Injectable Chemokine-Releasing Gelatin Matrices for Enhancing Endogenous Regenerative Responses in the Injured Rat Brain

by

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ABSTRACT

Brain injuries acquired from hemorrhage, ischemic strokes and trauma affect millions worldwide each year and often cause irreversible loss of neural tissue that disrupts vital neurological functions. Cell transplantation has traditionally been the strategy for achieving neuroregeneration but endogenous regenerative responses such as neurogenesis and neovascularization are increasingly recognized as an elegant alternative. These responses are directed toward injured tissues via chemokine signaling such as the stromal cell-derived factor-1α (SDF-1α)/CXCR4 pathway and offer the potential to replace lost neurovascular elements.

Endogenous regenerative responses are, however, not fully effective in the injured brain. Two prominent barriers are the loss of chemokine expression and disappearance of stroma following tissue loss in the brain lesion, which lead to sub-optimal engagement of endogenous regenerative responses and inability of recruited cells to infiltrate the lesion. The overall goal of this thesis was therefore to develop an injectable lesion-filling matrix that could re-establish chemokine release and stroma, thereby enhancing endogenous regenerative responses. Toward this goal, we demonstrated injectable gelatin-hydroxyphenylpropionic acid (Gtn-HPA) hydrogels as an appropriate scaffolding material that was permissive for proliferation, migration and differentiation of adult neural progenitor cells (aNPCs). We also synthesized dextran sulfate/chitosan polyelectrolyte complex nanoparticles (PCN), which could encapsulate SDF-1α efficiently and sustain its release for 4 weeks. When used in an in vitro migration assay to fill a core region that was surrounded by an aNPC-laden hydrogel construct, the resulting Gtn-HPA/SDF-1α-PCN matrix recruited aNPCs to accumulate around and migrate into the core region.

When injected into the brain lesion in a rat model of intracerebral hemorrhage, Gtn-HPA/SDF-1α-PCN matrix successfully increased the migration of endogenous neuronal precursors into the injured striatum and amplified neovascularization. Gtn-HPA/SDF-1α-PCN matrix also led to a newly formed vasculature within the lesion and supported infiltration by endogenous cells that included neutrophils expressing CXCR4 and VEGF. The neutrophil infiltrate did not spread to surrounding tissue or induce necrosis and compelled further investigation for their role in the injured brain. Importantly, Gtn-HPA/SDF-1α-PCN matrix reduced brain tissue loss and improved behavioral recovery. Overall, Gtn-HPA/SDF-1α-PCN matrix offered an opportunity to enhance endogenous regenerative responses and confer benefits to the injured rat brain.

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Chapter 1

Introduction

The adult brain of mammals in general is an organ that is highly sophisticated and specialized to handle bioelectrical processing and control various life-sustaining processes as well as voluntary thoughts, emotions and actions that largely determine the quality of life for the being. Amidst this high degree of sophistication and specialization lies an inherent susceptibility to injury arising from various conditions. High metabolic demand with little or no oxygen/nutrient reserves creates liability for hypoxic or ischemic injury in the event of compromised systemic blood flow or blocked artery. Strong dependence on tightly regulated transport of molecules and cells across the blood-brain-barrier sets up vulnerability to toxicity upon exposure to blood and immune cells in a hemorrhagic event. Lack of substantial mechanical strength further opens room for traumatic injury once excessive forces are transmitted to the brain in the event of a sudden impact or a compromised skull. Given the critical roles of the organ, there is no shortage of hope and ambition to heal the injured brain. Conventional wisdom has, however, pitted against the possibility of brain healing for a very long time. This conventional wisdom is best expressed by Spanish neuroscientist and Nobel laureate, Santiago Ramón y Cajal when he made the famous statement about the central nervous system: “Once the development was ended, the sources 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.”

True to his keen observation, there is still not a single treatment available a century later to reverse the damage inflicted by any form of brain injury. However, the science around the injured brain has grown tremendously since then. Much has been revealed about the molecular and cellular mechanisms driving the pathophysiology of brain injuries. The toolbox available to attempt protection, repair and regeneration in the injured brain has also undergone vast expansion to include tailored molecules, genetic manipulation, instructive biomaterials and multi-potent
stem and progenitor cells. Most importantly, seminal discoveries of endogenous neural stem and progenitor cells in the adult brain have sparked a new paradigm that the regenerative prospect of the injured brain is not as absolutely bleak as the “harsh decree” illustrated by Cajal. Overall, these advancements have given rise to a vast array of experimental therapies and immensely increase the possibility that a fruitful treatment can be materialized for the injured brain.

The key subject of this thesis is about enhancing endogenous regenerative responses in the wake of a brain injury. Compared to other strategies such as neuroprotection or transplantation of stem and progenitor cells, endogenous regenerative responses are much less commonly involved in experimental therapies. Many of the tools that are available in other strategies have also not been attempted to exploit the full benefit of endogenous regenerative responses. This is possibly because the discovery of the endogenous regenerative responses is relatively recent and existing knowledge about them is limited. Also, the scale of endogenous regenerative responses often pales in comparison to the extent of tissue loss and damage after brain injury. However, these current limitations should not be viewed as reasons to disregard the valuable resources that the injured brain is endowed with but as urging challenges to be resolved. Moreover, the notion of promoting the injured brain to heal itself is too appealing to completely disregard endogenous regenerative responses. Even if they prove to be insufficient to ultimately achieve complete healing, they can also be reasonably expected to increase the robustness of other forms of therapies. Simply put, the promise of endogenous regenerative responses is so great and yet, so little has been done to enable the injured brain to fully exploit them.

In this chapter, we provide an overview of several pertinent brain injuries, their pathophysiology and the state of the art in neuroregeneration in the brain. We also highlight the promises and limitations of endogenous regenerative responses in the injured brain. Finally, we outline the specific aspects of endogenous regenerative responses that we aim to target, the design criteria for tools that can achieve our aim and the approach we have taken to develop an appropriate injectable matrix to enhance endogenous regenerative responses in the injured brain.
1.1 Major Forms of Brain Injury

1.1.1 Hemorrhagic Stroke

Each year, stroke inflicts brain injuries on 15 million people worldwide, of whom around 5 million progress to become permanently disabled and another 5 million to death [1]. This makes stroke the leading cause of serious, long-term disability and 3rd leading cause of deaths (close after coronary heart diseases and cancer). Stroke is broadly classified into hemorrhagic and ischemic strokes. Of the these two subtypes, hemorrhagic stroke is associated with significantly higher morbidity and mortality and generally accounts for 8 – 18% of all strokes [2], although the figure can be substantially higher in certain ethnicities [3] and age groups [4]. Hemorrhagic stroke is an event where a blood vessel in the brain ruptures and leads to spontaneous bleeding. Depending on the location of the ruptured vessel and bleeding, hemorrhagic strokes can occur in the form of subarachnoid hemorrhage (SAH) or intracerebral hemorrhage (ICH). SAH describes the bleeding within the meninges. On the other hand, ICH refers to bleeding within the brain parenchyma, which can include the cerebral lobes, basal ganglia (striatum, globus pallidus and subthalamic nucleus), thalamus, cerebellum and the pons.

ICH is typically the more concerning type of hemorrhagic strokes, given that ICH is more common and affects many important structures within the brain. Hypertension is the primary cause of ICH. Chronic hypertension has been known to induce degenerative changes and reduction of compliance of the wall of blood vessels, thereby setting the stage for spontaneous rupture, especially near bifurcation of arteries where microaneurysms are prone to occur. Other causes also involve atypical blood vessels and include brain tumors, amyloid angiopathy, arteriovenous malformations, cavernous angiomas and arteriovenous fistulae. ICH may also occur non-spontaneously as: 1) a secondary injury event during hemorrhagic transformation of ischemic strokes when cerebral blood flow is restored to the affected brain tissues in thrombolytic therapies or; 2) a consequence of mechanical damage during traumatic brain injury.

The key concern in ICH is the mass effect of the hematoma or in other words, the pathological effects due to the hematoma exerting pressure or causing displacements to the surrounding. Management of ICH has therefore been centered on reducing the mass effect or the
hematoma itself. The use of ventricular catheters to drain cerebrospinal fluid and reduce intracranial pressure provides improvements to the neurological status of the affected individual [5]. American Heart Association guidelines also recommend anti-hypertensive medication to control elevated blood pressure. Surgical removal of the hematoma can also be an option, especially in the cases of large lobar hemorrhages (\(\geq 50 \text{ cm}^3\)) in young patients and cerebellar hemorrhages (>3 cm) that are accompanied with neurological deterioration [6]. Although craniotomy is conventionally performed to approach the hematoma, the surgical practice, for the obvious reason of reducing morbidity, appears geared toward the use of minimally invasive stereotactic aspiration of hematoma under the guidance of imaging modalities such as computed tomography [7]. To liquefy the hematoma and facilitate its complete removal, thrombolytic agents such as urokinase can also be infused into the hematoma [8]. Pilot studies on such techniques have revealed a 40% reduction in hematoma volume after surgery and a 40% in death rate [9].

Several pre-clinical models have been developed to facilitate experimental work on ICH. One model consists of inserting a microballoon into the brain and keeping it inflated for a period of time before its eventual removal [10]. This microballoon insertion model can recapitulate the physical mass effect of hematoma in ICH but does not reproduce chemical and biological aspects such as toxicity of hematoma and disruption of blood-brain-barrier. Another model involves the injection of blood into the brain to mimic the bleeding event in ICH. Several blood injection procedures have been attempted over the years and they include: single [11] or multiple [12] injections of isolated blood, use of a catheter to bridge the femoral artery to the brain [13] and allow bleeding into brain and the use of microinfusion pump to assist with the reproducibility of the rate and volume of blood injected [14]. Each of the procedures is an improvement over others in some ways but usually retains some disadvantages such as the inability to create bleeding under arterial pressure or the lack of reproducibility in the hematoma formed. In addition, they generally do not mimic the disruption of blood-brain-barrier, the spontaneity of intraparenchymal bleeding and the expansion of hematoma over time. A third model involves the injection of bacterial collagenase into the brain parenchyma and was discovered during a study to study the effects of proteolytic enzymes on the extracellular matrix in rats [15]. The injection of collagenase disrupts the basal lamina of blood vessels and results in spontaneous extravasation of blood into the surrounding brain tissue. Compared to the blood injection model, this model is
attractive for its simplicity as well as the ability to recapitulate hematoma expansion and continuous tissue loss over time [16]. Concerns have regularly been raised over the potential of the injected collagenase causing neurotoxicity, inflammation and therefore artifacts in the injury produced, but are proven to be unfounded by studies showing that the concentration of collagenase used does not cause neuronal toxicity [17] or inflammation [18].

1.1.2. Ischemic Stroke

Ischemic stroke is the dominant subtype of all strokes and affects as many as 800,000 people in the United States alone [19]. Although not as critically morbid and fatal as ICH, ischemic stroke still poses devastating repercussions on the neurological functions of the affected individual. The event underlying all ischemic strokes is the occlusion of blood vessels supplying the brain. This creates a disruption in the supply of oxygen and nutrients to meet the metabolic demands of the brain and often leads to severe distress or death for the affected brain tissue. Depending on the cause of the vessel occlusion, ischemic strokes can be grouped into thrombotic or embolic strokes. In thrombotic stroke, occlusion is caused by the formation of a thrombus (or blood clot) in the vessel. The formation of blood clots is generally induced by existing pathological states of the vessels such as atherosclerosis, vasoconstriction as well as inflammatory diseases. In embolic stroke, occlusion is caused by an embolus that travels into the brain after originating from somewhere else. The embolus is often a thrombus, especially in patients with cardiovascular complications, but may also be any sizeable bodies such as air, tumor, fat and bacteria clumps [20].

The only FDA-approved treatment for ischemic stroke is the use of tissue plasminogen activator (tPA), which lyses the blood clot occluding blood vessels in the brain and restores blood flow to the brain. Since its approval, only 1–6% of ischemic stroke patients are treated with tPA [21]. Part of the reason lies in the fact that tPA administration is only approved for a tight 3-hour window after the onset of stroke and a 4.5-hour window for a smaller selection of patients meeting stricter requirements. Another part lies in the reluctance of physicians [22], which may arise from the possible risks of tPA. One principal risk is that tPA may exacerbate hemorrhagic transformation, which may arise during reperfusion when blood flow is restored to the damaged
vasculature of the ischemic region. Such hemorrhagic transformation typically increases the morbidity and mortality and is of grave concern to the affected patients.

Several models have been developed to recapitulate the pathophysiology of ischemic stroke. In a model for thrombotic stroke, photochemicals are injected systematically followed by irradiation of the target cortical region through the intact skull [23]. The irradiation leads to platelet aggregation and subsequently, vessel occlusion in the target region but is faulted with prominent vascular injury that is usually not seen in acute ischemic strokes [24]. In another model that serves to mimic embolic stroke, blood clots are injected into the intracarotid arteries [25]. The model is attractive with its high degree of resemblance with actual embolic strokes and permits further investigation on the use of thrombolytic agents to lyse the blood clots. For modeling strokes arising from vasoconstriction, endothelin-1, a natural peptide that potently induces vasoconstriction, is injected into the brain next to a targeted artery [26]. The vasoconstriction causes a decline in blood flow to the territory of the targeted artery, thereby inducing a focal ischemic stroke. While the model is straightforward in terms of surgical procedures, the ischemia may not be sufficiently consistent due to high variability in the vasoconstriction response to endothelial and the return of blood flow after the effect wears off [27]. Among all models, the most widely used one is the intraluminal middle cerebral artery (MCA) occlusion model. The model involves threading a filament into internal carotid artery to occlude the MCA [28]. On top of being relatively simple and non-invasive, it results in a reproducible infarct with sizeable penumbra, enables precise control of the occlusion duration and allows for the investigation of transient and permanent occlusions.

