal osmolar concentration of sucrose (130 mM). After removal of the cerebellum, the two hemispheres were separated with a midsagittal cut, and each hemisphere was mounted for sectioning. Coronal slices (350 µm) at the level of the hippocampus were cut with a vibratome (VT1200S, Leica, Buffalo Grove, IL) and transferred into ACSF without sucrose. Slices were subsequently placed on tea paper, transferred to a holding chamber (37℃) for a 45 min-recovery period, and then stored at room temperature for up to 6 h.

Confocal imaging and analysis

Following recovery, acute cortical slices were transferred to an ACSF filled imaging chamber for confocal microscopy. GFP-positive grafts were imaged at 10x magnification using a confocal-scanning laser microscope (FluoView1000, Olympus, Center Valley, PA). Stacks of consecutive brightfield and confocal images were taken simultaneously at 10 µm intervals and acquired using an argon laser (488nm). All analyses were performed using ImageJ software (NIH, Bethesda, MD). A maximum intensity projection was generated from each Z stack and a region of interest (ROI) was drawn around the entire neocortex using the corpus callosum as the ventral border. The percentage of pixels with intensities more than two standard deviations above the background pixel intensity was quantified for each slice. Graft measurements were validated with automated cell counts performed on 5 randomly-chosen ROIs in control (n=9) and engrafted slices (n=16) using Volocity software (PerkinElmer, Waltham, MA). The number of GFP positive cells per ROI was quantified and normalized to the total number of DAPI positive. In subcortical regions, including the striatum, NeuN and DAPI positive cells were quantified per ROI using automated methods available through Fiji software (Schindelin et al., 2012).

Immunohistochemistry

Engrafted cells were phenotyped using standard immunohistochemistry. At eight weeks post-transplant, brains were removed and fixed in 4% paraformaldehyde/PBS after transcardial perfusion. Harvested brains were embedded in 2% agarose and sectioned coronally at 50 µm on a vibratome (Leica VT1000S, Leica, Buffalo Grove, IL). Free-floating sections were post-fixed in 4% PFA in PBS for 20 min, then permeabilized and immunoblocked at room temperature for 1h in PBS containing 2.5% goat or donkey serum and 0.2% Triton X-100. Slices were then incubated overnight at 4C with primary antibodies against the following antigens: GFP (1:1000, Molecular Probes, Grand Island, NY), Nestin (1:500, Millipore, Billerica, MA), Ki67 (1:200, Novoscastra, Buffalo Grove, IL), DCX (1:200, Santa Cruz, Dallas, TX), ßIII-Tubulin (1:1000, Millipore, Billerica, MA), NeuN (1:500, Millipore, Billerica, MA), and GFAP (1:1000, Millipore, Billerica, MA). After three washes in PBS, sections were incubated at room temperature for 2h with appropriate secondary antibodies conjugated to Alexa 594 or 488 (Molecular Probes, 1:300, Grand Island, NY). All antibodies were diluted in PBS. After several washes, slices were mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA) and examined with a confocal scanning-laser microscope. Confocal images from a single optical plane were acquired sequentially with two lasers (argon, 488nm; helium/neon, 543nm) at 20x magnification with optical zoom of 2 or 5. Image processing was carried out using ImageJ software.

Local field recordings and analysis

Acute cortical slices from uninjected controls (n=7) and animals injected with 80,000 cells per ventricle (n=8) were assayed. Field electrodes were placed at one of three positions along neocortical layer 2/3. All recordings were acquired under current clamp conditions in response to a single callosal stimulation delivered via bipolar electrodes. Threshold current amplitude (x) was determined empirically. Recordings were obtained in response to current steps of x, 2x, 4x, 6x, and 8x. Five trials were obtained at each step with a 10s intertrial interval. All analyses were performed with Clampfit software (Molecular Devices, Sunnyvale, CA).

Optical recordings

Optical responses to callosal stimuli were characterized in cortical slices (n=33) across four dose conditions in neonatal recipients. Slices (n=8) from injected animals without established grafts were also included in the study. Slices were dyed with di-3-ANEPPDHQ (Molecular Probes) in ACSF for 15 min, placed in an oxygenated interface chamber, and imaged using a fast CCD camera (NeuroCCD, RedShirtImaging, Decatur, GA) with 80 x 80 pixel matrix and 1 kHz frame rate. Epi-illumination was provided by a Xenon lamp driven by a stable power supply. With the 4x objective, the imaging field covered the area of 1.92 x1.92 mm including neocortex and underlying corpus callosum. Electrical stimulation (200 µA) was delivered by means of bipolar electrodes (World Precision Instruments, Sarasota, FL) placed on the corpus callosum. Three independent stimulation paradigms were applied (1 stimulus, 5 stimuli at 10 Hz, 5 stimuli at 40 Hz). Twelve trials were obtained per stimulation with 20 s elapsing between trials. Procedures were repeated for slices (n=18) obtained from adult injected brains. In an independent set of experiments, we obtained recordings 30 min after blockade of GABAergic signaling with picrotoxin (100 mM, Sigma, St. Louis, MO) in uninjected (n=5) and high engrafted (n=5) slices from neonatally injected brains.

VSD data analysis

Analysis of all optical data sets was performed with IGOR (Wavemetrics, Lake Oswego, OR) and software written on Matlab (Mathworks Inc., Natick, MA). Each data set represented the average of twelve trials of stimulation. Fluorescence values for each pixel in a frame were differential, represented as the difference (ΔF) between stimulation-evoked signal and basal signal in a reference frame. A reference frame was calculated as the average of 40 frames preceding the stimulation. Intensity measurements are reported as fractional fluorescence ($\Delta F/F$), or change in fluorescence divided by basal or resting fluorescence. Signal from an area unaffected by the stimulus was subtracted from fractional fluorescence and median filtering was applied to further reduce noise. For global assessment of cortical responses, normalized fractional fluorescence was averaged across pixels within large regions of interest (ROIs). Pixels

with intensities above 0.1% ΔF/F (≥ 4 SDs over noise levels) were defined as active. Neocortical ROIs were drawn manually using white matter as the ventral border and layer I as the dorsal border. Medial and lateral borders were drawn approximately 20 pixels from a defined vertical axis of columnar activation. Regional analyses of cortical responses were performed using semi-automated software. For each slice, a vertical axis of columnar activation was defined. Five bins with fixed dimensions were generated that were oriented perpendicularly to this vertical axis. In synaptic blockade experiments, responses collected at five minute intervals post treatment were normalized to a baseline response obtained prior to drug application. These values were further normalized to response from ACSF (vehicle only) treated groups.

Statistical analysis.

Unpaired two-tailed Student's t-test and One-Way ANOVA followed by Bonferonni post hoc tests were used, where applicable, to determine whether mean differences between groups were different and were considered significant when $P<0.05$. Data are reported as means ± SEM.

CHAPTER 3

Undifferentiated Neural Stem Cells Induce Microglia-Mediated Cytotoxicity

I. Introduction

Over the past decade, researchers have demonstrated that morphogenic gradients present in neurodevelopment can drive the *in vitro* specification of neuronal subtypes from stem cells. However, these precisely regulated developmental processes do not tolerate significant *in vivo* deviation, making neuronal replacement by transplanted NSCs a considerable challenge. Instead, NSC transplants yield predominantly undifferentiated grafts. The data described in Chapter 2 indicate that undifferentiated progeny can alter neural network function in the healthy postnatal brain (Weerakkody et al., 2013). However, it remains unclear how undifferentiated NSCs interface with cellular constituents of the host microenvironment, and whether these interactions can be generalized to NSCs of different developmental origins.

Numerous studies suggest that NSCs can modulate host immune function while retaining undifferentiated features (Pluchino et al., 2005). The majority of these studies have been performed in models of chronic inflammation in which peripheral immune cells are able to breach the blood-brain barrier. Grafted NSCs can minimize transendothelial migration of infiltrating cells and activate apoptotic pathways. Few data exist addressing the interactions between NSCs and resident microglia, which constitute the innate arm of the immune system. Given the emerging developmental role of microglia in sculpting neural circuits, it is important to understand how transplanted NSCs may influence these cells in both normal and pathological contexts (Wake et al., 2013). Recent evidence suggests that undifferentiated NSCs can activate microglia in the healthy brain via VEGF signaling (Mosher et al., 2012). However, downstream effectors and signaling components are undefined.

Here, we demonstrate that neuron loss is accompanied by microglia activation following C17.2 engraftment in the mature cortex. Furthermore, doxycycline, an inhibitor of microglial activation, can confer neuroprotection if administered within weeks of transplantation. Undifferentiated C17.2 cells constitutively secrete a host of proinflammatory cytokines, many of which are potent activators of microglia. Primary NSCs from the postnatal SVZ have a similar cytokine profile. We propose that these secreted factors are sufficient to induce microglial-mediated apoptosis in culture. Furthermore, TNFα is required for NSC-mediated toxicity.

Together, these data show that undifferentiated NSCs can stimulate inflammatory functions without direct antigen recognition, which have serious implications for both syngeneic and allogeneic grafts in the CNS.

II. Results

Progressive neuronal loss following NSC transplantation

Previously, we reported dose-dependent alterations in cortical excitability following the engraftment of undifferentiated NSCs (Weerakkody et al., 2013). GABAergic signaling was not the predominant mechanism for circuit dampening. Here, we propose an alternative explanation - the death of cortical neurons or reduction of cortical connectivity following transplantation may underlie network dysfunction. We investigated the possibility of neuronal depletion in deep layers using two independent methods *in vivo*, measurement of NeuN immunolabeling and N-acetylaspartate (NAA) concentrations. Engrafted cortices contained fewer endogenous NeuN+ cells relative to control at eight weeks post-transplant (32.00±2.25 vs. 42.57±1.10, $p<0.001$) **(Figs. 3.1a, b)**. Aberrant network function was not observed at two or four weeks **(Fig. 3.2**), which may be explained by the time-course of NSC engraftment following neonatal intraventricular transplantation (Weerakkody et al., 2013). Consistent with these observations, we found no significant difference in neuronal number between engrafted and control cortices up to four weeks post-transplant (40.86±1.41 vs. 36.17±0.87, $p>0.05$) **(Fig. 1b).** *In vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) was used to further corroborate the loss of neurons at eight weeks post-transplant. This method enables the quantification of cellular metabolites within a defined voxel. We quantified N-acetyl aspartate (NAA), a neuron-specific metabolite, in the cortex of engrafted and control animals and found a significant reduction at eight weeks post-transplant (2.552±0.270 vs. 4.591±0.300, $p=0.004$) **(Fig. 3.1c).**

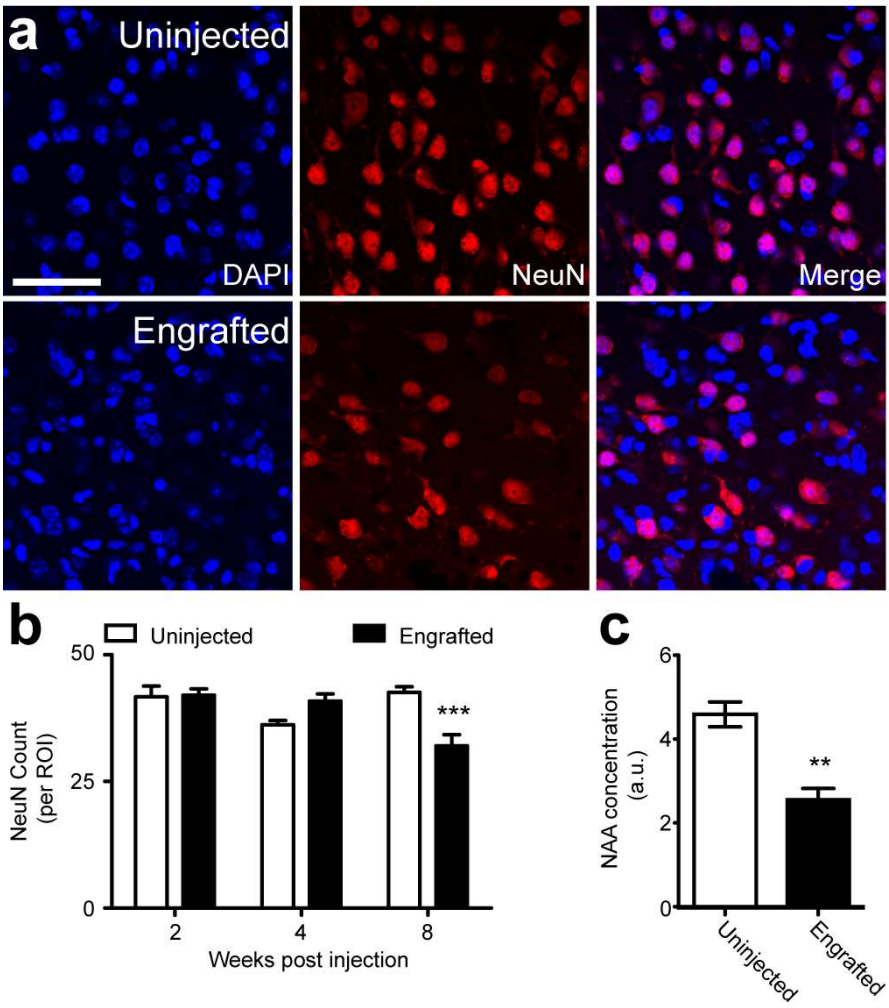

Figure 3.1. Engrafted cortices are depleted of endogenous neurons. (**a**) Confocal images showing NeuN immunolabeling at 8 weeks in uninjected and neonatally C17.2-injected brains. (**b**) Automated NeuN counts were performed on five randomly selected ROIs within deep layers of the somatosensory cortex. Histogram displaying mean counts in uninjected and engrafted cortices across 2, 4, 6, and 8 weeks. A minimum of n=6 hemispheres were analyzed per time point for each engraftment condition. (**c**) Concentration of neuronal metabolite N-acetylaspartate (NAA)(normalized to water peak) in defined cortical ROI of uninjected (n=5) and engrafted (n=3) animals , as measured by *in vivo* 1HMRS. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (Scale bar in **a**: 50 μm)

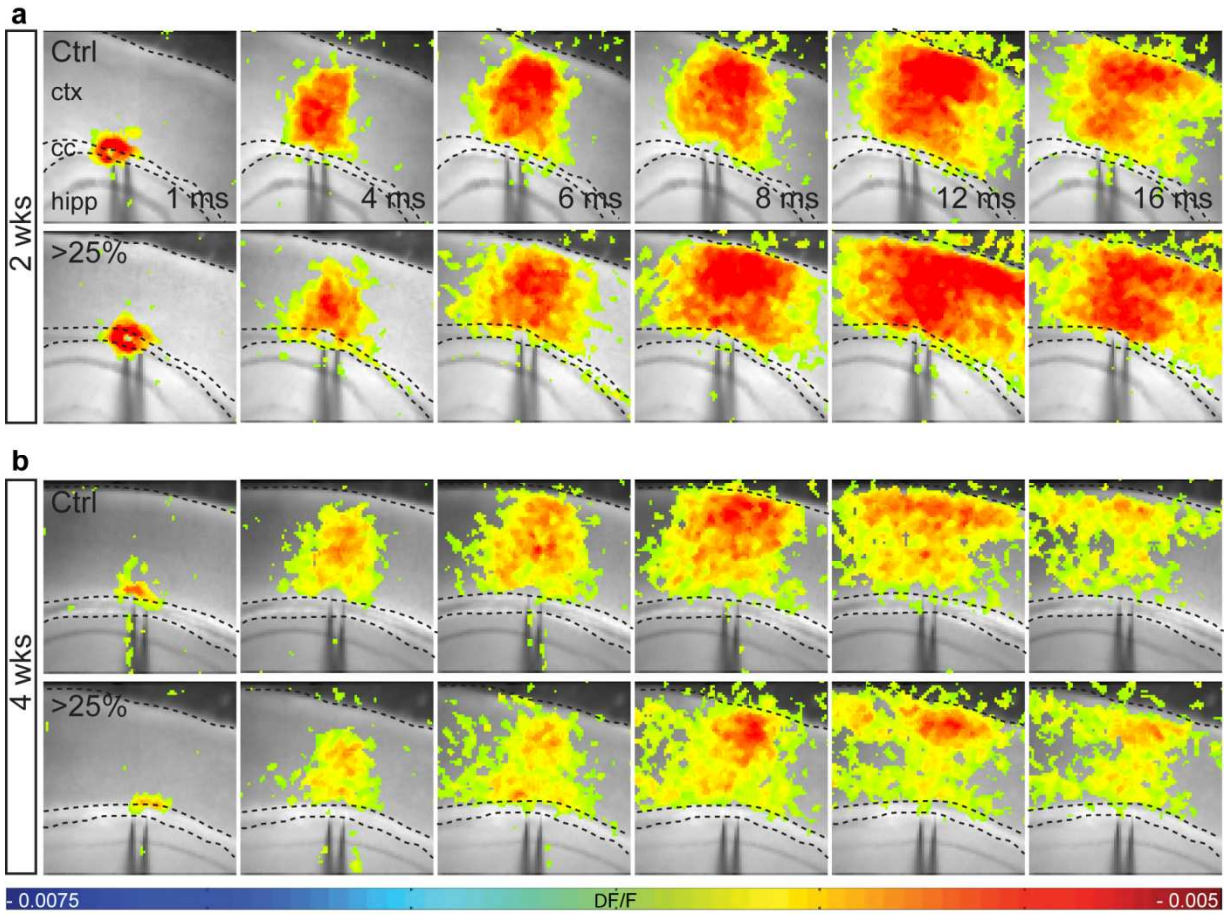

Figure 3.2. Cortical function is preserved up to 4 weeks following neonatal transplantation. Cortical activation profiles of uninjected (n=3) and highly engrafted (> 25% engraftment by area) (n=3) slices at **(a)** 2 and **(b)** 4 weeks post-transplant. Depolarization, depicted by warm colors, was elicited in response to a single callosal stimulation.

Neuroprotection conferred by microglial deactivation

Prior studies have demonstrated that allografts can survive in the mouse brain without immune rejection. Two potential reasons may account for long-term stability. First, the CNS is immunoprivileged as a result of the blood-brain barrier and other mechanisms that limit the invasion of peripheral immune cells (Bailey et al., 2006). Second, NSCs downregulate major histocompatibility complex (MHC) antigens, which allows them to evade recognition by the adaptive immune system (Hori et al., 2003). However, the innate immune system can also respond to allografts (Ideguchi et al., 2008; Chen et al., 2011). To investigate innate immune activation, we examined engrafted tissue for augmented levels of Iba1, a pan marker of microglia, and CD68, a marker of microglial phagocytosis. We observed a greater Iba1+ percent area in engrafted cortices at eight weeks (22.61±2.78% vs. 8.56±0.33%, $p<0.01$), suggesting elevated microglia numbers or activation-related microglial hypertrophy **(Figs. 3.3a,b)**. The percent area of CD68 immunoreactivity, which is associated with microglial activation, was also increased in at this time point (4.70±0.74% vs. 1.85±0.09%, $p<0.05$) **(Figs. 3.3c,d)**. The extent of immunolabeling in mock-injected cortices was not significantly different from uninjected cortices at eight weeks (Iba1, 9.68%±1.12 vs. 8.57%±0.33, $p=0.249$; CD68, 1.50±0.30% vs1.85±0.09%, $p=0.166$). Thus, the enhancement of Iba1 and CD68 staining was not a direct consequence of the injection procedure **(Fig. 3.4)**. We also investigated the time-course of microgliosis and did not detect any signs of activation at two weeks (Iba1, 16.37%±0.91 vs. 12.51%±0.53, $p>0.05$; CD68, 2.30±0.19% vs. 2.33±0.46%, $p>0.05$). However, Iba1 and CD68 immunoreactivity increased markedly by four weeks (Iba1, 40.15%±4.59 vs. 9.33±0.57%, $p<0.001$; CD68, 4.44±1.06% vs. 1.14±0.05%, $p<0.05$). We also examined brains for evidence of astrogliosis at eight weeks **(Fig. 3.5)**. GFAP immunolabeling in the cortex was enhanced following C17.2 engraftment.