1.1.3. Traumatic Brain Injury (TBI)

As the worldwide leading cause of death and disability for children and adults under the age of 45 years, TBI is another form of brain injury that draws significant attention, especially in sports and the military. TBI is a broad term to describe numerous events where physical disturbances are imposed on the brain. TBI can be classified into: 1) closed injury if the physical damage is incurred without the cranial space being compromised or; 2) penetrating injury if a foreign object physically invades the cranial space. It can also be classified into: A) focal injury if
the damage occurs in a specific location or; B) diffuse injury if the damage spreads over a wide area.

The cause of penetrating injury is usually clear; a foreign object (e.g. knife, bullet and shrapnel) pierces through the skull and into the brain matter. Such an event typically causes compression of the brain tissue as the foreign object competes for the constrained space within the skull, laceration of the brain tissue as well as hemorrhage when the blood vessels are mechanically ruptured. Closed injury can arise when an impact on the head produces contact and/or inertial forces on the brain [29]. Contact forces directly damage the impacted area and often lead to focal injuries. On the other hand, inertial forces result from sudden acceleration (and deceleration) of the head. Translation acceleration typically causes focal injuries (e.g. contusion and hemorrhage) due to differential movement of the brain relative to the skull while angular acceleration can cause either focal injury (e.g. laceration) or diffuse axonal injury due to differential movements of different brain structures.

As with ICH, treatment for TBI is limited to supportive measures. Intracranial pressure is monitored and if necessary, reduced by the placement of a drainage catheter into the ventricles or the use of diuretics to reduce fluid in the system [30]. Seizures may be prevented by the administration of benzodiazepines. Surgery may also be performed to remove foreign matter in the case of penetrating brain injury and to evacuate the hematoma in the event of a hemorrhage.

Preclinical models for the various types of TBI include: 1) fluid percussion model [31]; 2) controlled cortical impact model [32]; 3) weight drop model [33]; 4) penetrating ballistic injury [34] and; 5) shock tube model [35]. In the first three models, a physical impact is delivered, either with a fluid pressure pulse, a rigid impactor or a free falling guided weight, to exposed intact dura to cause a contusion. Of these, the controlled cortical impact model offers better control over the traumatic injury by allowing for easy adjustment of the time, velocity and depth of impact. Penetrating ballistic injury, on the other hand, is modeled with the use of an inflatable probe. To simulate the energy dissipation from a penetrating bullet round, the probe is inserted into the brain parenchyma, inflated and deflated rapidly and eventually withdrawn. Finally, to simulate TBI that typically results from bomb blasts, a compression-driven shock tube is used to replicate
blast waves and direct them to the brain. The model is shown to replicate diffuse axonal injury that is commonly observed with non-contact blast injury.

1.2 Post-Injury Sequelae

Many of the molecular and cellular processes in the complex environment of the brain are tightly regulated in a homeostatic state. In this regard, although the nature of the injury may vary substantially from hemorrhage, ischemia to trauma, the resulting disturbance to the homeostatic state triggers a similar chain of events. Furthermore, each form of injury usually develops to incorporate elements of other forms e.g. ischemia in ICH and hemorrhage in ischemic stroke and trauma. All in all, these cause the post-injury sequelae to share many common features.

1.2.1 Excitotoxicity and Ionic imbalance

With metabolic dysfunction (resulting from ischemia or impaired blood flow in hemorrhage or traumatic injury) and loss of energy stores, neurotransmitters are released while their reuptake is inhibited. As a result, excitatory amino acid neurotransmitters such as glutamate become excessively abundant in the extracellular environment [36]. Glutamate can bind to ionotropic N-methyl-D-aspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptors to induce calcium influx, which in turn activates lipid peroxidases, phospholipases and proteases and triggers the onset of various catabolic processes. It can also bind to: 1) ionotrophic glutamate receptors to mediate sodium influx and cell swelling or; 2) metabotropic glutamate receptors to trigger cell death [37]. Depolarization of excitatory neurons may also trigger the release of zinc, which is neurotoxic and mediates neuronal death [38].

1.2.2 Oxidative Stress

In addition to activating enzymes that drive the degradation of membranes and essential proteins, excessive glutamate stimulation and ionic imbalance can potently increase the production of reactive oxygen species (ROS). Specifically, more nitric oxide is produced with the heightened activity of nitric oxide synthase and more superoxide is generated by NADPH oxide synthase, xanthine oxidase, cyclo-oxygenase and leakage from the mitochondrial electron
transport chain. In the case of ICH, breakdown of heme may also give rise to iron, which catalyzes the Fenton reaction to produce highly reactive hydroxyl radicals [39].

The high production of ROS is poorly handled by the brain where endogenous levels of antioxidant enzymes (such as superoxide dismutase, catalase and glutathione) are low and easily exhausted. Also, the brain contains a high concentration of peroxidizable lipids which can be readily targeted by the ROS [40]. In addition to lipids, ROS also damages a variety of proteins, carbohydrates and nucleic acids. Their effects on DNA have particular serious consequences due to resultant over-activation of poly(ADP-ribose) polymerase-1 (PARP-1), which depletes ATP stores and leads to eventual cell death [41].

1.2.3 Necrosis and Apoptosis

Necrosis occurs when the primary injury (from the initial mechanical or ischemic insult) or the secondary damage is too severe or when ATP is too depleted to allow for apoptosis. In this mode of cell death, the lipid peroxidases, phosolipases and proteases activated through mechanisms mentioned above catalyze the autolysis of membranes and proteins in an unregulated fashion.

In situations where the cells are less severely injured, cell death may occur via apoptosis. The family of caspases plays a central role in mediating apoptosis. In caspase-mediated apoptosis, signaling may commence with members of the TNF family, namely tumor necrosis factor-alpha (TNF-α) and first apoptotic signal (FAS). These cytokines lead to the activation of initiator caspase 8, which in turn activates other caspases or pro-apoptotic factors (e.g. Bcl-2 associated X protein, BH3 interacting-domain death agonist, Bcl homologous antagonist killer, Bcl2 associated death promoter) to begin an amplificatory feedback loop of caspase activation. Following activation, effector caspases 3, 7 and 6 serve the role of proteases to degrade intracellular proteins and pave the way for cell death. Apoptosis may also occur via a caspase-independent pathway where activation of NMDA receptor leads to activation of PARP-1, which in turn results in the release of apoptosis-inducing factor from mitochondria [42].
1.2.4 Inflammatory mechanisms

Under normal physiologic conditions, the brain is largely isolated from systemic inflammatory cells but contains resident macrophages known as microglia which carry out similar functions such as immune surveillance, phagocytosis and productions of various cytokines to drive or regulate inflammation [43]. In the event of an injury, microglia are the first among the inflammatory cells to respond to the damaged tissue. Their production of ROS, pro-inflammatory cytokines and MMP-9, together with the initial insult to the cerebral vasculature and blood-brain-barrier, aids in the elevated expression of leukocyte adhesion molecules (e.g. intercellular adhesion molecule-1, E/P-selection) on the brain endothelial cells and the subsequent influx of circulating leukocytes into the brain [44].

Neutrophils arrive within a few hours from the brain injury and are among the first to infiltrate the brain [45]. They typically accumulate rapidly and may disappear within days via either apoptosis and phagocytosis by microglia/macrophages or active or reverse migration into the circulation [46]. Infiltration and accumulation of neutrophils in the acute setting after injury serve the critical role of clearing cellular debris in the damaged tissue but have also been generally viewed as a principal propagator of secondary damage. The neutrophil-mediated mechanisms underlying the observed secondary damage include the release of proteolytic enzymes such as elastase and MMPs and the production of pro-inflammatory cytokines to drive further cascades of inflammation. Neutrophils have been classically thought to induce bystander tissue damage with their oxidative burst (or rapid release of ROS), although a recent study now shows that brain injury such as stroke results in significant reduction in the oxidative burst of neutrophils, which in turn explains the susceptibility of stroke patients toward infections [47]. Instead of being completely pro-inflammatory and detrimental, neutrophils have now been shown to play diverse roles and can also mediate regenerative processes. In one study, neutrophils are found to mediate VEGF-induced focal angiogenesis [48] while in another, neutrophils are observed to express oncomodulin and essential for retinal ganglion cells to regenerate axons through the injured optic nerve [49].

Activated microglia/macrophages are the predominant cell types after the neutrophilic response. Similar to neutrophils, they serve the role of clearing debris (and neutrophils), which is critical to reduce additional damage and to prepare the environment for any plausible repair
and/or regeneration. They may also produce either secondary damage through the usual mechanisms of ROS, pro-inflammatory cytokines and proteases or neuroprotective effects with their secretion of neurotrophins such as FGF and TGF-β1. Current understanding organizes these opposing roles of microglia/macrophages into a simplified model where microglia/macrophages exist either as pro-inflammatory M1 state or the pro-regeneration/immunoregulatory M2 state [50, 51]. In one study looking at ischemic injury after MCAO, the microglia/macrophages appeared in the injury site mostly in the M2 state, but switched to the M1 state over time [52]. Such polarization dynamics may have implications on the brain injury and be possible intervention targets in the future.

T lymphocytes also infiltrate into the injured brain and contribute to the inflammatory processes. Different subtypes play substantially different roles. For example, CD4+ TH1 cells release pro-inflammatory cytokines and may contribute to the pathogenesis of brain injuries while CD4+ TH2 cells secrete anti-inflammatory cytokines and may aid in the resolution of inflammation [53]. Also, regulatory T cells modulate the activation, infiltration and cytokine production of inflammatory cells and are found to be neuroprotective in acute experimental stroke in mice [54].

1.2.5 Tissue loss: Atrophy and Liquefactive Necrosis

Loss of tissue volume is a salient feature of the injured brain. As mentioned above, numerous catabolic enzymes are produced by the dying neural cells and inflammatory cells and all of them contribute to process of liquefactive necrosis where cells and their associated extracellular matrix (ECM) are degraded and phagocytosed. In the case of diffuse injury and cell death, the affected region of the brain undergoes marked atrophy. In the case of a focal injury, the lesion is typically transformed into a fluid-filled cavity upon completion of the liquefactive process. In a significant departure from other non-CNS tissues, a collagenous scar tissue does not develop in the place the original neural tissue, unless the lesion opens up to the meninges and be invaded by meningeal fibroblasts that deposit a collagen-rich ECM to form a fibrotic scar.
1.2.6 Glial Scar Formation

In response to injury, astrocytes upregulate their expression of glial fibrillary acidic protein, S100 protein, vimentin and the intermediate filament protein plectin, undergo significant hypertrophy and transform into the reactive phenotype [55-58]. Reactive astrocytes amass around the lesion and have their processes stacked against the lesion boundary. The astrocytes then couple to one another via tight and gap junctions to form a barrier around the lesion [59].

The glial scar plays the critical role of sealing the site of injury from the rest of the brain. It does so with scavenging capabilities for glutamate and ions, thereby stemming excitotoxicity at the injury site [60]. It also aids in free radical scavenging and reduces oxidative stress in the environment [61]. On top of these, reactive astrocytes have also been known to produce chondroitin sulfate proteoglycans (CSPG) to create a diffusion barrier and prevent the spread of neurotoxic molecules [62].

1.2.7 Growth Inhibitory Molecules

A plethora of growth inhibitory molecules is expressed or secreted after brain injury. Inhibitory molecules derived from astrocytes include CSPGs [63] and ephrins [64]. Those derived from oligodendrocytes and myelin include Nogo [65], myelin-associated glycoprotein [66], oligodendrocyte myelin glycoprotein [67] as well as ephrin-B3 [68]. In the case of open lesions that are invaded by meningeal fibroblasts, sema IIIA may also be produced. Acting largely through the Rho GTPase signaling pathway that regulates the actin cytoskeleton [69], these inhibitory molecules robustly stall neurite outgrowth and mediate the collapse or repulsion of growth cones. The jarring of axonal regeneration in the injured brain has been mainly attributed to the hostile environment established by these growth inhibitory molecules.
1.3 Neuroregeneration

1.3.1 Goals

The ultimate goal of neuroregeneration is to restore the brain to its original state. Given the enormous complexity of the brain and thus the daunting nature of this goal, a realistic interpretation, based on the current state of the art, is that neuroregeneration aims to restore or initiate the path toward restoration for specific facets of the injured brain. The hope is for the advancement for each and every facet to be put together in a collective strategy to reverse the effects of injury on the brain.

Neuroregeneration can be considered on the axonal or cellular level. At the axonal level, neuroregeneration works on the premise that numerous neurons survive the brain injury but have suffered impairments to their axons. Specifically, affected neurons can lose their projections and synaptic connections if their axons are damaged or severed. In this regard, neuroregeneration strives to restore projections or synaptic connections to the appropriate target neurons. The target neurons can be the original synaptic partners if they have also survived the injury. They can also include new synaptic partners to exploit redundancy in brain circuitry and achieve the same nervous functions with new signal transmission pathways. While most neurons seem to retain the intrinsic ability to extend their axons and form synapses, they generally fail to do so due to the presence of various inhibitory molecules. The key challenge is therefore to derive strategies to overcome the highly inhibitory environment.

At the cellular level, neuroregeneration recognizes the loss of certain indispensable neural cells that have to be replaced in order for the original nervous functions to be restored. The focus here lies predominantly on neurons, which lack the same replicative ability as the two other principal neural cell types astrocytes and microglia. Loss of neurons is therefore deemed irreversible without any intervention. Multipotent stem and progenitor cells have generally been regarded as a promising resource to enable the replacement of loss neurons. However, reproducible in vivo differentiation of the stem and progenitor cells into neurons in general is non-trivial and the difficulty is compounded by the existence of neurons in numerous subtypes. Both axonal and cellular regeneration are inextricably linked. At times, successful projection and
synaptic connections may require replacement of neurons, especially when no or few target neurons remain after a severe brain injury. On the other hand, replacement of neurons will only be meaningful if the new neurons are integrated into the brain circuitry.

Understandably, neuroregeneration is an approach that complements and synergizes with neuroprotection. While neuroprotection aims to minimize the consequences of brain injury by salvaging any non-dying neural cells and tissues, its purpose is usually limited to a relatively narrow time window right after the onset of the brain injury. Also, it does not address irremediable damage. Neuroregeneration therefore complements neuroprotection by serving as an option in the chronic phase of the injury when neuroprotection becomes increasingly ineffective and by addressing the irremediable damage directly for the overall benefit of the injured brain. The synergy between neuroprotection and neuroregeneration arises from the fact that their efficacies are intimately tied to the state of the injured brain. In this regard, any benefit by one approach improves the tissue environment for the other to be more effective. For instance, transplantation of neural stem cells to replace lost cells may lead of suppression of post-injury immune responses, which reduce secondary damage to surrounding neurons and in turn produce a environment that is more conducive for the transplanted cells [70].

1.3.2 Tools: Molecular Agents

The role of molecular agents in neuroregeneration is generally focused on inducing axonal regeneration. Molecular agents can achieve this by enhancing the intrinsic ability of neurons to extend their axons and form synapses. For this, the pathway involving brain derived neurotrophic factor (BDNF)/tropomyosin-related kinase B (Trk B) is often implicated. BDNF has been widely known to induce axonal growth after injury [71]. It has also been found to increase markers of synaptogenesis [72] and result in structural instability in dendrites and spines so as to aid in morphological changes in accordance with synaptic activity [73]. To exploit this pathway, molecular agents, other than taking the form of BDNF itself, can include: 1) pharmaceutical drugs (e.g. niacin) that increase the endogenous expression of BDNF [74]; 2) small molecule agonist of the TrkB receptor [75] and; 3) antibodies that bind to and activate TrKB receptor [76]. Outside the BDNF/TrkB pathway, FGF-2 [77], NGF [78] and neurotrophin-3 [79] have also been used to promote axonal regeneration. In addition to using these molecular
agents one at a time, a combination may be used to achieve synergistic effects. For instance, the combined use of FGF-2, BDNF and NT-3 produced axon growth more than the sum of each neurotrophic factor alone [80].

Axonal regeneration may also be induced if growth inhibitors in the injured environment are overcome. Molecular agents can achieve this by taking the form of enzymes and dismantling the inhibitory signaling. Examples for this include phospholipase C that releases the Nogo receptor from neurons [81] or chondroitinase ABC to remove CSPG from the extracellular matrix [82]. They may also take the form of soluble receptors [83] and antibodies [84] to block the inhibitory signaling.

1.3.3 Tools: Cell transplantation

Given that mature neurons lack the ability to replicate and that the adult brain is only found relatively recently to contain resident neural stem and progenitor cells, neuroregeneration, at the level of cell/tissue reconstitution, has relied heavily on cell transplantation. Over the years, numerous types of stem cells have been isolated, processed and transplanted via intravenous administration or direct injection into the injured brain. They include neurons [85], embryonic stem cells [86], neural stem cells [87], bone marrow-derived mesenchymal stem cells [88], adipose-derived stem cells [89] and umbilical cord blood-derived stem cells [90]. Since the discovery of induced pluripotent stem cells (iPSCs) that can be generated by reprogramming somatic cells, neural stem cells have also been derived from iPSCs and transplanted into the brain [91]. Each type of cells has been shown to provide benefits along the lines of: 1) replacing neurons, astrocytes, oligodendrocytes and endothelial cells; 2) increased angiogenesis; 3) improvements in neurological functions and; 4) regulating post-injury immune responses [70]. However, particular types of cells are expectedly more attractive in terms of their source and lineage restriction. Specifically, iPSCs are immensely appealing given that they can be isolated without donor site morbidity or ethical concerns and that they are autologous cells that do not evoke immune responses upon transplantation. On the other hand, neural stem cells are attractive for having a lineage that is appropriately restricted to the three principal neural cell types found in the brain.
Cell transplantation is a highly promising strategy to induce neuroregeneration, but can be associated with several downsides. For instance, preparations for cell transplantation, which may include expanding the number of cells and differentiating them to the desired phenotype, can be highly costly, laborious and technically challenging. Many cell types also pose the risk of detrimental immune complications after their transplantation. Furthermore, control over the cells is promptly lost after their transplantation. The transplanted cells may differentiate inappropriately into undesirable cell types or fail to survive over time due to their sudden introduction in large numbers into the harsh environment in the post-injury brain tissue [92] and anoikis (dissociation-induced programmed cell death) in the structureless cavitary lesions.

1.3.4 Tools: Biomaterials

Biomaterials are non-viable materials intended to interact with biological systems. One application of biomaterials has been to re-establish structure in injury sites where liquefactive necrosis characteristically leads to cavitary lesions. To date, various scaffolds and hydrogels, derived either from natural polymers such as hyaluronan [93] and gelatin [94], or synthetic polymers such as PLGA [95] and polycaprolactone [96] have been developed for this purpose. Of particular interest is the class of injectable hydrogels that can: 1) be implanted (injected) in a minimally invasive fashion; 2) conform effectively to an irregularly shaped lesion; and 3) undergo safe cross-linking in situ after injection to impart resistance to in vivo degradative processes. Injectable hydrogels, which have been successfully injected to induce regeneration in the injured brain, include peptide nanofiber scaffold [97], xyloglucan [98], matrigel [99] and alginate [100]. These scaffolds and hydrogels have been shown to ameliorate injuries such as distortion of cerebral cortex around the defect as well as support axonal regeneration across the injury gap. However, most of them can be relatively biologically inert such that they are often used with biological factors or cells to improve interactions with the native tissue.

In addition to serving as a physical structure in the injured brain, biomaterials have been particularly useful in steering molecular agents through the complex aspects of the injured brain, ensuring that they preserve their integrity and function as well as meet spatial and temporal requisites. For example, nogo-66 receptor antibody had been conjugated to hyaluronic acid hydrogels that were implanted within both brain ischemic lesions. Through degradation,
the hydrogels helped to gradually release the bioactive antibodies to block inhibitory signaling via the nogo-66 receptor and enhance axonal growth [101].

Biomaterials have also been particularly useful in improving the survival of transplanted cells, presumably by serving as a support to reduce anoikis and protecting the cells from the harsh environment in the injured brain. In a rat model where focal cerebral ischemia led to a cystic cavity in the cerebral cortex, matrigel made the critical difference in allowing transplanted human neural precursor cells to survive 8 weeks after transplantation and mediate a \(~50 - 60\%\) reduction in infarct cavity volume [102]. In another example, VEGF-releasing PLGA microspheres provided physical support for the transplanted neural stem cells and the in growth of blood vessels, ultimately leading to the formation of a de novo tissue within the stroke cavitory lesion [103]. The value of scaffolding biomaterials is also convincingly illustrated in a study where polyglycolic acid scaffolds were implanted into cystic lesions resulting from a unilateral hypoxia-ischemic brain injury [104]. In this instance, only cystic lesions implanted with scaffolds enabled neurons derived from neural stem cells grafted into the lesion to innervate the contralateral hemisphere as well as host neurons from contralateral hemisphere to innervate the lesion. Given these successful applications, it is reasonable to expect biomaterials to become regular features of cell-mediated neuroregeneration.

1.4 Endogenous Regenerative Responses

1.4.1 Neurogenesis

The mammalian brain has long been believed to stop producing any nascent neurons soon after birth until the paradigm was overturned by the discovery that neurogenesis persists into adulthood [105, 106]. The adult mammalian brain is now widely known to contain NSPCs in both the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus. These endogenous NSPCs can: 1) generate neurons throughout life; 2) produce neuroblasts or immature neurons in the SVZ and SGZ that respectively migrate considerable distances into the olfactory bulb (OB) or a short distance into the granule cell layer of the hippocampus; and 3) contribute to new neurons that functionally integrate into local neural
circuitry. The endogenous NSPCs are regulated by their local neurogenic niches which encompass i) cellular components; ii) biological molecules; and iii) extracellular matrix. This dependence on environmental signals allow endogenous NSPCs to behave accordingly when redirected to a different environment (as exemplified by SGZ progenitors which, upon being grafted into SVZ, behave like SVZ progenitors[107]) and even more importantly, respond to perturbations to their local environments.

Since their discovery in the adult brain, endogenous NSPCs have been extensively reported to respond to various forms of brain injury such as hemorrhagic stroke [108], ischemic stroke [109] and traumatic brain injury [110]. Their ability to respond has moreover been observed across many species ranging from rodents [111, 112] to humans [113, 114] and in both the young and the old [115-117]. The responses of endogenous NSPCs proceed as follows. First, brain injury potently increases the proliferation of endogenous NSPCs through various pathways such as FGF [118], Notch [119], Wnt and erythropoietin [120]. Second, it strikingly induces cells (e.g. astrocytes, microglia [121], and endothelial cells [122]) in the injury site to express chemoattractive signals (e.g. SDF-1α [123], angiopoietin 1 [122] and 2 [124], osteopontin [125] and monocyte chemoattractant protein-1 [126]) which direct NSPCs to migrate towards the damaged tissue. Third, the NSCs differentiate into the region-specific types of neurons (e.g. striatal medium-sized spiny neurons [109]) destroyed by the injury, form synapses with neighboring neurons and integrate functionally into the local circuit [127]. Notably, these responses can last for as long as 16 weeks after the injury [121, 125].

These responses undertaken by endogenous NSPCs have not only proven to offer cell replacement for the purpose of neuroregeneration, but have also been associated with improvements in functional behavior after brain injury [128, 129]. This association has further been scrutinized in a transgenic model where endogenous neuronal progenitors derived from NSPCs in the SVZ could be specifically ablated after stroke injury [130]. In this study, the removal of endogenous neuronal progenitors that would typically migrate out of the SVZ into the injured striatum led to both increased lesion size and aggravated behavioral deficits, thus demonstrating the importance of endogenous NSPCs in ameliorating the detrimental effects of brain injury. All in all, the entirety of the endogenous regenerative responses (from proliferation, migration, differentiation to integration into brain circuitry), their persistence as well as their
definitive role in determining the outcomes of brain injury strongly motivate their use in novel therapies to promote neuroregeneration.

1.4.2 Neovascularization

In the aftermath of brain injury, a highly parallel set of events is also known to occur with endothelial progenitor cells (EPCs). In response to brain injury (as well as injury in numerous other tissues), EPCs mobilize from the bone marrow into the circulation and travel into the injury site. The mediators for this mobilization and recruitment of EPCs overlap significantly with those for the post-injury neurogenic response and include SDF-1α, erythropoietin and VEGF [131]. At the same time, elevated expression of pro-angiogenic factors stimulate the proliferation and migration of endothelial cells in existing vessels. The vasculogenesis resulting from recruited EPCs and angiogenesis mediated by newly generated endothelial cells collectively contribute to neovascularization in the injured brain [132].

There are several postulated roles for neovascularization in the injured brain. One plausible role is to increase blood flow and thereby, the delivery of oxygen and nutrients to the compromised brain [133]. Another possible role is to provide for the passage of phagocytic cells and facilitate the removal of debris after brain injury. Recently, there is also an emerging concept that considers neuronal and vascular elements as a neurovascular unit. This has largely been informed by observations where the development of neural tube and embryonic blood vessels is highly integrated [134] and neuronal progenitors migrate in close association with blood vessels [129]. As such, blood vessels may play the essential role as a guiding cellular scaffold as well as a signaling partner in neuroregeneration. It is also highly plausible that neovascularization contributes to functional recovery after brain injury. By far, several reports have indicated that treatments which increase neovascularization improve behavioral outcomes [132].

1.4.3 Tools to Manipulate Endogenous Stem and Progenitor Cells

Endogenous regenerative responses constitute an elegant and appealing resource for neuroregeneration. Unlike non-autologous cells, the endogenous cells involved do not trigger host
immune reactions that may lead to catastrophic consequences in the brain or suppress stem cell differentiation and compromise the efficacy of the treatment. Even when compared to autologous cells or iPSCs, endogenous cells avoid the complications and significant costs typically associated with isolating/purifying/processing cells for transplantation.