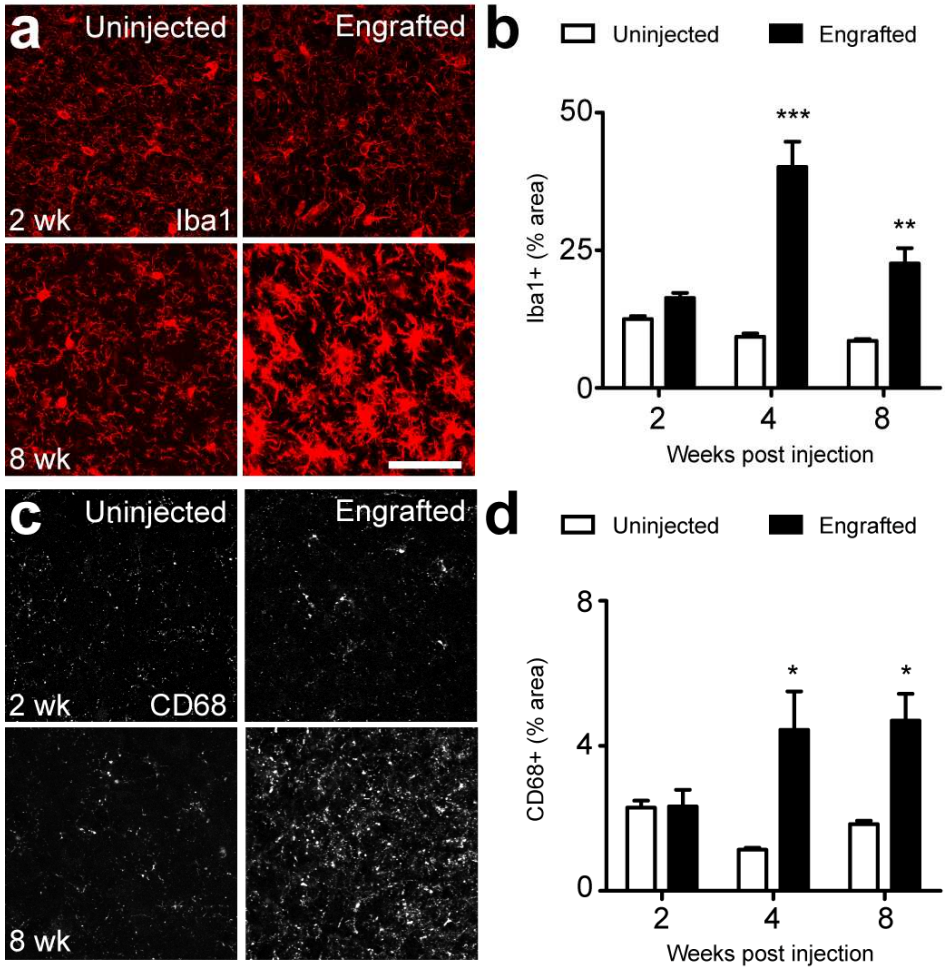

Figure 3.3. Transplanted NSCs induce persistent microglial activation. (**a**) Maximum projection showing microglial Iba1 immunolabeling at 2 and 8 weeks in uninjected and neonatally C17.2-injected brains. The ramified morphology of Iba1+ microglia in engrafted cortices is evident at 2 weeks whereas amoeboid morphology, a hallmark of activated microglia, is seen at 8 weeks. (**b**) Histogram displaying percent area of Iba1 immunoreactivity within selected ROIs across 2, 4, 6, and 8 weeks. A minimum of n=8 hemispheres were analyzed per time point for each engraftment condition. (**c**) Maximum projection showing CD68 (phagocytic marker) immunolabeling at 2 and 8 weeks in uninjected and neonatally C17.2 injected brains. (**d**) Histogram displaying percent area of CD68 immunoreactivity within selected ROIs across 2, 4, 6, and 8 weeks. A minimum of n=6 hemispheres were analyzed per time point for each engraftment condition. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (Scale bar in **a**: 50 μm)

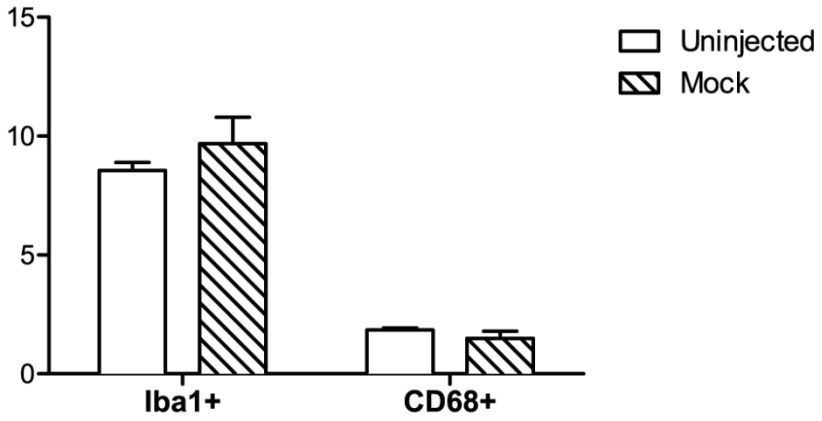

Figure 3.4. Injection procedure does not induce microgliosis. Histogram displaying percent area of Iba1 and CD68 immunoreactivity within selected ROIs at 8 weeks in uninjected and mock-injected cortices. A minimum of n=8 hemispheres were analyzed for each engraftment condition. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

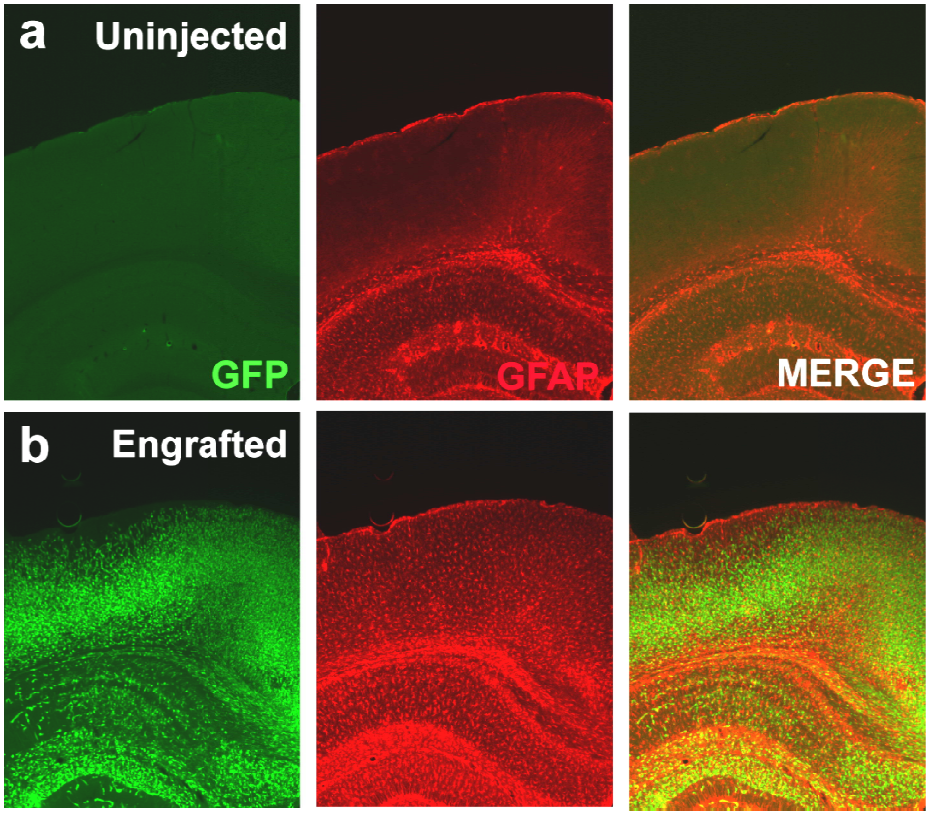

Figure 3.5. NSC engraftment induces significant astrogliosis. Fluorescence images of GFAP immunoreactivity in coronal sections of the (a) uninjected and (b) engrafted brain at 8 weeks. A minimum of n=3 hemispheres were analyzed for each engraftment condition.

To test whether neuroinflammation contributed to host cell death, we treated transplanted and control animals with doxycycline, which is known to reduce microgliosis and astrogliosis. Control animals treated with doxycycline three weeks post-transplant showed a reduction in neuronal number, as indicated by NeuN immunolabeling (33.67±1.00 vs. 42.57±1.10, $p<0.001$) **(Fig. 3.6a)**. Prior studies suggest that microglia provide trophic support to layer V projection neurons in early postnatal development (Ueno et al., 2013). At this time, inactivation of microglia by minocycline or transient ablation in a transgenic model leads to apoptosis in the normal brain. Consequently, we administered doxycycline at a later time point of six weeks, which induced a similar neuronal loss (34.67±0.82 vs. 42.57±1.10, $p<0.001$) **(Fig. 3.6a)**. Interestingly, engrafted animals treated at three and six weeks exhibited neuronal survival comparable to untreated controls (three weeks, 41.78±1.83 vs. 42.57±1.10, $p>0.05$; six weeks, 39.56±0.96 vs. 42.57±1.10, $p>0.05$) **(Fig. 3.6b)**. These data suggest that inflammatory blockade in transplanted animals can promote neuroprotection.

Proinflammatory cytokine profile of NSCs

There are several possible mechanisms contributing to NSC-mediated microglial activation in the host brain. Allogenic transplants can elicit an innate immune response as previously discussed (Ideguchi et al., 2008; Chen et al., 2011). However, recent data suggests that even syngeneic transplants derived from the rodent forebrain can activate microglia in recipient mice through the expression of soluble factors (Mosher et al., 2012). We explored the possibility that NSCs constitutively secrete immunomodulatory factors by analyzing the composition of NSC supernatants obtained after three days *in vitro* (DIV). C17.2 cells were cultured as an adherent monolayer, as previously described (Weerakkody et al., 2013). Primary NSCs from the neonatal SVZ were propagated as neurospheres in the presence of mitogenic factors. Luminex multiplex analysis of supernatants from C17.2 and primary SVZ-derived cells revealed a number of cytokines involved in the regulation of microglial activity **(Fig. 3.7)**. Among these were potent activators of proinflammatory functions, including IL-1α, IL-1β, IL-6, MIP-1α, MIP-1β, and VEGF. In addition, IP-10, M-CSF, and MCP-1 were present, which are

chemoattractants for microglia. Earlier studies suggest that cytokine expression is unique to NSCs with undifferentiated features (Mosher et al., 2012)

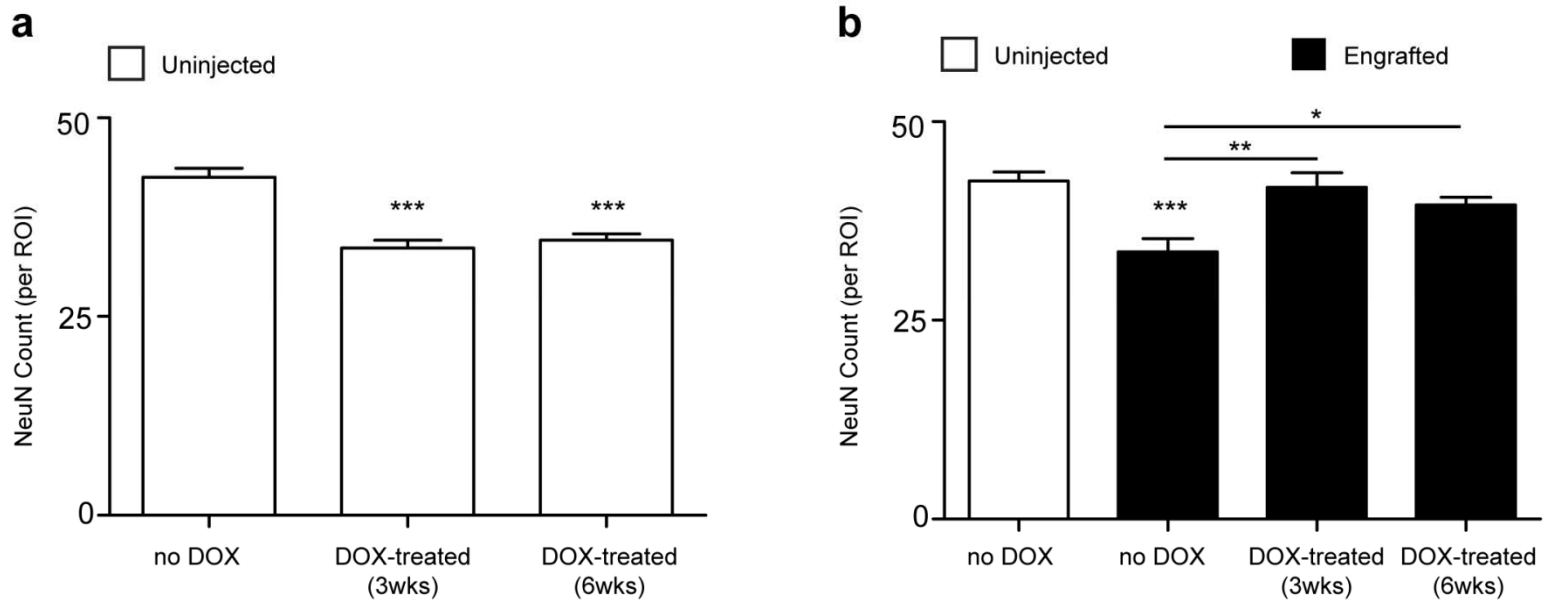

Figure 3.6. Doxycycline treatment protects against neuronal loss in the engrafted cortex. (a) Histogram depicting the effect of Doxycycline (Dox), a known anti-inflammatory agent, in uninjected animals following treatment at three weeks and at six weeks. Treatment resulted in a marked reduction of endogenous neurons for both treatment regimens. **(b)** Histogram showing the effect of doxycycline in engrafted animals following treatment at three and at six weeks. Treatment restored neuron numbers to those of untreated control independent of Dox timecourse. Neurons were quantified by NeuN immunolabeling in five randomly selected ROIs per hemisphere. A minimum of n=9 hemispheres were analyzed per condition. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

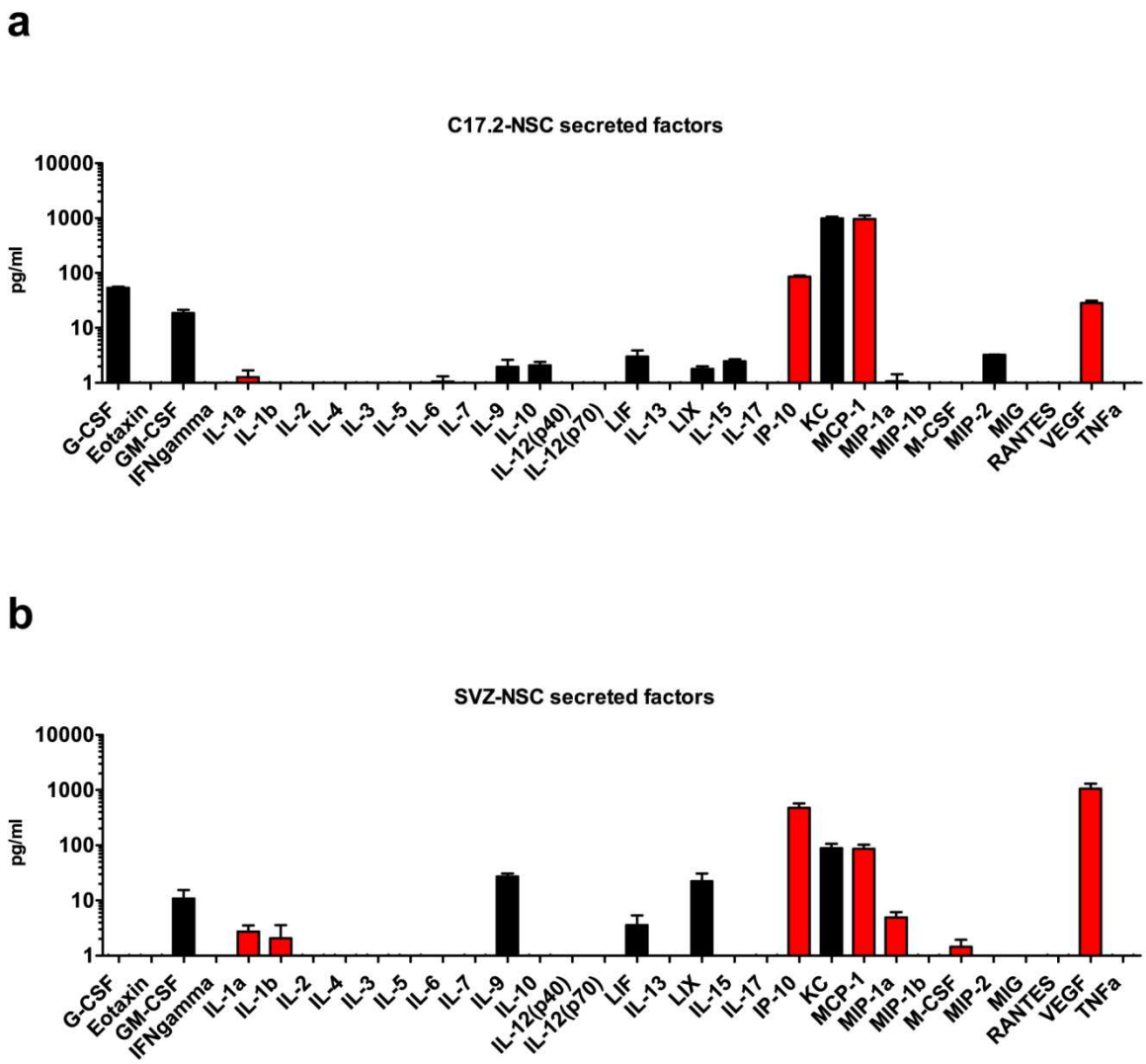

Figure 3.7. Undifferentiated NSCs secrete proinflammatory cytokines. Multiplex cytokine analysis of conditioned media (CM) obtained from cultured (**a**) C17.2 cells and primary progenitors isolated from the (**b**) P0 mouse subventricular zone (SVZ). CM was collected 48 h post-plating for analysis from independent passages of C17.2 cultures (n=4) and SVZ cultures (n=8). Red bars on histogram represent factors involved in proinflammatory microglial functions.

Microglial-mediated apoptosis induced by NSC factors in vitro

To investigate whether secreted factors are sufficient to induce microglia-mediated cell death, an *in vitro* co-culture system was used. Rat cortical neurons were harvested from the embryonic rat brain (E18), cultured for two weeks, and subsequently treated with a panel of NSC supernatants and control media formulations. All neuronal cultures showed basal level of apoptosis, as quantified by phosphatidylserine exposure (**Fig. 3.8a**). Moreover, the percentage of apoptotic cells in cultures treated with C17.2 and SVZ supernatants were comparable to those treated with unconditioned medium (C17.2 (1:5 dilution), 6.33±1.20% vs. 10.67±2.33%, $p>0.05$; SVZ, 7.33±0.88% vs. 10.67±2.33%, $p>0.05$). Thus, NSC-derived factors alone are not sufficient to induce cell death. In an independent experiment, primary microglia obtained from P4 rat pups were plated on neuronal cultures at DIV 14. Co-cultures were treated with the same panel of conditioned NSC and unconditioned supernatants. As expected, co-cultures treated with a known activator of microglia, lipopolysaccharide (LPS), exhibited more apoptosis relative to LPS-treated neuronal cultures (25.00±1.95% vs. 12.33±3.48%, $p<0.01$) and unconditioned co-cultures (25.00±1.95% vs. 12.00±2.31%, $p<0.01$) (**Fig. 3.8a**). Similarly, NSC-conditioned supernatants increased cell death in co-cultures as compared with neuronal cultures (C17.2 (1:5 dilution), 24.00±1.73% vs. 6.33±1.20%, $p<0.01$; SVZ, 19.50±3.12% vs. 7.33±0.88%, $p<0.05$). It should be noted that C17.2 supernatants were diluted in order to reveal differences in cell viability in the presence and absence of microglia. Co-cultures treated with LPS and SVZ supernatants exhibited augmented levels of necrosis as compared to stimulated neuronal cultures (LPS, 23.00±5.29% vs. 1.67±0.88%, $p=0.016$; SVZ, 5.00±1.16% vs. 1.33±0.33%, $p=0.038$). However, co-cultures treated with SVZ supernatants did not undergo more necrotic cell death as compared to unconditioned co-cultures (5.00±1.16% vs. 9.00±2.89%, $p=0.268$) **(Fig. 3.8b)**. Overall, these data suggest that NSC-derived factors can induce cell death in a microglia-dependent manner.

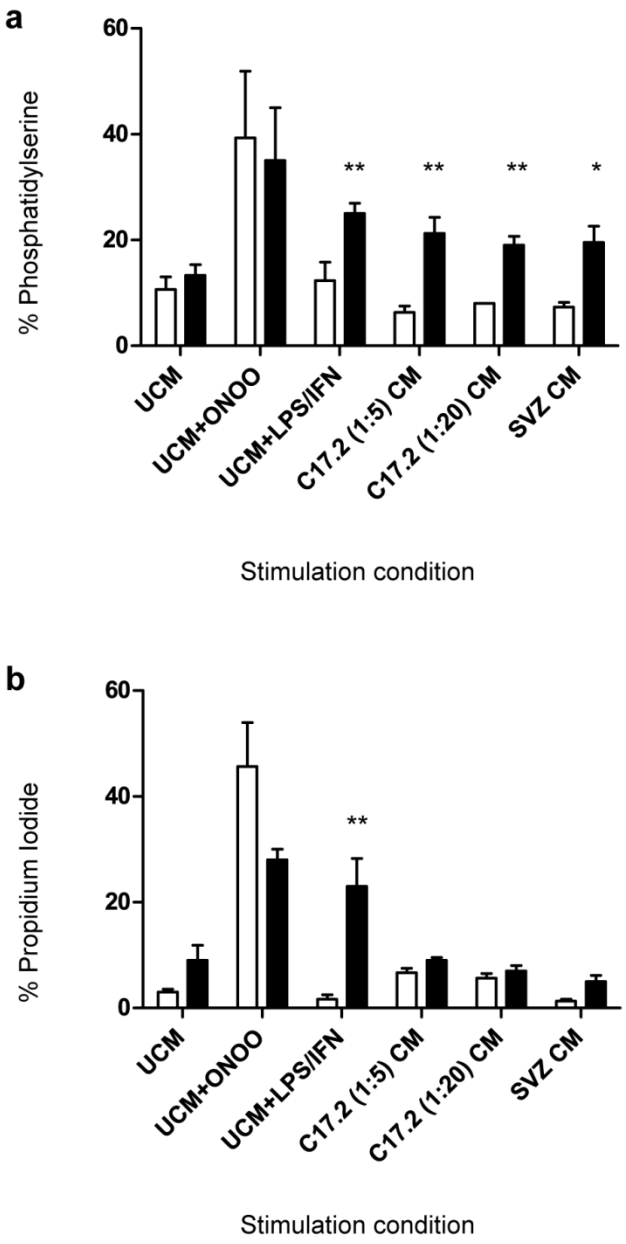

Figure 3.8. Microglia enhance NSC-induced apoptosis *in vitro*. Neuronal and co-cultures were treated with control or conditioned NSC media for 48 h. **(a)**Histogram depicting percentage of apoptotic cells, as measured by phosphatidylserine immunostaining. **(b)**Histogram depicting percentage of necrotic cells, as measured by propidium iodide permeation. White bars represent neuron-only cultures and black bars represent co-cultures containing both neurons and microglia. A minimum of n=3 cultures were analyzed per condition. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Requirement of TNFα signaling for toxicity