However, there exist several limitations that prevent these endogenous regenerative responses from mediating appreciable neuroregeneration after brain injury. First, the endogenous regenerative responses are largely driven by the expression of various cytokines in the damaged tissue. In certain injuries, cytokine expression may be limited in duration [135]. As the damaged tissues die and disappear, cytokine expression is limited in both scale and amount to the surviving tissue in the periphery of the lesion [136]. Overall, these limitations are likely to cause the endogenous regenerative responses to proceed in a sub-optimal fashion. Second, the liquefaction of dying tissue often results in a cavitary lesion and limits the migration of endogenous cells up to the peri-lesion [137]. Third, the number of endogenous NSPCs resulting from injury-induced neurogenesis, albeit substantially higher than basal level, is inadequate relative to the massive loss of neuronal cells after ischemic injury. Lastly, as much as 80 – 90% of the neurons derived from the endogenous NSPCs recruited to the damaged tissue die in the long term [109, 122]. The poor survival has been attributed to caspase-mediated apoptosis [121].

Given the huge potential of the injury-induced endogenous regenerative responses, several tools have been developed to overcome the limitations or stimulate the responses. Molecular agents have successfully been utilized to target the various aspects of endogenous regenerative responses, e.g. 1) increasing proliferation of endogenous NSPCs through the use of TGF-α [138, 139], Notch ligand [140], epidermal growth factor [141]; 2) enhancing the recruitment of NSPCs towards the damaged tissue by SDF-1β [122], angiopoietin [122] and EPO [142]; 3) enhancing neovascularization with SDF-1α [143]; and 3) inhibiting apoptotic death with caspase inhibitors [121]. Biomaterials have also contributed to the effort to manipulate the endogenous cells. The polymer, polyethylene glycol, has been used to facilitate intranasal delivery of TGF-α, enabling the mitogen to increase the pool of endogenous NSPCs via a non-invasive route [144]. Hyaluronan/methyl cellulose hydrogels have also been applied on the cortex to provide sustained delivery of EGF [145] and EPO [146] to stimulate the proliferation of the endogenous NSPCs after stroke injury. Many of the tools above have also been shown to
result in functional improvements. Overall, it is clear that the development of tools to enhance endogenous regenerative responses is a compelling strategy to benefit the injured brain.

1.5 Outline of research

Brain injury typically leads to the liquefaction of affected neural tissue, which in turn results in the loss of chemokine expression and disappearance of stroma within the lesion. These two commonly observed deficiencies limit the robustness of the endogenous regenerative responses and the ability of the involved endogenous cells to migrate into the lesion. The overall goal of this research is to address these two deficiencies and thereby enhance endogenous regenerative responses. Our approach toward this goal is to develop a matrix to fill the lesion and concurrently replaces both elements of chemokine release and stromal support. The overall hypotheses are that such a lesion-filling matrix can: i) influence or enhance neurogenesis and neovascularization in the surrounding brain tissue and; ii) re-establish a physical framework to enable recruited endogenous cells to migrate into the otherwise structureless brain lesion. The design criteria guiding the development of the matrix include: 1) the need for injectability to enable the matrix to be delivered via a minimally invasive route and therefore be applicable to deeply seated brain lesions and; 2) achieving both elements of chemokine release and stromal support by modular design so that each element can be optimized individually.

The first stage of the research involves in vitro evaluation of gelatin hydroxylphenylpropionic acid (Gtn-HPA) hydrogel as a candidate biomaterial to formulate the desired matrix for filling brain lesions. This recently developed biomaterial is selected for investigation because: i) Gtn-HPA is poised to fulfill the criterion for injectability given its ability to transition from a pre-gel solution into a semi-solid hydrogel; 2) Gtn-HPA permits independent tuning of gelation rate and crosslinking degree, a flexibility that allows control of the material properties and the injection procedure without causing constraints to each other; 3) Gtn-HPA offer additional benefit of undergoing covalent crosslinking which may be useful to appropriately resist liquefactive processes in brain lesions. The evaluation takes into consideration of the eventual need for Gtn-HPA hydrogels to provide a permissive environment within the lesion for endogenous cells to mediate neuroregeneration and is geared toward assessing the ability of Gtn-
HPA hydrogels support proliferation, migration and differentiation of adult neural progenitor cells.

In the second stage, we investigate the use of dextran sulfate/chitosan (DS/CS) polyelectrolyte complex nanoparticles (PCN) as independent vehicles to encapsulate and deliver SDF-1α, a chemokine that is widely implicated in driving the endogenous regenerative responses after brain injury. DS/CS PCNs are chosen for their facile preparation and their similarities with heparan sulfate-based growth factor sequestration in natural extracellular matrices. We assess whether the encapsulation and loading efficiencies are in line with reported dosage of SDF-1α for in vivo applications and examine their release kinetics. Both the hydrogel and nanoparticle modules are combined to formulate Gtn-HPA/SDF-1α-PCN matrix. Prior to its in vivo application, we exploit the well-controlled environment of an in vitro three-dimensional migration assay to examine the ability of Gtn-HPA/SDF-1α-PCN matrix to recruit adult neural progenitors, the chemotactic nature of the recruitment as well as the profile of MMPs involved in migration into Gtn-HPA/SDF-1α-PCN matrix.

In the third stage, we conduct a pilot study to explore the stereotactic injection of Gtn-HPA/SDF-1α-PCN matrix into a rat brain lesion resulting from ICH injury, as part of the transition from in vitro experimentation to in vivo application. The collagenase-induced ICH model is chosen for its ease of implementation, the reproducibility of injury and more importantly, for the overall relevance of ICH in the sense that this form of brain injury is clinically managed by stereotactic surgical procedures which can be readily adopted for implanting injectable matrices. We specifically seek to validate the delivery of Gtn-HPA/SDF-1α-PCN matrix into the injured brain via the needle-based injection procedure and the location of the implanted matrix relative to the lesion and surrounding host tissue. We also evaluate Gtn-HPA/SDF-1α-PCN matrices composed to varying wt% Gtn-HPA to identify the optimal formulation for further investigation. Success criteria include persistence of the matrix, maintenance of interface with surrounding host tissue and permissiveness toward infiltration by endogenous cells.

In the final stage, we investigate the use of Gtn-HPA/SDF-1α-PCN matrices as a form of treatment for brain injury in a rat ICH model. Gtn-HPA/SDF-1α-PCN matrices are compared against aCSF as well as Gtn-HPA and Gtn-HPA/PCN matrices. We assess the effects on
behavioral outcome and overall tissue loss, which are two fundamental metrics used to determine the beneficial value of a treatment for brain injury. We specifically seek to verify our hypothesis by examining post-injury neurogenesis and neovascularization as well as the infiltration of matrix-filled lesions by endogenous cells. Alongside, we also examine common injury-induced responses such as gliosis and neutrophil/macrophage/microglia-mediated inflammation. Overall, the study is structured to provide a conclusion on the two overall hypotheses of this thesis and provide a comprehensive picture on the effects of Gtn-HPA/SDF-1α-PCN matrices on the injured brain.
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Chapter 2

Characterization of Injectable Gtn-HPA Hydrogels as Supporting Matrices for Neural Progenitors

2.1 Introduction

Neural stem and progenitor cells (NSPCs) can self-renew or differentiate into the three principal central nervous system (CNS) cell types (neurons, astrocytes and oligodendrocytes). When generated endogenously or transplanted into the brain, they can potentially mitigate tissue loss after injuries such as hemorrhage, ischemic stroke or trauma. However, the environment at injury sites in the brain is highly unfavorable for the introduction of these cells. The progression of a CNS injury dictates the dissolution and clearance of necrotic tissue resulting in a cavitary lesion. Without structural support within the lesion, transplanted NSPCs lack cell-matrix interactions and may suffer from anoikis [1]. Of the cells that survive, most lack a matrix to be spatially retained and organized within the lesion and typically leave the lesion for the surrounding viable host tissue [2]. For similar reasons, endogenous NSPCs migrating towards the lesion usually fail to infiltrate the lesion core [3]. Within the lesions, transplanted or endogenous NSPCs are also exposed to hostile factors [4] such as oxidative stress, hypoxia and inflammatory responses and tend to survive poorly. Collectively, these conditions reduce the potential of NSPCs to mediate meaningful regeneration in the brain lesions.

For these reasons, injectable hydrogels are increasingly important in therapeutic approaches focusing on NSPC-mediated regeneration in cavitary lesions after brain injuries. Injectable hydrogels provide the potential means to reestablish structure in the cavitary lesions and modulate the hostile environment. Their injectable nature enables their introduction with minimal disruption of surrounding tissue and upon gelling in situ, optimal conformation to the
cavity. Notable benefits (e.g. increased survival of transplanted NSCs and reduction in lesion cavity [5]) have also been demonstrated with the use of injectable hydrogels in brain lesions. Injectable hydrogels which crosslink non-covalently upon thermal or ionic stimuli (e.g. Matrigel [5] and self-assembling peptide hydrogels [6]) have attracted much attention over the years due to their cytocompatibility and availability. However, such hydrogels may suffer from potential mechanical instability or premature dissolution and are often passive matrices for the transplanted cells. Therefore, there is growing interest in hydrogels with cytocompatible covalent crosslinking chemistries [7, 8] as well as those with features that can actively influence NSPCs in aspects relevant for neural tissue regeneration [9, 10].

Recently, a class of biopolymers [11-14], including gelatin-hydroxyl-phenylpropionic acid (Gtn-HPA) [15], was developed in order to enable safe in vivo covalent crosslinking via enzyme-mediated oxidative coupling of phenol moieties conjugated along the polymers. During crosslinking, the concentrations of the enzyme (horseradish peroxidase, HRP) and the oxidant (hydrogen peroxide, H₂O₂) independently control the gelation rate and the degree of crosslinking respectively. An injectable formulation of these biopolymers can potentially be fine-tuned to: i) meet procedural requirements; ii) optimize conformity to irregularly-shaped cavitary defects; and iii) produce a matrix which matches the mechanical behavior of surrounding neural tissues. Among them, Gtn-HPA is of particular interest for neural applications given the role of its tunable mechanical stiffness in promoting neurogenic differentiation of mesenchymal stem cells (MSCs) [16].

Towards the ultimate goal of using injectable Gtn-HPA hydrogels in brain injury sites to support endogenous and transplanted NSPCs in brain injuries, the objective of this study was to investigate whether Gtn-HPA hydrogels would serve as appropriate scaffolding materials for adult neural progenitor cells (aNPCs). Specifically, we evaluated cytocompatibility of Gtn-HPA and examined the effects of the hydrogels on the oxidative stress resistance of the aNPCs. We further investigated if Gtn-HPA hydrogels would provide a stromal framework that was permissive for the proliferation, migration and differentiation of aNPCs, given the importance of these processes in tissue regeneration. Finally, we tracked the survival of aNPC-derived neurons and astrocytes within Gtn-HPA hydrogels.
2.2 Materials and Methods

2.2.1 Preparation of Gtn-HPA Hydrogels

As previously described, Gtn-HPA conjugate was synthesized via a general carbodiimide/active ester-mediated coupling reaction [15]. Specifically, 3.32g of HPA (Sigma Aldrich) was dissolved in 250ml of 40 vol% N,N-dimethylformamide (in deionized water), followed by addition of 3.20g of N-hydroxysuccinimide and 3.82g of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The solution was stirred for 5 hr at room temperature. 150ml of 6.25 wt% Gelatin (MW = 80-140 kDa; pI =5; Wako Japan) solution was then added to the solution, which stirred overnight at room temperature. Using dialysis tubes with molecular cut-off of 1 kDa, the solution was dialyzed against 0.1M sodium chloride solution for 2 days, 25 vol% ethanol (in deionized water) for 1 day and deionized water for 1 day. The purified solution was eventually lyophilized and stored at 4°C until use. 90% of the amine groups in gelatin were conjugated with HPA. To prepare Gtn-HPA hydrogels (GH-0.85, GH-1.0, GH-1.2 and GH-1.7), 2 wt% solution of Gtn-HPA, either alone or added with cells at the required density, was sequentially mixed with 0.1 U/ml HRP (Wako USA), unless specified otherwise, and 0.85, 1.0, 1.2 or 1.7 mM H_2O_2 (Sigma) respectively.

2.2.2 Rheological Measurement

Measurements were performed with a TA instruments AR-G2 rheometer using cone and plate geometry of 40mm diameter and 2° angle. For each measurement, 750µl of Gtn-HPA containing 0.03 U/ml HRP and varying concentrations of H_2O_2 was applied to the bottom plate immediately after mixing. The upper cone was lowered to a measurement gap of 54µm. A layer of silicone oil was also applied during the experiment to prevent evaporation. All measurements were taken at 37°C in the oscillation mode with a constant strain of 1% and frequency of 1 Hz. To estimate the gelation rate, the time at which gel point (as defined by the crossover between storage modulus, G’ and loss modulus, G’") occurred was measured.
2.2.3 In vitro Degradation Assay for Gtn-HPA Hydrogels

100μl gels were incubated in 2ml of phosphate-buffered saline (PBS) containing 10U/ml type I collagenase (Invitrogen) and incubated at 37°C on an orbital shaker at 150rpm. Samples were collected at every hour for six hours and analyzed for degradation products from Gtn-HPA hydrogels using the bicinchoninic assay (Thermo Fisher Scientific). Degradation rate constants were derived by fitting the data for mass loss into an inverse exponential model.