Several signaling pathways may underlie microglial-mediated toxicity in culture. Two major proinflammatory pathways include p38 MAPK and NFκB, which induce cell death via downstream release of nitric oxide (NO) and tumor necrosis factor alpha (TNFα). To assess the role of these of pathways, we measured levels of soluble NO **(Fig. 3.9a)** and TNFα **(Fig. 3.9b)** in co-cultures 48 h post stimulation. Detectable levels of NO were only observed in co-cultures treated with unconditioned media + peroxynitrite, which suggests this molecule does not drive microglial-mediated apoptosis *in vitro*. Conversely, TNFα levels were elevated in response to treatment with LPS and C17.2 conditioned media (LPS, 191.80±14.69 pg/ml vs. 22.29±3.61 pg/ml $p<0.001$; C17.2 (1:5 dilution), 173.30±16.40 pg/ml vs. 22.29±3.61 pg/ml, $p<0.001$). Blockade of TNFα activity in co-culture using a soluble receptor (sTNFR1) reduced toxicity to levels observed in co-cultures treated with unconditioned media (LPS, 17.60±1.17% vs. 12.40±2.29% $p=0.080$; C17.2 (1:5 dilution), 12.00±2.65% vs. 12.40±2.29% $p=0.951$; SVZ, 6.00±1.00% vs. 12.40±2.29% $p=0.088$) **(Fig. 3.9c)**. Taken together, these data suggest that NSCs can alter microglial functions via activation of canonical proinflammatory signaling pathways, which have cytotoxic effects in the healthy host brain.

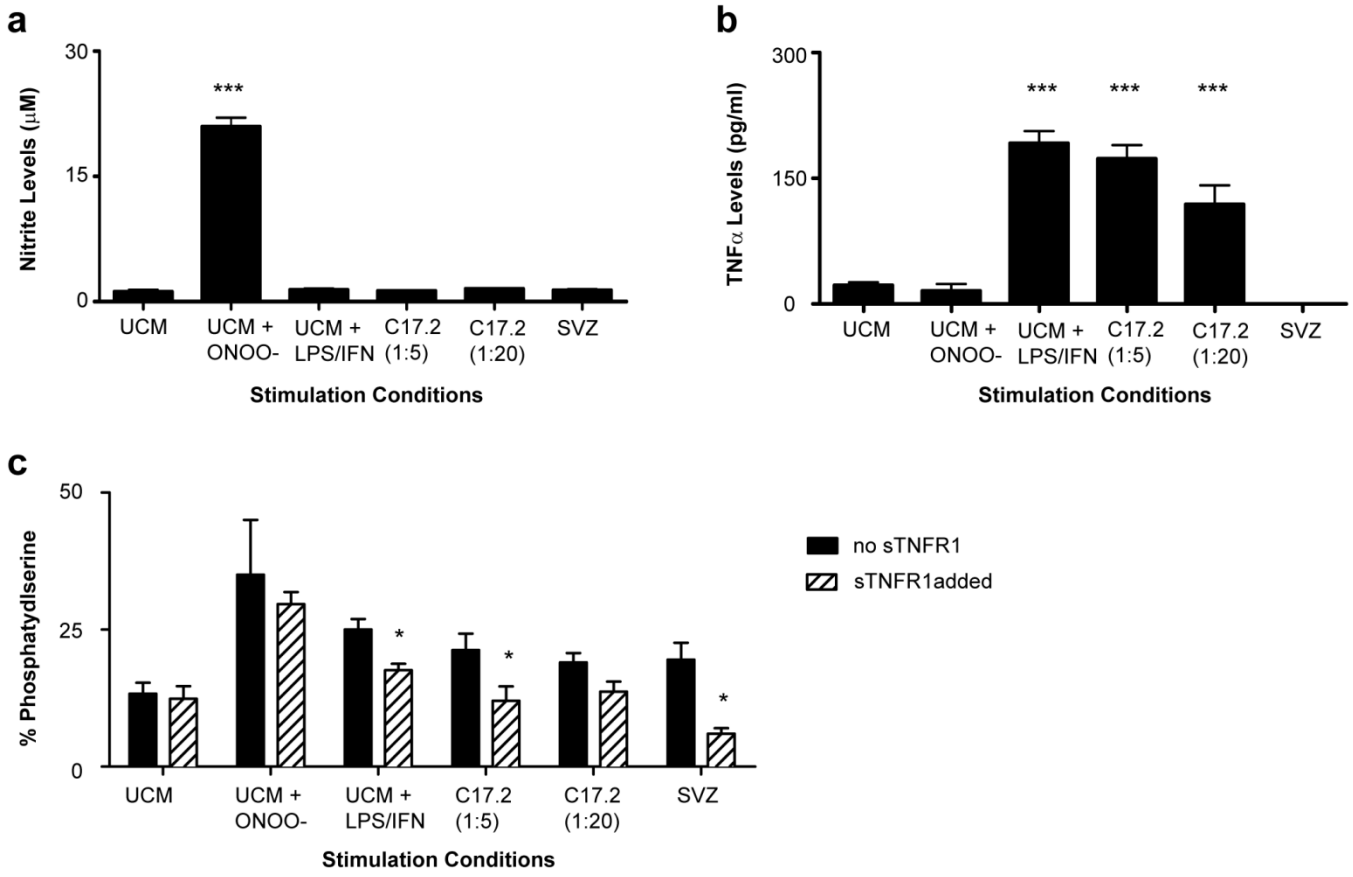

Figure 3.9. TNFα is required for microglia-mediated cytotoxicity.

Figure 3.9. TNFα is required for microglia-mediated cytotoxicity. (a) Nitrite concentration in co-cultures 48h post stimulation with control and NSC supernatants. **(b)** TNFα production is elevated in co-cultures 48h following treatment with conditioned C17.2 supernatants. **(c)**Histogram showing percentage of apoptotic cells after48 h treatment with a soluble receptor to TNFα receptor (sTNR1) A minimum of n=3 cultures were analyzed per condition. Data are means ± SEM (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

III. Discussion

Our results are similar to earlier data citing the role of innate immune signaling in host-graft interactions (Mosher et al., 2012). Microgliosis was observed in response to the engraftment of undifferentiated C17.2-NSCs in the healthy rodent cortex and potentially contribute to neuronal loss. To directly test the influence of NSC-secreted factors on the host microenvironment, we used a mixed culture of neurons, astrocytes, and microglia. The direct contribution of microglia to cytotoxicity was isolated in this *in vitro* system. We observed marked neuronal apoptosis following the application of supernatants obtained from cultured C17.2-NSCs and primary SVZ-NSCs alike. Elevated apoptosis in C17.2 and SVZ-treated conditions could be attributed to downstream TNFα activation.

Graft rejection as a therapeutic concern

Various sources of transplantable stem cells for CNS therapies have been identified and applied in preclinical models of neurological disease (Lindvall and Kokaia, 2010). These include immortalized NSC lines, primary fetal and adult NSCs, and ES-derived NSCs, all of which produce allogeneic grafts (i.e. the donor is genetically distinct from recipient). A concern raised by allografts is immune activation due to major histocompatibility complex (MHC) mismatch. Graft survival may be increased through immunosuppressive treatment (Pearl et al., 2011). Alternatively, iPSC technology may circumvent the need for immunosuppression and alleviate concerns related to graft rejection (Guha et al., 2013). Conflicting data suggest that the immunogenicity of iPSCs in a syngeneic host depends on the specific cell types generated (Zhao et al., 2011; Araki et al., 2013). T-cell recognition of donor antigens is classically thought to mediate graft rejection (Pluchino and Cossetti, 2013). However, innate immune processes can also play a prominent role in graft rejection. Recent evidence suggests that resident microglia can eliminate allogeneic targets (Chen et al., 2011). In addition, there may be circumstances in which host inflammation is not sufficient to induce graft loss (Ideguchi et al., 2008). Here, we report robust and stable engraftment of transplanted C17.2-NSCs despite persistent microglial activation in an immunocompetent host.

Mechanism of microglial-mediated toxicity

We propose that NSCs can regulate microglial activity via cytokine release. Previously, undifferentiated NSCs were found to have a secretory profile that is distinct from that of their differentiated progeny (Mosher et al., 2012). We corroborated these earlier findings by performing a multiplex cytokine analysis on conditioned NSC supernatants. C17.2 cells and primary SVZ-NSCs constitutively express a number of proinflammatory factors (IL-1α, IL-1β, IL-6, MIP-1α, MIP-1β, VEGF, IP-10, and M-CSF) that are known to recruit and activate microglia. Thus, the secretory activity of undifferentiated NSCs is a potential concern for both allogeneic and syngeneic transplants.

Interestingly, we did not observe direct effects of NSC-secreted factors on neuronal survival. Rather, changes to cell viability *in vitro* only occurred in the presence of activated microglia. Furthermore, our *in vivo* data indicate that microglial activation precedes neuronal loss, supporting the idea that effects may be microglial-mediated. Emerging evidence indicates that microglia regulate cell number in the developing cortex (Cunningham et al., 2013) and play a prominent role in synaptic refinement (Kettenmann et al., 2013). Induction of inflammatory processes during cortical development can enhance apoptosis and reduce synaptic connectivity. However, neonatal C17.2 transplantation preserves neuron number up to four weeks, which is consistent with VSD activation profiles at early time points. These data suggest that reductions in circuit excitability and neuronal number within deep cortical layers are not caused by an early defect in neuronal production or synaptic remodeling.