2.2.4 Neural Progenitor Cell Culture

aNPCs were kindly provided by the University of California, San Diego and had been isolated from the hippocampi of adult female Fischer 344 rats as previously described [17]. Cells were grown on poly-ornithine/laminin (P/L)-coated flasks in N2 media [DMEM/F12 media with 1% N2 supplement (Invitrogen) and 1% Penicillin-Streptomycin (Sigma)] containing 20ng/ml recombinant human fibroblast growth factor (FGF)-2 (R&D Systems) as a monolayer culture to achieve a high degree of population homogeneity as compared to neurosphere culture [18]. At approximately 80% confluency, cells were subcultured using accutase (Sigma).

2.2.5 Adhesion Assay

Gtn-HPA conjugate and hyaluronic acid-tyramine (HA-Tyr) conjugate were dissolved in PBS and mixed at different ratios to a final concentration of 2wt%. 200μl of the mixture was added into each well in a 24-well plate and crosslinked with 0.03U/ml HRP and 1700pM H2O2 for 30 min. aNPCs were seeded onto hydrogel- or P/L-coated 24-well plates at 75,000/cm² and incubated for 4 hours to allow for cell attachment. Each well was then aspirated and washed once with PBS to remove any unattached cells. Cells attached on hydrogels were then harvested using an enzyme cocktail containing 1000U/ml collagenase and 400U/ml hyaluronidase (Sigma) while those attached on P/L-coated wells were harvested using accutase. The picogreen DNA quantification assay (Invitrogen) was used to determine the number of attached cells.
2.2.6 Evaluation of Viability

aNPCs [(5,000 cells/cm² on P/L-coated 4-well chamber slides or 4×10⁵/ml within 100μl of Gtn-HPA hydrogels in transwell cell culture inserts (6.4mm diameter, 3.0μm pore, polyethylene terephthalate membrane, BD Biosciences)] were incubated with 2μM calcein AM and 4μM ethidium homodimer-1 in N2 media for 1 hour at 37°C, 5% CO₂. They were further incubated with PBS for 30 min at room temperature before imaging with epifluorescence microscope (Olympus BX60). Cells in three randomly selected fields of view under 20x objective lens magnification were counted.

2.2.7 Oxidative Stress Resistance Assay

aNPCs were encapsulated at 4×10⁵/ml within 100μl of 0.1wt% collagen type I (BD Biosciences), 2 wt% alginate (FMC BioPolymer; crosslinked with 25mM CaCl₂) or 2 wt% Gtn-HPA (crosslinked at 1mM H₂O₂). Collagen and alginate were chosen as control hydrogels to encapsulate aNSCs because they are commonly used, well-characterized biomaterials with highly cytocompatible crosslinking [19, 20] that would not pose detrimental effects on the aNPCs prior to the oxidative stress challenge, and could each partially mimic Gtn-HPA in aspects such as mass density and cell adhesiveness. Following 24 hours of culture, aNPCs were exposed to media containing H₂O₂ for 4 hours to induce oxidative stress and measured for survival. The concentration range of H₂O₂ was rationalized at 50 – 500μM to impose sufficient oxidative stress on the aNPCs even in the presence of phenol red in DMEM/F12 media and possible shielding by the three-dimensional matrices around the cells. To investigate the effect of HRP/H₂O₂ crosslinking on the bioreduction capacity of aNPCs, cells encapsulated within Gtn-HPA were either compared directly with cells encapsulated within collagen hydrogels or extracted from Gtn-HPA hydrogels with 500U/ml collagenase type I and plated on P/L surfaces to be compared with control monolayer cultures. To investigate if soluble HPA could prevent the cytotoxic effects of H₂O₂, aNPCs were fed with fresh media containing HPA before the addition of H₂O₂. Survival or bioreduction capacity of aNPCs was measured using the MTS assay (Promega) according to the manufacturer’s instructions. For relevant measurements, background absorbance determined from cell-free hydrogels was subtracted prior to analysis.
2.2.8 Evaluate of proliferation and migration

aNPCs (5000/cm² on P/L-coated tissue cultureware or 1×10⁵/ml within Gtn-HPA hydrogels) were maintained in N2 media containing 10ng/ml FGF-2. At 4 and 7 days post-plating, accutase or 1000U/ml collagenase was added to harvest monolayer and hydrogel-encapsulated aNPCs respectively. The number of cells harvested was quantified using the Picogreen Assay.

2.2.9 Evaluation of differentiation

aNPCs (5,000 cells/cm² onto P/L-coated 4-well chamber slides or 4×10⁵/ml within Gtn-HPA hydrogels in transwell cell culture inserts) were treated with differentiation media for 6 days. The differentiation conditions included: 1) 1μM retinoic acid (RA; Sigma) and 5μM forskolin (For; Sigma) for neuronal differentiation; 2) 50ng/ml leukemia inhibitory factor (LIF; Millipore) and 50ng/ml bone morphogenetic protein (BMP)-4 (Peprotech) for astrocytic differentiation; and 3) 1μM RA and 1% fetal bovine serum (FBS; Invitrogen) for mixed differentiation.

2.2.10 Immunofluorescent Staining

aNPCs in monolayer culture or Gtn-HPA hydrogels were fixed with 4% paraformaldehyde at room temperature for 15 min and 1 hour respectively. The Gtn-HPA hydrogels were further cryoprotected in 25% sucrose solution, flash-frozen in isobutane (-30°C), embedded in Tissue Tek O.C.T compound (Sankura Finetek) and cryosectioned (50μm). All staining procedures were carried out at room temperature unless mentioned otherwise. Samples were blocked and permeabilized with 10% donkey serum (Sigma) and 0.3% Triton X-100 in PBS for 2 hours. Samples were then incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. After washing in PBS/0.3% Triton X-100, samples were incubated with secondary antibodies for 2 hours and eventually with DAPI (1:50,000, Invitrogen) to counterstain cell nuclei. The primary antibodies used were mouse monoclonal anti-β-tubulin III (TuJ1, 1:5000, Covance) to identify neurons and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:2000, Dako) to identify astrocytes. The secondary antibodies used were Dylight 488-conjugated donkey anti-mouse and anti-rabbit IgG (1:500, Jackson ImmunoResearch).
Images were collected using epifluorescence microscope (Olympus BX-60). Controls, in which undifferentiated aNPCs were stained or primary antibodies were replaced with relevant IgG (Dako), resulted in no detectable staining. Cells were counted in three randomly selected fields of view for each well of a chamber slide (monolayer culture) or each of three cryosections (culture within Gtn-HPA hydrogel). Cells with pyknotic nuclei were excluded from counting.

2.2.11 Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using RNeasy Mini Kit (Qiagen, USA) and measured with NanoDrop ND-1000 to determine concentration and purity. RNA was then reverse transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer’s protocol. For quantitative real-time polymerase chain reaction (qRT-PCR), reactions were prepared with 10μl Fast SYBR Green Master Mix, 250nM of each forward and reverse primer, 500ng cDNA and sufficient nuclease-free water to achieve 20μl in total and were performed in duplicates on Applied Biosystems StepOnePlus PCR System. Samples were analyzed for the genes TuJ1 and GFAP with normalization to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression level of each target gene was compared to day 0 controls and the relative fold change was calculated as $2^{\Delta\Delta Ct}$ [21]. Forward and reverse primers for GAPDH, TuJ1 and GFAP were summarized in Table 1.

2.2.12 Statistical Analysis

A sample size of 3 – 6 was used in all experiments. Values are expressed as mean ± standard error mean (SEM). Analyses of variance (ANOVA) and Student’s t-test were used for statistical analysis. Statistical significance was set at p<0.05.
2.3 Results

2.3.1 Rheological Measurements of Gtn-HPA Hydrogels

All Gtn-HPA hydrogels (crosslinked to varying degrees with 0.85, 1.0, 1.2 and 1.7mM H₂O₂ and termed as GH-0.85, GH-1.0, GH-1.2 and GH-1.7 respectively) reached gel point in 40 – 45 seconds when crosslinked with the same HRP concentration of 0.03U/ml. On the other hand, the crosslinking degree, which was controlled by H₂O₂ concentration, significantly influenced the storage moduli of Gtn-HPA hydrogels (one-factor ANOVA, p<0.0001). With increasing crosslinking degrees, G' was found to increase from 449 Pa to 1717 Pa (Figure 2.1).

![Figure 2.1: Storage moduli of Gtn-HPA hydrogels changed significantly with the crosslinking degree controlled by H₂O₂ concentration (n=3, p<0.0001, ANOVA).](image_url)
2.3.2 *In vitro* Degradation Profiles of Gtn-HPA Hydrogels

Varying crosslinking degrees also resulted in concomitant changes in the degradation rates of Gtn-HPA hydrogels. When degraded with type I collagenase, Gtn-HPA hydrogels with higher crosslinking degrees underwent a slower rate of mass loss (Figure 2.2). The effect of crosslinking degree on degradation rate constant was significant (one-factor ANOVA, p<0.01), with the rate constant decreasing from 94 to 38 %/hr over the selected crosslinking degrees (Figure 2.2 insert).

![Figure 2.2: Mass loss for Gtn-HPA hydrogels upon enzymatic degradation by 10U/ml collagenase I (n=3). (Insert) Degradation rate constants were derived from an inverse exponential model and changed inversely with the crosslinking degree (p=0.0003, ANOVA).](image)
2.3.3 Adhesion of Neural Progenitor Cells on Gtn-HPA Hydrogels

Gtn-HPA hydrogels served as adhesive matrices for aNPCs, yielding an attachment of 83.1% that was as high as well-established substratum coatings such as P/L (Figure 2.3). We further examined the efficacy of Gtn-HPA in promoting aNPC adhesion at lower densities by preparing a “titrated” series of 2wt% composite hydrogels with varying weight ratios of Gtn-HPA : HA-Tyr (Hyaluronic acid-Tyramine, a minimally adhesive polymer which could be crosslinked via the same mechanism as Gtn-HPA). Hydrogels comprising of HA-Tyr alone resulted in a low level of aNPC adhesion (6.2%). Proportions of Gtn-HPA at 10% or 20% produced little or no enhancement to aNPC adhesion. However, further increments of Gtn-HPA to 30% and 40% gave rise to steep increases in aNPC adhesion (Fisher’s post-hoc test, p<0.001). Once the proportion of Gtn-HPA in the composite hydrogels was increased to 40% or higher, aNPC adhesion reached the optimal level seen with P/L or 100% Gtn-HPA hydrogels.

![Figure 2.3: aNPC adhesion on 2wt% composite hydrogels with varying ratios of Gtn-HPA : HA-Tyr (n=6).](image-url)
2.3.4 Viability of Neural Progenitor Cells within Gtn-HPA Hydrogels

The viability of aNPCs encapsulated within Gtn-HPA hydrogels was measured to assess the cytocompatibility of Gtn-HPA. Despite exposure to H$_2$O$_2$ during the enzyme-mediated oxidative crosslinking process, the viability of encapsulated aNPCs remained as high as the control cultures that were spared of any hostile handling (~95-97% for cultures on P/L and within GH-0.85, GH-1.0 and GH-1.2). Only GH-1.7 resulted in a slight decrease in cell viability to ~93%.

2.3.5 Oxidative Stress Resistance of Neural Progenitor Cells within Gtn-HPA Hydrogels

The high tolerance of encapsulated aNPCs towards H$_2$O$_2$ used in the crosslinking process prompted further evaluation of their oxidative stress resistance. Using GH-1.0 as representative Gtn-HPA hydrogels, aNPCs were challenged with H$_2$O$_2$ one day after their encapsulation and compared with similar aNPC cultures within collagen and alginate hydrogels. Two-factor ANOVA revealed a significant dependence of metabolic viability on H$_2$O$_2$ concentration (p<0.0001) and more importantly, the type of hydrogel (p<0.0001) (Figure 2.4). Specifically, aNPCs encapsulated within collagen and alginate hydrogels were vulnerable to the oxidative stress induced by H$_2$O$_2$ and suffered massive cell death, as evidenced by MTS assay and

![Figure 2.4: Metabolic viability of aNPCs upon challenge with cytotoxic levels of oxidative stress (n=3). aNPCs encapsulated within Gtn-HPA hydrogels, as represented by GH-1.0, maintained a significantly higher level of viability when compared to those in collagen and alginate hydrogels (** p<0.01, Fisher's post-hoc test)
increased appearance of necrotic cells, especially towards the higher H₂O₂ concentrations. In contrast, the metabolic viability of aNPCs encapsulated within Gtn-HPA hydrogels remained unchanged by H₂O₂ concentrations up to 200µM, with cell death becoming more apparent only at 500µM. The viability of aNPCs within Gtn-HPA hydrogels was therefore higher than those within collagen and alginate at nearly every level of oxidative stress investigated (Fisher’s post-hoc test, p<0.01), particularly at 500µM H₂O₂ where metabolic viability within Gtn-HPA hydrogels persisted at ~84% as opposed to ~8% and ~15% in collagen and alginate hydrogels respectively.