Our results also indicate that TNFα is required for microglial-mediated effects on cell viability. A rise in TNFα is a hallmark of acute and chronic neuroinflammation as well as various neuropathologies including ischemic stroke, Alzheimer and Parkinson diseases, amyotrophic lateral sclerosis, and multiple sclerosis (McCoy and Tansey, 2008). TNFα binds to TNFR1 to exert its neurotoxic effects. Receptor binding displaces a silencer on intracellular death domain (DD) of TNFR1. In its place, an adaptor protein TNFR-associated DD (TRADD) binds, which allows TNFR1 to associate with two DD containing proteins, Fas-associated death domain (FADD) and receptor-interacting protein kinase 1 (RIP1). FADD mediates TNFR1-induced

activation of caspase-8 and/or caspase-10 that leads to apoptosis. RIP1 induces a necrotic program of death via reactive oxygen species. In our experimental system, we observed evidence of elevated apoptosis in the presence of activated microglia.

Though our data support the notion that microglia adopt a neurotoxic phenotype, microglial secretory activity can vary depending on the pathologic context (Hanisch and Kettenmann, 2007). In agreement with this, doxycycline treatment had differential effects on neuronal survival in control and transplanted brains. Microglia in control brains were required for the survival of deep layer neurons, consistent with earlier findings (Ueno et al., 2013). Our results further suggest that microglial support may be necessary even after critical periods of circuit refinement and remodeling. In contrast, doxycycline treatment in transplanted mice restored neuron numbers to untreated control levels. Many reports have surfaced citing that grafted NSCs can release neurotrophic factors that increase cell viability, axonal sprouting, and remyelination in the diseased brain (Kokaia et al., 2012). Microglial deactivation in this context may potentially unmask the trophic effects of undifferentiated NSCs.

Effect of the diseased microenvironment on NSCs

The diseased microenvironment can modify the immunophenotype of transplanted cells and exacerbate their proinflammatory functions. In the healthy brain, allografts are well tolerated due to minimal surface expression of MHCI/II antigens and the costimulatory molecules CD40, CD80, and CD86 (Hori et al., 2003; Imitola et al., 2004). Consistent with these findings, we observed persistent C17.2 engraftment in a healthy, immunocompetent host. However, upon exposure to IFNγ or TNFα, precursor cells upregulate MHC and costimulatory marker expression and are no longer able to evade adaptive immune surveillance (Imitola et al., 2004; Mammolenti, 2004). Proinflammatory cytokines, including IFNγ or TNFα, can also enhance the expression of toll-like receptors (TLR2, TLR4) in NSCs and how these cells respond to TLR2 and TLR4 agonists (Covacu, 2009). In turn, activation of these receptors results in production of additional proinflammatory cytokines. Thus, NSCs may be primed for cytokine production during neuroinflammatory or traumatic conditions. There is also evidence that inflammation can halt the

differentiation of endogenous and transplanted NSCs (Carpentier and Palmer, 2009). NSCs sustained in an undifferentiated state may have prolonged impact on their host environment via cytokine release.

In summary, undifferentiated NSCs can have a profound influence on the host microenvironment through intrinsic secretory mechanisms. Proinflammatory factors released by NSCs were found to increase microglial-mediated neuronal death *in vitro* via canonical signaling that relied on TNFα production. These effects may generalize to the transplantation setting and to other types of NSCs. Collectively, these data suggest that innate immune signaling initiated by transplanted NSCs may occur without antigen recognition, which raises the concern that even undifferentiated syngeneic grafts may lead to inadvertent host cell loss.

IV. Materials and Methods

Cell culture

C17.2 cells. The C17.2 line was derived after v-myc immortalization of progenitor cells isolated from the postnatal mouse cerebellum (Ryder et al., 1990). NSCs were maintained as an adherent monolayer on uncoated 10-cm dishes at 37℃ and 5% CO_2 and passaged at a ratio of 1:10 by trypsinization twice per week. Growth medium contained 83% Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5g/L) and 1mM sodium pyruvate, 10% fetal bovine serum (FBS), 5% horse serum, 1% Glutamax, and 1% penicillin-streptomycin-fungizone (all from Gibco, Grand Island, NY). Lentiviral-mediated labeling of C17.2 cells was performed as described previously to enable reliable graft identification (Watson et al., 2006). A self-inactivating (SIN) lentiviral vector driving constitutive EGFP expression from the human elongation factor 1α promoter (EF1α) was generated using standard triple transfection approach (Watson et al., 2006). C17.2 cells were transduced for 12 h in conditioned medium containing the vector (SIN.EF1 α.EGFP) at multiplicities of infection (MOIs) of approximately 10. Labeled populations were sorted for EGFP on the FACSVantage SE cell sorter (BD Biosciences, San Jose, CA) and expanded *in vitro* following recovery for two additional passages prior to transplantation. EGFP has been used as a reporter gene in a number of other transplantation studies without observable

alteration to donor cell physiology (Englund et al., 2002; Alvarez-Dolado et al., 2006; Koch et al., 2009).

Primary SVZ cells. Primary SVZ cells were obtained from *GFP C57BL/6J* mice at postnatal day 1-2. Tissue was microdissected bilaterally from the subventricular zone of the brain and collected in ice cold growth media containing 98% DMEM/F12 with 1x B27 (Life Technologies, Carlsbad, CA), EGF (20 ng/mL), and bFGF (20 ng/mL). To promote cell survival, Y-27632 dihydrochloride (ROCK inhibitor; 10uM, Sigma, St. Louis, MO) was included in the media at the time of isolation but removed for subsequent passages. Isolated tissue was mechanically dissociated by repeated trituration with a glass pasteur pipette. Neurospheres were maintained in uncoated T75 culture flasks at 37˚C in 5% CO_2 and passaged every 3-4 days by mechanical dissociation.

Primary neurons. Primary cortical neurons were obtained from Sprague-Dawley rat brains at embryonic day 18.5. Cortices were carefully removed and trypsinized in DMEM containing 0.027% trypsin at 4C for 20 min. Cells were resuspended in neuronal growth media containing 97% Neurobasal medium, 1x B27, and 1% Glutamax. Prior to plating, 30mm glass bottom cell culture dishes (MatTek, Ashland, MA) were coated with poly-L lysine (100 ug/mL) and preincubated with neuronal growth media for 1h. Cells were then seeded at a density of 3E5 cells/dish and maintained as an adherent monolayer at 37℃ in 5% CO_2.

Primary microglia. Mixed primary glia were obtained from Long-Evans rats at postnatal day 2-4. Forebrains were carefully removed, the isolated tissue was mechanically dissociated by repeated trituration, and the resulting suspension was passed through a 40 µm mesh cell strainer (BD Biosciences, San Jose, CA). Cells were collected by centrifugation and resuspended in growth medium containing 78% DMEM, 10% FBS, 10% horse serum, 1% Glutamax. 1% penicillin-streptomycin-fungizone and ROCK inhibitor (10uM) were included at the time of isolation to promote cell survival, but both were removed after 24 h. Cells were plated onto T75 plastic culture flasks coated with poly-L lysine (100 µg/mL) and maintained as an adherent monolayer at 37℃ in 5% CO_2.

Co-cultures. Co-cultures were established when both the primary neuronal and primary microglial cultures reached 14 DIV. Microglia were isolated from mixed primary glial cultures by agitation for 30 min at 250 rpm and 37˚C. Primary microglia were then resuspended in neuronal growth media and seeded directly on the neuronal cultures at a density of 6E4 cells/dish. Co-cultures were maintained at 37℃ in 5% CO_2.

Supernatant collection and analysis

C17.2 supernatants were derived from cultures grown in DMEM/F12 with 1% FBS, EGF (20 ng/mL), bFGF (20 ng/mL), and 1% Glutamax. C17.2 supernatants were obtained 48 h after plating and were supplemented with 1x B27 and 1% Glutamax following collection. SVZ supernatants were derived from cultures maintained in growth media containing 98% DMEM/F12 with 1x B27, EGF (20 ng/mL), and bFGF (20 ng/mL). They were collected at passages occurring every 3-4 days and were supplemented with 1% FBS, 1x B27, and 1% Glutamax following collection. All supernatants were stored at -80˚C. A Luminex assay (Millipore, Billerca, MA) was used according to manufacturer instructions for multiplexed quantification of a panel of pro- and anti-inflammatory cytokines present in NSC supernatants.

Stimulation

Neuron only cultures and co-cultures were stimulated at 15 DIV, 24H after establishing the co-cultures. 10% of the growth media was replaced with control media formulations or NSC supernatants. Unconditioned media containing DMEM/F12 with 1% FBS, 1X B27, 1% Glutamax, EGF (20 ng/mL), and bFGF (20 ng/mL) served as a negative control. Lipopolysaccharide (500 ng/mL, Sigma Aldrich, St. Louis, MO) and interferon γ (50 ng/mL, Sigma Aldrich, St. Louis, MO), which have been shown to activate microglia, were added to unconditioned media as a positive control for microglial activation. Peroxynitrite ($ONOO^-$; 20 µM, Cayman Chemical, Ann Arbor, MI), a potent oxidant, was added to cultures that had received unconditioned media 24 h after initial stimulation as a positive control for cell death. Experimental conditions included SVZ or C17.2 supernatants. Preliminary data showed that cultures could not tolerate C17.2 conditioned media,

so C17.2 supernatants were diluted 1:5 and 1:20 in unconditioned media in order to obtain meaningful data in subsequent cell viability assays and mechanistic analyses. For rescue experiments, soluble rat TNFR (200 ng/ml, R&D Systems, Minneapolis, MN) was added directly to cultures immediately prior to the addition of control or NSC conditioned media. Assays for cell viability and microglial activation were performed 48 h after stimulation.

Fixed and live-cell fluorescent staining

To assess cell viability following stimulation, cultures were immunolabeled for markers of cell identity or apoptosis. Cultures were fixed in 4% PFA/PBS for 20 min, permeabilized in 0.3% Triton X-100/PBS for 15 min, and immunoblocked for 1h in 4% goat serum/PBS. Cultures being stained for phosphatidylserine were not permeabilized. Cultures were then incubated overnight at 4˚C with primary antibodies against the following antigens: Iba1 (1:1000, Wako Chemicals USA, Richmond, VA), Tuj1 (1:1000, Neuromics, Edina, MN), and phosphatidylserine (1:500, Cell Signaling, Danvers, MA). Cultures were washed and then incubated in PBS containing appropriate secondary antibodies conjugated to Alexa 594 or 488 at room temperature for 2h (1:1000, Molecular Probes, Grand Island, NY). Cultures were washed four more times, with DAPI (1:1000, Life Technologies, Carlsbad, CA) included in the second wash. Coverslips were mounted in Prolong Gold Antifade Reagent (Life Technologies, Carlsbad, CA).