The increased resistance of aNPCs against oxidative stress could stem from either an “extracellular” effect mediated by the presence of protective agents such as free radical scavengers or an “intracellular” effect where NPCs were preconditioned by prior exposure to sub-cytotoxic levels of oxidative stress [22]. Since aNPCs would have been transiently exposed to H₂O₂ during their encapsulation within Gtn-HPA hydrogels, we determined if the higher oxidative stress resistance was due to an altered state in the aNPCs arising from preconditioning. In the MTS assay which examined the dehydrogenase-dependent bioreduction of the tetrazolium salt, aNPCs encapsulated within GH-1.0 (and therefore had been exposed to oxidative crosslinking) exhibited 27.4% higher bioreduction capacity than aNPCs that had been encapsulated within collagen hydrogels and thus not been exposed to HRP/H₂O₂ crosslinking (t-test, p<0.01, Figure 2.5).

Figure 2.5: Bioreduction capacity of aNPCs measured by dehydrogenase-dependent bioreduction of tetrazolium salts when the cells were in either i) Gtn-HPA (+) or collagen hydrogels (-) (n=3); or ii) monolayer culture (n=6). Prior exposure to HRP/H₂O₂ crosslinking led to a significant increase in bioreduction capacity (** p<0.01, *** p<0.001, Student’s t test).
To confirm that the higher bioreductive capacity was not due to differences in the culture microenvironment (i.e., Gtn-HPA versus collagen) during assay, aNPCs within Gtn-HPA hydrogels were also extracted via enzymatic digestion of the hydrogels and plated on P/L to be compared with control cultures. Again, aNPCs with prior exposure to HRP/H2O2 crosslinking displayed 32.4% higher bioreductive capacity (t-test, p<0.0001).

A fair amount of HPA moieties on Gtn-HPA might remain uncoupled after crosslinking and be responsible for protecting encapsulated aNPCs from oxidative stress by scavenging radicals generated from H2O2 or by increasing the activity of cellular enzymes that aids in breaking down H2O2 [23]. We tested this alternative hypothesis by challenging aNPC cultures on P/L with H2O2 in the presence of soluble HPA to determine if the cytotoxicity effects of oxidative stress could be ameliorated. 50μM H2O2 produced a low level of cytotoxicity that decreased aNPC viability to 77%. The presence of soluble HPA, even up to 10-fold in excess of H2O2, failed to produce any recovery in aNPC viability (Figure 2.6). Similarly, soluble Gtn-HPA conjugates, providing approximately ~279μM HPA immobilized on the soluble gelatin polymers, did not significantly reduce the cytotoxic effects arising from 50μM H2O2 (data not shown). It was therefore likely that HPA did not directly mediate the increased oxidative stress resistance observed in aNPCs encapsulated within Gtn-HPA hydrogels. Collectively, the above findings suggested that exposure to the HRP/H2O2 crosslinking of Gtn-HPA could provide a preconditioning effect on aNPCs and increase their oxidative stress resistance.

![Figure 2.6: Effects of soluble HPA on the viability of aNPCs that were challenged with oxidative stress induced by 50μM H2O2 (n=3-5).](image)
2.3.6 Proliferation of Neural Progenitor Cells within Gtn-HPA Hydrogels

aNPCs encapsulated within Gtn-HPA hydrogels increased significantly in number by day 4 and 7 (two-factor ANOVA, p<0.0001) in response to mitogen FGF-2 (Figure 2.7). The proliferation rate within the Gtn-HPA hydrogels was, however, generally lower compared to monolayer culture on P/L (Fisher’s post-hoc test, p<0.0001). The crosslinking degree of Gtn-HPA also exerted a significant influence on the rate of proliferation (two-factor ANOVA, p<0.0001). By day 4, proliferation of aNPCs was evidently in an inverse trend with the crosslinking degree i.e. each higher crosslinking degree resulted in a significantly lower rate of proliferation. By day 7, proliferation within the hydrogel with the highest crosslinking degree (i.e., GH-1.7) continued to be the lowest (Fisher’s post-hoc test, p<0.0001) while those in the other three crosslinking degrees exhibited no significant difference.

![Figure 2.7: Proliferation of aNPCs cultured on P/L surfaces or within Gtn-HPA hydrogels by day 4 and 7 (n=4). aNPCs in Gtn-HPA hydrogels proliferated more slowly as compared to monolayer cultures (p<0.0001, Fisher’s post-hoc test). Among Gtn-HPA hydrogels, higher crosslinking degrees significantly decreased the rate of proliferation (p<0.0001, ANOVA).]

2.3.7 Migration of Neural Progenitor Cells within Gtn-HPA Hydrogels

When cultured in the mixed differentiation condition, aNPCs encapsulated in GH-1.0, GH-1.2 and GH-1.7 were noted to form neurospheres with processes extending outwards (Figure
(aNPCs in GH-0.85 mostly remained as individual cells and were excluded from the following analysis). Immunofluorescence staining for immature neuronal marker TuJ1 revealed that the processes in all hydrogels were TuJ1⁺ and therefore originated from cells of neuronal lineage. DAPI nuclei staining further revealed that the nature of these processes changed as the crosslinking increased. In moderately crosslinked Gtn-HPA hydrogels (i.e., GH-1.0 and GH-1.2), these processes contained multiple nuclei along their length and were likely to be migratory chains of young neurons (Figure 2.8B & C). On the other hand, processes in the heavily crosslinked Gtn-HPA hydrogels (i.e., GH-1.7) did not contain any nucleus and were neurites extending from the neurospheres (Figure 2.8D).

![Figure 2.8](image)

Figure 2.8: Chain migration from neurospheres. (A) Phase contrast of neurospheres formed after 6 days in mixed differentiation condition. Representative immunofluorescence images showing neuronal marker TuJ1 (green) and DAPI nuclei (red pseudocolor) staining in neurospheres within (B) GH-1.0, (C) GH-1.2 and (D) GH-1.7. Bar=50µm.
This was also reflected in our measurements of process length and particularly, process width, which gave a strong indication if entire cells were present in the process. One-factor ANOVA revealed significant effects of crosslinking degree on both process length (p<0.0001) and width (p<0.001) (Figure 2.9 A & B). Between GH-1.0 and GH-1.2, the increment in crosslinking did not affect the process width but caused a reduction in process length (Fisher’s post-hoc test, p<0.05). This agreed with the immunostaining findings that the increment in crosslinking from GH-1.0 to GH-1.2 did not prevent chain migration from occurring but did impede the extent of migration of the immature neurons along these chains. On the other hand, between GH-1.2 and GH-1.7, both process length and width were significantly reduced (Fisher’s post-hoc test, p<0.0001 and p<0.001 respectively). This was also in line with the immunostaining findings that further increment in crosslinking from GH-1.2 to GH-1.7 eliminated chain migration and only permitted the formation of neurites.

Figure 2.9: (A) Width of processes extending from neurospheres (n=4), showing that increases in the crosslinking degree only produced a significant reduction at GH-1.7 (*** p < 0.001, Fisher’s post-hoc test). (B) Length of processes extending from neurospheres (n=4), which decreased significantly at each increment in the crosslinking degree (* p<0.05, *** p<0.001, Fisher’s post-hoc test).
2.3.8 Differentiation of Neural Progenitor Cells within Gtn-HPA Hydrogels

We first examined the influence of Gtn-HPA hydrogels on gene expression for neuronal marker TuJ1 and astrocytic marker GFAP when aNPCs were cultured in various differentiation conditions. Using GH-1.0 as representative Gtn-HPA hydrogels, encapsulated aNPCs were noted to exhibit higher gene expression for the lineage-specific markers when compared to those cultured on P/L [i.e. enhancements in gene expression for i) TuJ1 in neuronal differentiation condition (Figure 2.10A); ii) GFAP in astrocytic differentiation condition (Figure 2.10B); and iii) both markers in mixed differentiation condition (Figure 2.10C)].

We further performed immunofluorescence staining for TuJ1 and GFAP and quantified the proportion of aNPCs that differentiated into neurons and astrocytes. In general, Gtn-HPA hydrogels constituted a permissive environment for aNPCs to differentiate specifically in response to the given stimuli. aNPCs encapsulated in Gtn-HPA hydrogels differentiated mostly into neurons (60\% vs 3\% astrocytes) in neuronal differentiation condition (Figure 2.11A-B), mostly astrocytes (57\% vs 12\% neurons) in astrocytic differentiation condition (Figure 2.11C-D) and a mixture of neurons and astrocytes (43\% and 18\% respectively) in mixed differentiation

Figure 2.10: QRT-PCR measurements of gene expression (fold change from Day 0) for neuronal marker TuJ1 and astrocytic marker GFAP in aNPCs cultured on P/L surfaces and within GH-1.0 (n=3-4) after 6 days in (A) neuronal, (B) astrocytic and (C) mixed differentiation conditions (\** p<0.01, \*** p<0.001, Student's \(t\) test).
Figure 2.11: Immunofluorescence staining for TuJ1 and GFAP after 6 days in (A-B) neuronal, (C-D) astrocytic and (E-F) mixed differentiation conditions. (A, C, E) Representative immunofluorescence images of aNPCs cultured on P/L surfaces and within GH-1.0 (Bar=20µm). (B, D, F) Quantification of aNPCs stained positive for TuJ1 and GFAP (n=3) (** p<0.01, Fisher’s post-hoc test).
condition (Figure 2.11E-F). The proportions of neurons and astrocytes derived from aNPCs resulting from neuronal and astrocytic differentiation conditions were similar between P/L and Gtn-HPA hydrogels. In mixed differentiation condition, aNSCs within Gtn-HPA hydrogels produced 2.0-fold more neurons (Fisher’s post-hoc test, \( p<0.01 \)) and 1.5-fold more astrocytes (Fisher’s post-hoc test, \( p<0.01 \)) than those on P/L. Finally, we determined if the crosslinking degree of Gtn-HPA hydrogels would affect the differentiation of aNPCs. In all differentiation conditions and across all four selected crosslinking degrees, one-factor ANOVA did not reveal a significant effect in the crosslinking degree of Gtn-HPA hydrogels on neuronal or astrocytic differentiation.
2.3.9 Survival of Progenitor-derived Neurons and Astrocytes within Gtn-HPA Hydrogels

In addition to the direct influence on the lineage commitment of aNPCs, we examined if Gtn-HPA hydrogels might exert selective effects over the course of differentiation. We specifically applied neuronal or astrocytic differentiation conditions and tracked the cell number and cell death to determine if Gtn-HPA hydrogels would preferentially promote proliferation or survival in progenitors of a particular lineage. In measuring cell number over the 6 days of culture (Figure 2.12A), we observed significantly smaller increases in cell number in Gtn-HPA hydrogels as compared to P/L in both neuronal and astrocytic differentiation conditions. These resembled the trend for proliferation during expansion culture with FGF-2. Two-factor ANOVA analysis on the increases in cell number within Gtn-HPA hydrogels over 6 days did not demonstrate any significant effect in the type of differentiation condition, suggesting that Gtn-HPA did not favor proliferation of progenitors of one lineage over the other. In terms of cell survival (Figure 2.12B), a modest increase in cell death was observed within Gtn-HPA hydrogels when compared to P/L. Interestingly, two-factor ANOVA analysis on cell death within Gtn-HPA hydrogels further revealed a significant effect in the type of differentiation condition (p<0.0001). While cell death was minimal in neuronal differentiation condition and remained below 10% throughout all 6 days, a slight but significant increase in cell death was observed in astrocytic differentiation condition at day 4 and 6 (t test, p<0.01).

![Figure 2.12: Selective effects of Gtn-HPA hydrogels on aNPC proliferation and survival in neuronal and astrocytic differentiation conditions. (A) Fold increase (relative to day 0) in cell number and (B) cell death over the course of differentiation on P/L surfaces and within GH-1.0 (n=4, ** p<0.01, Student’s t test).](image)
2.4. Discussion

Gtn-HPA hydrogels, with their independent tuning of gelation rate and crosslinking degree, have been shown to be promising biomaterials for delivering and influencing stem cells [16, 24, 25]. Moreover, other data in our lab (not shown) have revealed that the hydrogels display minimal swelling characteristics (a mere 7% increase in the diameter of MSC-laden GH-0.85 hydrogels and an 8% decrease in the diameter of MSC-laden GH-1.7 hydrogels after 7 days of culture) that are appropriate for applications in the CNS where fluctuations in interstitial pressure should be avoided. Our study further revealed several physical and chemical aspects of Gtn-HPA hydrogels that were notable for their potential role as injectable matrices to support endogenous or transplanted aNPCs. First and foremost, the storage modulus of Gtn-HPA hydrogels changed sensitively with the crosslinking degree and could be tuned to levels that were appropriately close to that of neural tissue in the adult brain (400 - 1000Pa [26, 27]). Studies on biomaterial-based implants in a variety of tissue types (e.g. heart [28, 29] and spinal cord [30]) or on the extreme case of silicon-based neural electrodes implanted in the brain [31] have suggested that mechanical mismatch between implant and surrounding tissue could result in strain along the implant-tissue interface, inflammatory responses or even tissue laceration. With the capability to match the storage modulus of surrounding neural tissue in the adult brain, Gtn-HPA can constitute appropriate implants that avoid complications arising from mechanical mismatch.