Cultures were also subjected to live dye imaging to assess necrosis. Cultures were briefly washed with artificial cerebrospinal fluid (ACSF) (Weerakkody et al., 2013) and then incubated for 30 min with propidium iodide (1:1000) and Hoescht 3342 (1:2000) (both from Molecular Probes, Grand Island, NY). Following two more washes with ACSF, coverslips were wetmounted in ACSF and imaged immediately. Image processing for both fixed and live staining was conducted using ImageJ software.

Measurement of soluble factors

The mechanism of microglial-mediated cell death was investigated by measuring levels of two main downstream effectors of activated microglia, nitric oxide (NO) and tumor necrosis

factor alpha (TNFα), in stimulated co-cultures. Media was collected from co-cultures 48 h after stimulation and stored at -80˚C until the assay was conducted. NO concentrations were measured using the Griess Reagent System (Promega, Madison, WI), and TNFα levels were assessed using a rat- TNFα ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer instructions.

Neonatal transplantation

Cells were harvested for transplantation as previously described (Watson et al., 2006). Briefly, cells were trypsinized and washed twice in PBS before final resuspension in PBS to yield a final concentration of 40,000 cells/µl. Only viable cells, determined using trypan blue exclusion, were included in cell counts. During the transplantation procedure, the heads of cryoanesthetized neonatal (P0-2) C57BL/6 mice were transilluminated and approximately 2 µl of cell suspension or PBS was slowly injected into each lateral ventricle with a finely drawn glass micropipette. The angle of injection is such that the needle does not penetrate through the somatosensory cortex, but instead enters through caudal aspect of the brain to minimize tissue damage. Injected pups were warmed up and returned to maternal care after recovery. All procedures were approved by the Institutional Care and Use Committee at the Children's Hospital of Philadelphia.

Microglial inactivation in vivo

To assess the contribution of microglia to neuronal loss *in vivo*, uninjected and C17.2 injected C57BL/6 mice were treated with doxycycline, a pharmacological agent known to inhibit microglial activation. Doxycycline was administered in Dox Diet food (BioServ, Frenchtown, NJ). Treatment either began at 3 weeks post-transplant for a treatment period of 5 weeks or began at 6 weeks post-transplant for a period of 2 weeks. Regardless of the treatment timeline, all animals were sacrificed at 8 weeks of age. All procedures were approved by the Institutional Care and Use Committee at the Children's Hospital of Philadelphia.

NAA spectroscopy

In-vivo 1H MRS was performed on 2 healthy controls and 6 C17.2 engrafted mouse brains. In-vivo 1H MRS was performed on 9.4 T horizontal bore scanner (Varian, Palo Alto, CA, 25 G/cm gradients). A 20 mm inner diameter quadrature birdcage coil (M2M, Cleveland, OH) was used for signal transmit and receive. Multi-slice spin echo T2-weighted images were acquired for planning the voxel. Single voxel ^{1}H-MRS was performed using a PRESS sequence by placing a voxel of 1 mm x 3 mm x 3.5 mm on the cerebral cortex covering both hemispheres of the brain with the following acquisition parameters: TR=3000 ms, TE=12.68 ms (min), number of averages=256, complex point=4096 and spectral width=4000 Hz. Water suppression was performed using VAPOR technique. After completing water suppressed spectrum, an unsuppressed water spectrum was also acquired with 8 number of average to compute metabolite to water ratios. We have also acquired spectrum from the thalamus region of the brain from the engrafted mouse brain.

In-vivo ^{1}H-MRS data was analyzed using a LC-model spectroscopic data analyzing software to measure concentration of the metabolites (NAA and Cho) in the control and engrafted mouse brain. Only the metabolites with <20% SD or CRLB from the LC-Model were used for the data analysis.

Immunohistochemistry

Immunohistochemistry was performed to assess cell viability and neuroinflammation invivo at two, four, and eight weeks post-transplant. Mice were transcardial perfused, brains were removed, and tissue was fixed in 4% paraformaldehyde/PBS. Harvested brains were embedded in 2% agarose and sectioned coronally at 50 µm on a vibratome (Leica VT1000S, Leica, Buffalo Grove, IL). Equivalent sections were chosen from each animal at the level of the hippocampus for further analysis. Free floating sections were post-fixed in 4% PFA/PBS for 20 min, then permeabilized and immunoblocked at room temperature for 1h in PBS containing 2.5% goat or donkey serum and 0.2% Triton X-100. Slices were then incubated overnight at 4C with primary antibodies against the following antigens: NeuN (1:500, Millipore, Billerca, MA), Iba1 (1:1000,

Wako Chemicals USA, Richmond, VA), CD68 (1:200, AbD Serotec, Kidlington, UK), and GFAP (1:1000, Millipore, Billerca, MA). After three washes in PBS, sections were incubated at room temperature for 2h with appropriate secondary antibodies conjugated to Alexa 594 or 488 (1:500, Molecular Probes, Grand Island, NY). All antibodies were diluted in PBS. After several washes, one including DAPI (1:1000, Life Technologies, Carlsbad, CA), slices were mounted in Prolong Gold Antifade Reagent (Life Technologies, Carlsbad, CA) and examined with a confocal scanning-laser microscope. Confocal images from a single optical plane were acquired sequentially with two lasers (argon, 488nm; helium/neon, 543nm) at 40x magnification with optical zoom of 2. Image processing was carried out using ImageJ software. Engraftment was quanitified as %GFP+ area in each injected cortical hemisphere after blind analysis of neuronal count and microglial activation. No signs of microgliosis or microglial activation were observed in mock injected brains.

Statistical analysis

Unpaired two-tailed Student's t-test and One-Way ANOVA followed by Bonferonni post hoc tests were used, where applicable, to determine whether mean differences between groups were different and were considered significant when $P<0.05$. Data are reported as means ± SEM.

CHAPTER 4

Conclusions and Future Directions

I. Modulation of Host Cellular and Circuit Functions by NSCs

The results described in Chapters 2 and 3 indicate that a well-characterized NSC line (C17.2) can exert detrimental effects on host circuit and cellular function. These findings are illustrated in a model in Figure 4.1. We propose that transplanted C17.2 cells constitutively secrete several cytokines and chemokines implicated in the activation and recruitment of microglia. These proinflammatory factors induce canonical NFκB or p38 MAPK signaling, resulting in the production of TNFα. TNFα subsequently stimulates caspase activation in neurons and induces exposure of phosphatidylserine as these cells undergo apoptosis. Neuronal death can further trigger reactive microgliosis, which drives progressive neurotoxicity. The loss of endogenous neurons via microglial phagocytosis impairs neural network function.

Consistent with this proposed mechanism, C17.2 conditioned supernatants enhanced microglial-mediated cytoxicity *in vitro*, an effect that could be blocked with sTNFR1 pretreatment. Interestingly, these effects generalized to primary SVZ-NSCs. Thus, it is possible that undifferentiated NSCs, independent of origin, possess immunomodulatory properties. Transplanted C17.2 cells similarly influenced host inflammatory functions. Evidence of microgliosis was found in the engrafted brain by four weeks. Microglial activation preceded neuronal loss *in vivo*, which was not detected until eight weeks post-transplant. Furthermore, treatment with doxycycline, which suppresses microglial activation, was neuroprotective.

There was a linear correlation between engraftment level, host cell loss, and diminished cortical excitability. Host networks tolerated quantities of ectopic C17.2 cells equivalent to those previously achieved with primary, ESC, or iPSC derived transplants, but network activity was particularly susceptible to very high levels of engraftment. These levels altered gross measures of network function, including the amplitude, spatial extent, and velocity of propagating excitatory potentials. Although columnar activity was preserved, the lateral spread of activity was diminished in superficial cortical layers. We attribute these global changes to preferential engraftment and neuronal depletion in deep cortical layers. Previous reports indicate that interactions between supragranular and infragranular layers are critical for the propagation of cortical activity (Wester

and Contreras, 2012). Thus, a substantial layer-specific loss of endogenous neurons was sufficient to induce widespread functional alterations.

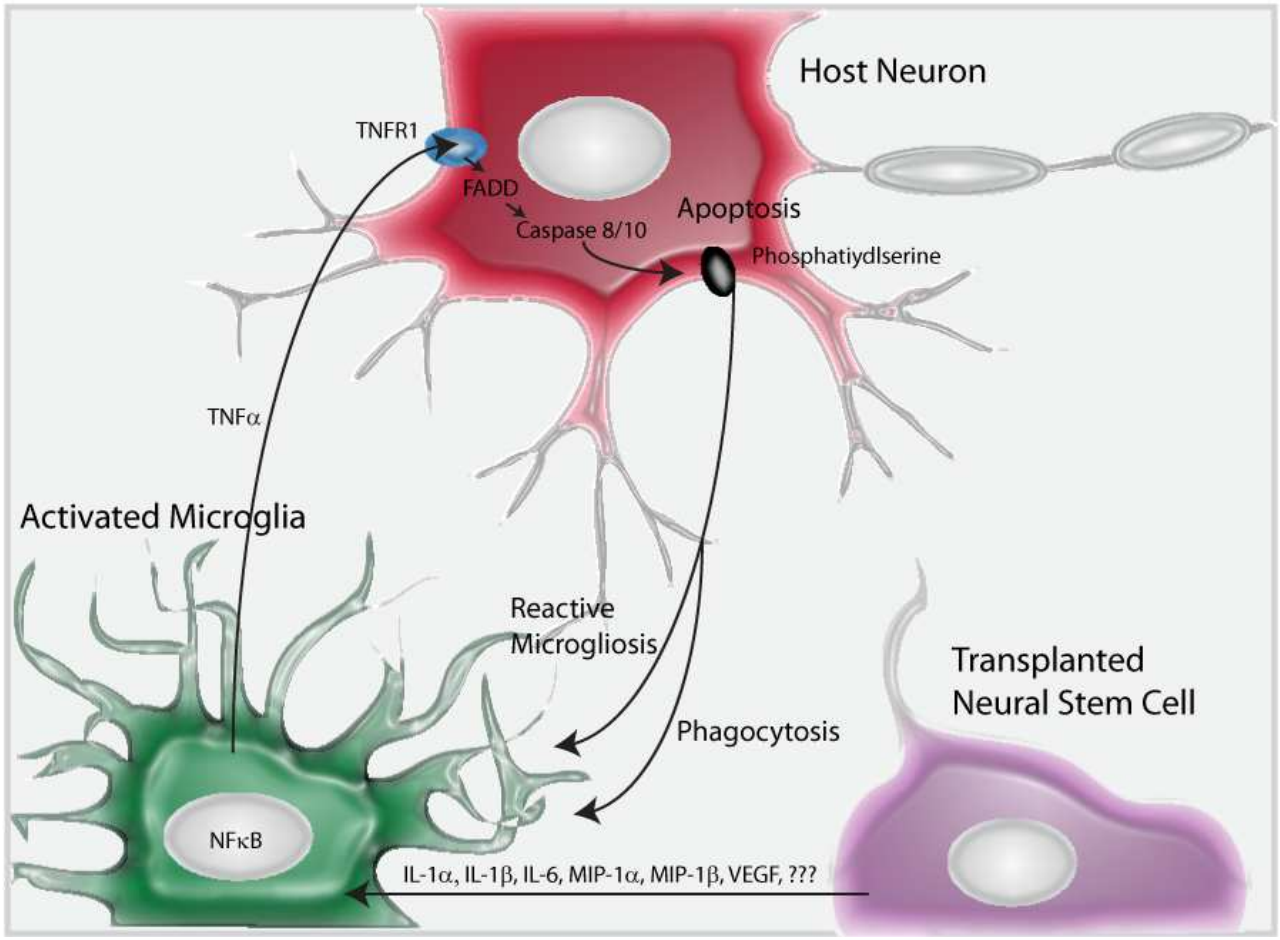

Figure 4.1. Hypothetical Mechanism Driving NSC-Induced Neuronal Loss in the Transplanted Brain