The enzyme-mediated covalent crosslinking of Gtn-HPA, which involved the use of H₂O₂, was shown to be highly cytocompatible with aNPCs. Our supposition is that such high cytocompatibility could arise from the rapid consumption of H₂O₂ during the oxidative crosslinking process. This might be verified in the future with probes such as dihydroorhodamine or 3′-(p-Aminophenyl) fluorescein [32] to track the concentration of H₂O₂ and free radicals, although the probes, which would consume H₂O₂ to provide fluorescent signals, would need to be employed in an appropriate fashion that would not severely interfere the HRP/ H₂O₂ crosslinking of Gtn-HPA. In addition to the high cytocompatibility, the encapsulated aNSCs unexpectedly displayed a dramatically increased resistance against oxidative stress. In the lesion environment after brain injury, resistance against oxidative stress can be beneficial. During CNS injury, endogenous antioxidant mechanisms, such as superoxide dismutases, dehydrogenases and glutathione peroxidases, are perturbed, leading to insufficient clearance of oxygen radicals [33].
Such excessive oxidative stress has been postulated to contribute to the poor survival of transplanted neural stem and progenitor cells [34]. Two approaches exist to tackle the issue of low cell survival due to oxidative stress. One is to modulate the oxidative environment with antioxidants or free radical scavengers. Biomaterials, which can be functionalized [35] or loaded [36] with these protective agents, generally adopt this approach. The other is to induce the transplanted cells to become more resistant by preconditioning them with sub-cytotoxic levels of oxidative stress [22] or by overexpressing pro-survival signals [37]. Our study using Gtn-HPA is the first to demonstrate the role of a biomaterial and its associated crosslinking chemistry in preconditioning aNPCs and increasing their resistance against oxidative stress to attain better cell survival. Gtn-HPA and other biomaterials utilizing similar enzyme-mediated oxidative coupling may therefore present a one-step approach for preconditioning and transplantation of NSCs as well as a variety of other cell types (e.g. MSCs [38], cardiomyocytes [39] and retinal pigment epithelium cells [40]) that could be preconditioned to achieve increased oxidative stress resistance. Importantly, our findings emphasize the need to look beyond the conventional role of crosslinking and examine additional biological benefits that crosslinking may plausibly provide.

We also found Gtn-HPA to exemplify the strategy of employing cytocompatible crosslinking chemistries on appropriate extracellular matrix (ECM) derivatives such as gelatin to yield an injectable matrix which can crosslink covalently in situ and mimic natural ECM in its interactions with aNPCs. While cell adhesive molecules have to be added to certain biomaterials to promote adhesion of neural cells and prevent anoikis [9, 41], Gtn-HPA is inherently abundant in arginine-glycine-aspartic acid (RGD) sequences and in our study, strongly promoted aNPC adhesion even when diluted to lower densities with minimally adhesive materials. Therefore, it is likely that Gtn-HPA can readily prevent anoikis when used alone or in mixtures with other biomaterials to support aNPCs in CNS injury sites. Likewise, Gtn-HPA inherently carries cleavage sites for matrix metalloproteinases (MMPs) and is amenable to remodeling by cells. In our observations, aNPCs, which are known to express MMPs [42], were able to proliferate as well as to give rise to neuronal cells that migrated into the surrounding Gtn-HPA matrix in chains. Chain migration is a vital mechanism by which neural progenitors from neurogenic niches such as the subventricular zone may deviate from stereotypical migratory routes (e.g. rostral migratory stream towards the olfactory bulb) and instead, home towards brain injury sites [3, 43, 44]. By being permissive for chain migration of aNPCs and their differentiated progeny, Gtn-
HPA hydrogels may be well poised as injectable matrices for use in brain cavitary defects to allow the endogenous migratory mechanisms to proceed in a similar manner as the brain ECM.

In addition to providing a permissive environment for proliferation and migration, Gtn-HPA hydrogels offers control over these cellular behaviors via its tunable crosslinking. Our study showed that while proliferation persisted in all Gtn-HPA hydrogels, those with higher crosslinking degrees were able to decrease the extent of proliferation. Similar effects have previously been reported [20] and attributed to increased mechanical stiffness. Given the degradability of Gtn-HPA, the concomitant increases in the resistance to degradation could have also played a contributing role. The tunable crosslinking of Gtn-HPA hydrogels also influenced the migration of aNPCs. In hydrogels of the lowest crosslinking degree (GH-0.85), it was possible that aNPCs migrated and dispersed efficiently, therefore failing to form neurospheres that our study used as reference points to track aNPC movement. In the other Gtn-HPA hydrogels examined in the study, neurosphere formation was observed and increasing levels of crosslinking were seen to impede and even completely arrest chain migration from the neurospheres. Again, crosslinking of matrices affects both mechanical stiffness and degradation rate, which can in turn regulate cell migration [45] and thus explain the influence of tunable Gtn-HPA hydrogels on the migration of aNPCs.

Gtn-HPA hydrogels influenced aNPCs in several ways over their course of differentiation. Compared to P/L, Gtn-HPA hydrogels enhanced gene expression for TuJ1 and/or GFAP for all the differentiation conditions examined, with the enhancements being specific to the type of differentiation condition. As seen in immunofluorescence staining, Gtn-HPA hydrogels also induced encapsulated aNPCs to produce a higher proportion of neurons and astrocytes in mixed differentiation condition, although such increases were not observed for neuronal and astrocytic differentiation conditions. It appears plausible that in the presence of strong, specific stimuli (e.g., RA/For in neuronal differentiation condition or LIF/BMP-4 in astrocytic differentiation condition), the majority of aNPCs was already directed towards the relevant lineages and was expressing relatively high levels of the relevant genes. Gtn-HPA hydrogels mainly acted to further increase the gene expression for these committed cells, thus explaining why enhanced gene expression did not translate into a higher proportion of neurons or astrocytes. On the other hand, in the presence of mild, mixed stimuli, aNPCs were driven less strongly...
towards a particular lineage commitment. In this context, Gtn-HPA-induced enhancement in gene expression was able to serve as an additional factor to promote lineage commitment in aNPCs and therefore, resulted in higher proportions of neurons and astrocytes.

Regarding the higher efficiencies of differentiation for aNPCs within Gtn-HPA hydrogels in mixed differentiation condition, we had noted that the increase in the resulting proportion of neurons was greater than that for astrocytes. This was consistently observed across all the examined crosslinking degrees, which had been selected in this study to tune Gtn-HPA hydrogels to be soft matrices (449 – 1717 Pa) approximating adult brain tissue. At the same time, we also observed that when compared to P/L-coated polystyrene (~10^9 Pa [46]), Gtn-HPA hydrogels exerted a selective effect where aNPCs driven towards neuronal lineage exhibited a higher survival than those towards astrocytic lineage. These observations on the bias that Gtn-HPA hydrogels had for neuronal cells were in line with similar studies where soft substrates promoted neuronal differentiation [20, 47, 48] or induced poor survival among aNPCs over astrocytic differentiation [49]. Further investigation will be necessary to verify if the bias in Gtn-HPA hydrogels is driven by substrate modulus. Finally, it should be noted that these effects of Gtn-HPA hydrogels on aNPCs over their differentiation might be extended into animal models given the injectable nature of Gtn-HPA. It is of great interest to examine these effects in vivo and more importantly, to exploit them in actively directing NSPCs to mediate brain tissue repair and regeneration.

In conclusion, brain lesions may benefit from implanted matrices to support endogenous or transplanted NSPCs. Towards the aim of using Gtn-HPA hydrogels for such purposes, this work demonstrates that Gtn-HPA, which is an injectable hydrogel that undergoes covalent crosslinking via an enzyme-mediated process, features high cytocompatibility and adhesive support for aNPCs. We also show that beyond the role as a supporting matrix, Gtn-HPA presents an approach to augment the oxidative stress resistance of encapsulated aNPCs for enhanced cell survival. Gtn-HPA hydrogels are capable of modulating proliferation and migration of aNPCs via their tunable crosslinking. Furthermore, Gtn-HPA hydrogels can exert bias for neuronal cells by promoting neuronal differentiation and maintaining a higher level of cell survival over neuronal differentiation.
2.5 References


Chapter 3

Development of Gtn-HPA/SDF-1α–PCN Matrices for Recruitment of Neural Progenitor Cells

3.1 Introduction

With the discovery of endogenous stem cells and the factors influencing their behaviors, tissue regeneration using endogenous stem cells has become a viable approach for tissue engineering. By recruiting endogenous stem cells into compromised tissues and directing them to replace lost cells, provide paracrine signals or modulate the microenvironment, this approach is an attractive alternative to conventional cell transplantation therapies in terms of the potential to avoid exogenous cells and their associated costs/complications. Depending on the persistence of cell generation and homing, endogenous stem cells can also be continuously, albeit gradually, introduced into the compromised tissues. In cases where the blood supply is impaired, this may orchestrate regenerative processes that are more congruent with the physiological provisions than the sudden introduction of cells in large numbers by transplantation [1]. So far, such an approach has been successfully attempted in tissues such as cartilage [2] and muscle [3].

For the adult brain that has long seemed to lack regenerative capacities, evidence has now surfaced to underscore the possibility of tissue regeneration using endogenous stem cells. After brain injury, endogenous neural progenitor cells (NPCs) from stem cell niches in the lateral ventricles [4] and hippocampus [5] as well as from local resident pools found throughout the brain parenchyma [6] become activated. Injured neural tissues release chemotactic cues for recruiting endogenous NPCs [7] and retain an adequately intact stroma where the recruited NPCs reside and generate both glia [8] and region-specific neurons [9]. The newly differentiated neurons may further integrate into existing brain circuitry [10]. Notable advances have been made towards exploiting the endogenous NPCs for repairing/regenerating injured neural tissues. These
include the use of caspase inhibitors to improve the survival of newly formed neurons [11] and mitogens to amplify the number of progenitors [12].

However, making similar advances in cavitary brain lesions has remained highly challenging. Formed after the death and clearance of neural tissues, cavitary brain lesions are characteristically depleted of most cells and stroma and constitute a predominantly structureless environment [13]. As a result, cavitary brain lesions, unlike injured neural tissue, lack an inherent ability to recruit endogenous NPCs, even though the NPCs typically accumulate within close proximity around the cavity [14]. It is therefore imperative to establish chemotactic cues within the cavity to recruit the NPCs as well as fill the cavity with a structural support that is permissive to the migration of recruited NPCs. This will essentially require multi-faceted scaffolding materials that can concurrently fulfill both aspects and recruit NPCs into themselves.

In this study, we develop matrices from gelatin-hydroxylphenylpropionic acid (Gtn-HPA) hydrogels and stromal cell-derived factor-1 alpha (SDF-1α) polyelectrolyte complex nanoparticles (PCNs). Gtn-HPA hydrogels are injectable scaffolds that cater for tunable gelation rate and crosslinking degree [15]. The tunable gelation rate facilitates minimally invasive injection of Gtn-HPA solutions into target locations within the brain prior to their covalent crosslinking in situ to form hydrogels. The tunable crosslinking degree, as shown in our recent study [16], tailors Gtn-HPA hydrogels to match the storage modulus of adult brain tissues and be permissive matrices to the proliferation, migration and differentiation of adult rat hippocampal NPCs (aNPCs). On the other hand, SDF-1α is known to mediate homing of endogenous NPCs to sites of brain injury [7]. The use of PCNs, which can entrap cationic proteins facilely without affecting their bioactivity and serve as biocompatible delivery vehicles for the proteins [17], further offers control over the release of SDF-1α. We hypothesize that by simultaneously providing NPC-compatible structural support and sustained release of SDF-1α, Gtn-HPA/SDF-1α-PCN matrices can recruit NPCs into themselves. These matrices may ultimately be valuable in enabling brain cavitary lesions to mobilize endogenous NPCs. We investigated this in vitro by formulating Gtn-HPA/SDF-1α-PCN matrices as cylindrical cores that interfaced with annular tissue simulant containing aNPCs and their neuronal progeny. We then examined the migration of cells towards and into the matrices as well as the underlying mechanisms.
3.2 Materials and Methods

3.2.1 Preparation of SDF-1α PCNs

Dextran sulfate (DS; M<sub>w</sub> 500kDa; Fisher Scientific, Pittsburgh, PA) was dissolved in deionized water. Chitosan (CS; M<sub>w</sub> 10kDa; Polysciences, Warrington, PA) was dissolved in 0.175% acetic acid and the solution was adjusted to pH 5.5 using 1M NaOH. 5% Trehalose and 15% Mannitol were prepared using deionized water. All solutions were sterilized by filtration prior to use. Lyophilized recombinant rat SDF-1α (Peprotech, Rocky Hill, NJ) was reconstituted in 5% Trehalose and stored at -20°C until use. Preparation of SDF-1α PCNs was performed on a magnetic stirrer placed within the sterile environment of a tissue culture hood. Mixing was done in a sterile cryogenic vial using a micro flea stir bar. To prepare SDF-1α PCNs with a DS:CS:SDF-1α ratio of 1:0.33:0.2, 25μl 0.5mg/ml recombinant rat SDF-1α was mixed sequentially with 25μl 2.5mg/ml DS and 20.8μl 1mg/ml CS for 30 min each. Finally, 35.4μl 15% mannitol (Sigma Aldrich) was added and mixed for 10 min. The PCNs were centrifuged at 15,000g for 20 min at 4°C. The PCNs were washed and centrifuged twice in 5% mannitol prior to lyophilization. To vary DS:CS or DS:SDF-1α ratios, the concentration of SDF-1α or the volume of CS was adjusted accordingly. Blank PCNs were similarly prepared without SDF-1α.