Figure 4.1. Hypothetical Mechanism Driving NSC-Induced Neuronal Loss in the Transplanted Brain. A model depicts the theoretical mechanism underlying the depletion of endogenous neurons following transplantation of the C17.2 NSC line. Transplanted NSCs secrete a number of potent immunomodulatory factors that activate canonical proinflammatory (NFκB and P38 MAPK) signaling in resident microglia. Microglial activation leads to production of tumor necrosis factor alpha (TNFα). TNFα binds TNFR1 which triggers recruitment of Fas-Associated protein with Death Domain (FADD) and subsequent activation of caspase 8 and 10 apoptotic pathways. Apoptosis induces phosphatidylserine exposure, which serves signal to initiate microglial phagocytosis and reactive microgliosis.

II. Implications for NSC Therapy in the CNS

These findings have direct implications for the clinical use of NSC transplantation. Our results suggest that inflammatory responses, apart from those already present in the pathological environment, can be induced by undifferentiated NSCs and may contribute to further neurotoxicity. Doxycycline treatment can inhibit microglial activity and consequently, protect the host brain against neuronal loss. Thus, patients may benefit from pharmacological agents that target innate immune mechanisms in addition to traditional immunosuppressive drugs which minimize graft rejection.

The data show that a substantial amount of NSC engraftment is required to severely impair neural function in the host brain. These levels far exceed those previously obtained from primary NSCs (Magnitsky et al., 2008; Neri et al., 2008; Chaubey and Wolfe, 2013). However, subtle changes to network function, specifically to the integration of afferent inputs, were induced by smaller densities of ectopic donor cells. These experiments were performed with undifferentiated NSCs, which exert immunomodulatory actions not demonstrated by differentiated cell types (Mosher et al., 2012). Therefore, it is possible that the host brain can tolerate greater numbers of differentiated cells. Further studies will be needed to increase the efficiency of *in vivo* differentiation to determine if that can reduce the immunomodulatory effect of transplanted cells.

Previous studies have demonstrated that transplanted NSCs can acquire basic functional properties of mature neurons, such as action potential firing and postsynaptic currents (Englund et al., 2002; Koch et al., 2009). However, complete functional integration requires more complex physiological properties, including the ability to adopt and regulate the activity of host circuits. Voltage dye imaging permits the analysis of entire neuronal ensembles, which are the substrate of higher order brain functions (Peterka et al., 2011). Using this method, we identified a number of functional abnormalities introduced by the engraftment of undifferentiated NSCs. Such network-level assessments of host activity should be incorporated into clinical trials using NSC therapy to detect global alterations.

III. Future Directions

The results described in this dissertation are the first to demonstrate the complex interplay between nonintegrating NSCs and endogenous neural networks in the normal brain. The data establish the intrinsic immunomodulatory nature of undifferentiated NSCs and provide useful parameters for their transplantation. Nonetheless, these findings were obtained using a single transplantation paradigm and only begin to elucidate the mechanism underlying network alterations. Additional experiments are needed to further clarify the mechanism for circuit defects and to determine whether results are generalizeable.

The idenfitication of the specific factors responsible for microglial-mediated cytotoxicity remains an experimental goal. We found that C17.2 and SVZ-derived NSCs released a number of cytokines (IL-1α, IL-1β, IL-6, MIP-1α, MIP-1β, and VEGF) implicated in microglial activation, but it is unclear which, if any, of these factors are sufficient to induce neuronal death *in vivo*. NSC-secreted VEGF was previously found to induce microgliosis *in vivo* (Mosher et al., 2012), making it a likely candidate. Cytokines may be immunodepleted from supernatants to investigate their role in microglial activation *in vitro*. However, there are reported disadvantages of immunodepletion columns, including variable depletion efficiency between proteins (Tu et al., 2010) and the accompanying loss of nontargeted proteins (Bellei et al., 2011). Alternatively, small interfering RNAs (siRNAs) may be used for the targeted silencing of cytokine expression *in vitro.* NSCs can be manipulated *ex vivo* with this approach and subsequently transplanted to assess the *in vivo* effect of downregulated cytokine release. We should still consider the possibility that a combination of NSC factors is required to activate microglia signaling, in which case genetic knockdown strategies can be prohibitively difficult.

Future investigation may also eliminate the possibility that microgliosis observed post-transplant is a direct consequence of graft immunogenicity. To address this issue, syngeneic transplants can be performed in an immunocompetent host. Moreover, to verify that the adaptive immune system does not mediate host neuronal depletion, allogeneic transplants can be repeated in immunodeficient mice. In addition, immunolabeling of CD3 antigen, a pan T-cell

marker, can confirm whether peripheral lymphocytes have invaded host tissue. These experiments are planned and the dissertation will be amended if the results are informative.

Further research will be needed to establish a direct causal relationship between neuronal loss and cells of the innate immune system. Our *in vitro* data suggest that activated microglia are sufficient to induce neuronal apoptosis in the presence of NSC-secreted factors. However, we detected both microglial activation and astrogliosis in the engrafted brain. The contribution of microglia to host cell death was tested using doxycycline. Doxycycline is a synthetic antibiotic of the tetracycline inhibitors group, with reported effects on microglial activation (Yrjänheikki et al., 1998)**.** However, it remains unclear whether doxycycline treatment specifically targets microglial function. There is evidence that this agent can also suppress astrogliosis, which would confound the *in vivo* results (Jantzie et al., 2005). A genetically targeted approach for microglial ablation would be preferable and several transgenic lines have been developed (Duffield et al., 2005; Parkhurst et al., 2013). For example, transgenic CD11b.DTR mice have a diphtheria toxin (DT) inducible system that transiently depletes microglia and tissue macrophages (Duffield et al., 2005). The transgene insert contains a fusion of the simian diphtheria toxin receptor (DTR) and green fluorescent protein under the control of the human ITGAM (integrin alpha M) promoter (CD11b). More recently, Cx_3Cr1^{CreER} mice were generated to express tamoxifen-inducible Cre recombinase (CreER) in microglia under the control of the fractalkine receptor (Cx_3Cr1) promoter (Parkhurst et al., 2013). Using $Cx3Cr1^{CreER}$ to drive DTR expression, microglia can be selectively ablated from the brain following DT administration. However, microglial elimination in these models is inefficient and ablation protocols may inadvertently induce microgliosis. It has been shown recently that astrocytes have phagocytic capabilities and can refine connectivity during development (Chung et al., 2013). In addition, the selective induction of astrogliosis can lead to local synaptic perturbations that alter network excitability (Ortinski et al., 2010). Thus, clarifying the contribution of reactive astrocytes to network defects in the transplanted brain is important but the complexities of the interactions appear to preclude simple experimental analyses. The challenge is that astrocytes serve a number of vital

functions in the developing and mature brain, thus even their conditional removal may introduce deleterious effects.

In addition to elucidating the inflammatory mechanisms involved, future investigation should characterize the phenotypes of the lost and remaining host neurons. In our studies, we determined that neurons were depleted from deep layers due to preferential engraftment in these regions. Although circuit excitability was severely dampened, we cannot conclude that excitatory neurons were selectively depleted. A diversity of neuronal subtypes exists in deep layers and it remains unclear if a particular subtype is more susceptible to microglial-mediated cytotoxicity. Immunolabeling with subtype-specific antibodies will reveal any differential loss. Alterations to functional connectivity may be assessed using conventional electrophysiological approaches. We assayed neural networks because they underlie higher order functions. However, cellular approaches may reveal changes to intrinsic and active membrane properties of surviving host neurons. For example, MGE transplantation studies have shown that the normal brain homeostatically regulates synaptic strength and number in response to changes in functional connectivity introduced by ectopic cells (Southwell et al., 2012)

It remains unclear whether our results are generalizeable to the diversity of NSCs available for transplantation, which may vary substantially in differentiation potential. Primary SVZ-NSCs reproduced the cytotoxic effects of C17.2-NSCs *in vitro*. However, we could not evaluate their effect *in vivo* because the graft density was well below that needed to perturb circuits. SVZ-NSCs, along with a panel of other primary NSCs and established lines, may be transplanted to assess their immunomodulatory properties *in vivo*. Microglial activation can also be evaluated in the presence of differentiated NSC progeny to determine whether these effects are unique to undifferentiated NSCs. An inducible expression system may be utilized to precisely regulate the differentiation of grafted NSCs. A significant obstacle to these studies is the limited survival and migration of primary transplants (Magnitsky et al., 2008; Neri et al., 2008; Chaubey and Wolfe, 2013). The sparse engraftment of these donor cell types precludes a circuit-level analysis of their effects. Thus, while the levels of engraftment achieved in these contexts may be

sufficient to induce a proinflammatory response, it is unclear whether these levels will be great enough to cause significant neuronal depletion and network dysfunction.

In conclusion, while this work provides substantial progress in understanding and predicting the functional effects of NSC transplantation, a great deal of further study is needed to fully elucidate the cellular mechanisms which underlie network-level changes. Until methods are developed to enhance the engraftment of primary, ESC, and IPSC-derived cells to levels achieved with established NSC lines, it will remain difficult to accurately compare their functional impact on the host brain. At the same time, increasing NSC engraftment is important if these cells are to be used therapeutically, presenting a serious conundrum to translational medicine. Nonetheless, by identifying mechanisms that drive NSC-induced alterations in network activity, the results described herein provide the first step toward understanding the complex interactions between ectopic NSCs and host cells, and also establish important transplantation parameters.

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