3.2.2 Characterization of SDF-1α PCNs

Mean particle size and polydispersity index (PDI) were determined by dynamic light scattering using a 90Plus/ZetaPALS particle analyzer (Brookhaven Instruments, Holtsville, NY). The amount of encapsulated SDF-1α and DS was determined by analyzing the supernatant from each centrifugation step using bicinchoninic acid (BCA) protein assay (Fisher Scientific) and toluidine blue colorimetric assay (EMD Millipore, Billerica, MA) respectively. Toluidine blue colorimetric assay was performed by mixing 40μl of the collected supernatants with 1ml 35μg/ml toluidine blue (in deionized water) and measuring its absorbance at 595nm. Mass of PCNs was estimated from the mass of encapsulated SDF-1α and DS together with the added mass of CS (assumed to be fully reacted given that DS was in excess relative to CS). Encapsulation efficiency and loading efficiencies were calculated using Equation 3.1 and 3.2.
Encapsulation efficiency (%) = \frac{\text{Mass of SDF-1α encapsulated}}{\text{Total mass of SDF-1α added}} \times 100 \quad \text{Equation 3.1}

Loading efficiency (%) = \frac{\text{Mass of SDF-1α encapsulated}}{\text{Mass of PCNs}} \times 100 \quad \text{Equation 3.2}

To measure release kinetics, approximately 50μg of lyophilized SDF-1α PCNs was suspended in 400μl of a release buffer that comprised of 50% PBS and 0.02% sodium azide. The suspension was transferred to a Protein Lobind tube (Eppendorf, Hauppauge, NY) and incubated at 37°C with shaking at 100 rpm. At each time point, the suspension was centrifuged at 15,000g for 20min at 4°C. Following this, 120μl of supernatant was collected and replaced and the suspension was returned to incubation at 37°C. All collected supernatant was stored in -20°C and analyzed together using BCA protein quantification assay.

3.2.3 Preparation of Gtn-HPA conjugate

Gtn-HPA conjugate was synthesized from gelatin (Wako Pure Chemical Industries, Japan) and HPA (Sigma Aldrich) as previously described in Section 2.2.1.

3.2.4 Neural Progenitor Cell Culture

NPCs were cultured as previously described in Section 2.2.4.

3.2.5 Characterization of Gtn-HPA/PCN Matrices

For all Gtn-HPA hydrogels in this study, 2% Gtn-HPA solution was crosslinked using 0.1U/ml horseradish peroxidase (HRP; Wako Chemical USA, Richmond, VA, USA) and targeted concentrations of H₂O₂ (Sigma Aldrich). To incorporate PCNs within Gtn-HPA hydrogels, PCNs were mixed into Gtn-HPA solutions prior to gelation. To investigate degradation rate, 50μl Gtn-HPA solution incorporating PCNs (0 - 1mg/ml) was crosslinked with HRP and 1mM H₂O₂ in
cylindrical molds and incubated with 10U/ml type IV collagenase (Life Technologies) at 37°C. Samples collected every hr were measured for degradation products from Gtn-HPA/PCN matrices using the BCA assay. The data for mass loss was fitted into an inverse exponential model to determine degradation rate constants as previously described [16]. To investigate cytocompatibility of PCNs, 100μl Gtn-HPA solution containing NPCs (4×10⁵/ml) and PCNs (0 – 1mg/ml) was crosslinked with HRP and 1mM H₂O₂ in 96-well tissue culture plates. After 1 or 3 days, metabolic viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

3.2.6 Three-Dimensional Migration Assay

Assembly of Annular + Core (A+C) Construct

A three-dimensional construct consisting of a NPC-laden annulus and an acellular core was assembled to evaluate the migration of NPCs toward and into the core. To create the core, 236μl Gtn-HPA solution was crosslinked with HRP and 0.95mM H₂O₂ in a cylindrical mold and incubated for 1hr at 37°C. The Gtn-HPA core was transferred to a 24-well tissue culture plate (Figure 3.1A & B). To form the annulus, 141μl of 0.8mg/ml type I collagen solution and 423μl of 0.8mg/ml type I collagen solution containing 10ng/ml FGF-2 and 1×10⁶ NPCs/ml were sequentially added around the core and incubated for 30min and 2hr respectively at 37°C. 1ml neurogenic medium i.e N2 medium containing 1μM retinoic acid and 5μM forskolin (Sigma Aldrich) was finally added, followed by an 80% change every other day.

Figure 3.1: (A) Schematic illustrating the procedure to assemble A+C constructs. (B) Gross appearance of A+C construct in a 24-well plate.
Characterization of cell accumulation at the interface

After 7 days, A+C constructs were fixed overnight with 4% paraformaldehyde at 4°C. The constructs were incubated with 20ng/ml DAPI for 1hr at room temperature to stain for cell nuclei and washed thoroughly with PBS prior to imaging using an epifluorescence microscope (Olympus BX-60). Micrographs were computed into intensity plots using ImageJ software. For each sample, four regions in the annulus, spanning 1mm along the interface and 0.5mm from the interface, were randomly selected from the micrographs. DAPI fluorescence in the selected regions was integrated using ImageJ software and averaged to quantify cell accumulation at the interface.

Characterization of cell migration across the interface

At day 4 and 7, ten fields of view along the annulus/core interface were imaged using a Phase Contrast Microscope (Olympus IX51). For each sample, the total number of migrated cells (i.e., cells that traversed the interface into the core and/or their daughter cells after proliferation) was counted and normalized by the length of interface imaged. To determine migration distance into the core, the perpendicular distance of each cell body from the interface was measured.

Study on effects of SDF-1α PCNs

Gtn-HPA core containing SDF-1α PCNs that equated to 5μg/ml SDF-1α or ~25μg/ml PCNs were compared with control Gtn-HPA cores that were blank or contained soluble SDF-1α or PCNs at matching concentrations. To ascertain the specific involvement of SDF-1α in cell accumulation and migration, the medium bath for the A+C constructs was supplemented with 25μg/ml AMD3100 (Sigma Aldrich). Dose responsiveness was examined with a range of SDF-1α-PCN doses that equated to 0 - 20μg/ml SDF-1α or 0 - 100μg/ml PCNs and with matching doses of soluble SDF-1α or PCNs. To distinguish chemotactic recruitment due to SDF-1α concentration gradients from chemokinetic effects, medium bath for the A+C constructs was supplemented with 1.31μg/ml soluble SDF-1α.

Study on secondary effects of PCNs

DS, CS, low molecular weight DS (DSL; Mw 9 - 20 kDa; Sigma Aldrich) and heparin (Mw 9 - 21kDa; Sigma Aldrich) were either incorporated within the Gtn-HPA core or added to the medium bath. In each experiment, the doses of DS, DSL and heparin were matched by mass.
to the DS component within the PCN group in comparison and the dose of CS to the CS component.

Study on crosslinking degree of Gtn-HPA/SDF-1α-PCN matrix cores

The influence of crosslinking degree on migration of NPCs was examined in the A+C constructs using Gtn-HPA/SDF-1α-PCN matrix cores crosslinked with 0.85, 0.95, 1.05 or 1.20 mM H₂O₂.

Study on MMPs used for cell migration into Gtn-HPA/SDF-1α-PCN matrix cores

For broad-spectrum MMP inhibition, medium bath was supplemented with 10μM GM6001 (EMD Millipore). To inhibit individual MMPs, specific MMP inhibitors with high selectivity were used at a concentration five-fold of their known half maximal inhibitory concentration. The specific MMP inhibitors (and their working concentrations) were: i) MMP-2 inhibitor (60nM; Santa Cruz Biotech, Santa Cruz, CA, USA); ii) MMP-3 inhibitor (29.5nM; Sigma Aldrich); iii) MMP-8 inhibitor I (20nM; EMD Millipore); iv) MMP-9 inhibitor I (25nM; EMD Millipore); and v) MMP-13 inhibitor (40nM; EMD Millipore). Matching volumes of DMSO were added to culture medium for controls.

3.2.7 Three-Dimensional Proliferation Assay

A bilayer construct was assembled to determine how Gtn-HPA matrices of varying compositions influenced the proliferation of NPCs in an overlying collagen matrix. To assemble this construct, 100μl of Gtn-HPA solution was crosslinked with HRP and 0.95mM H₂O₂ in a 48-well tissue culture plate and incubated for 1hr at 37°C. 100μl of 0.8mg/ml type I collagen solution containing 10ng/ml FGF-2 and 1×10⁶ NPCs/ml was then added on top of the Gtn-HPA matrix and incubated for 2hr at 37°C (Figure 3.2). 250μl of neurogenic medium was finally added, followed by an 80% change every other day.
Figure 3.2. Schematic of bilayer constructs

At day 0, 4 or 7, 200μl of the medium was replaced with 1000U/ml type I collagenase to extract the encapsulated cells. The number of cells was determined using the Picogreen DNA quantification assay (Invitrogen). To study the effects of SDF-1α PCNs, Gtn-HPA core containing SDF-1α PCNs that equated to 5μg/ml SDF-1α or ~25μg/ml PCNs were compared with control Gtn-HPA cores that were blank or contained soluble SDF-1α or PCNs at matching concentrations. To study secondary effects of PCNs, DS, CS, low molecular weight DS (DSL; Mw 9 – 20 kDa; Sigma Aldrich) and heparin (Mw 9 – 21kDa; Sigma Aldrich)] were either incorporated within the Gtn-HPA cores or added to the medium bath.

3.2.8 Fluorescence Immunohistochemical Analysis

After 1 or 7 days of culture, A+C constructs were fixed overnight in 4% paraformaldehyde at 4°C. The constructs were further cryoprotected via overnight incubation with 12.5% and 25% sucrose solutions. The constructs were then detached from the culture well, flash-frozen in isobutane (-30°C) and embedded in Tissue Tek O.C.T compound (Sankura Finetek). The embedded constructs were further cut into 50μm cryosections and mounted on Superfrost Plus Slides (Fisher Scientific). All staining procedures were performed at room temperature unless specified otherwise. Sections were blocked and permeabilized with 5% donkey serum/0.3% Triton X-100/PBS for 2hr and then incubated with primary antibodies overnight at 4°C. After washing with 0.3% Triton X-100/PBS, sections were incubated with secondary antibodies for 2hr and eventually with 10ng/ml DAPI (Invitrogen) for 5 min to counterstain cell nuclei. All antibodies were diluted in 5% donkey serum/PBS. The primary and secondary antibodies used are listed in Table 3.1. Sections were subsequently imaged using epifluorescence microscope (Olympus BX-60). Controls performed with relevant IgG (Dako) in place of the primary antibodies resulted in no detectable staining. To quantify the percentage of
cells positive for each marker, three randomly selected fields of views from cryosections of four independent hydrogel constructs were analyzed.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Cells Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Monoclonal IgG1 Anti-Nestin (Millipore MAB353)</td>
<td>1:2500</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Neural Progenitors</td>
</tr>
<tr>
<td>Mouse Monoclonal IgG2a Anti-β-tubulin III (TUJ1; Covance MMS-435P)</td>
<td>1:2000</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Neurons</td>
</tr>
<tr>
<td>Rabbit Polyclonal Anti-Glial Fibrillary Acidic Protein (Dako Z0334)</td>
<td>1:2000</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>Mouse monoclonal IgG1 Anti-CNPase (Abcam ab6319)</td>
<td>1:1000</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Oligodendrocytes</td>
</tr>
<tr>
<td>Rabbit polyclonal Anti-Ki67 (Abcam ab15580)</td>
<td>1:100</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Proliferating cells</td>
</tr>
</tbody>
</table>

Table 3.1 List of Primary and Secondary Antibodies used in Fluorescence Immunohistochemical Analysis

3.2.9 Statistical analysis

A sample size of 3–8 was used in all experiments. Values are expressed as mean ± standard error mean (SEM). One-Factor Analysis of Variance (ANOVA) followed by Fisher’s protected least square difference post-hoc test and Student’s t test were performed accordingly. Statistical significance was set at $p < 0.05$.  

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3.3 Results

3.3.1 Characterization of SDF-1α PCNs

PCNs formulated with DS and CS displayed a mean particle size of \(466 \pm 6\) nm (PDI: \(0.28 \pm 0.03\)). To achieve sustained release of SDF-1α, the chemokine was entrapped within PCNs. Within the range investigated, both the entrapment and loading efficiencies were minimally influenced by the proportion of CS relative to DS but increased significantly with the proportion of SDF-1α relative to DS \((p < 0.001, \text{ Fisher's post-hoc test; Figure 3.3A & B)}\). Overall, high entrapment efficiency of \(75 - 91\%\) and loading efficiency of \(7 - 31\) wt% were achieved. SDF-1α PCNs formulated with the DS:CS:SDF-1α ratio of 1:0.33:0.2 released 44.7% of their SDF-1α load in a sustained linear fashion over the 28-day evaluation period (Figure 3.3C) and were used in all subsequent studies.

![Figure 3.3: (A) Entrapment efficiency and (B) loading efficiency of SDF-1α PCNs formulated using different DS:CS and DS:SDF-1α ratios \((n=3)\). (C) Cumulative SDF-1α release from SDF-1α PCNs formulated with the DS:CS:SDF-1α ratio of 1:0.33:0.2 \((n=3)\). PCNs were incorporated within Gtn-HPA hydrogels at concentrations ranging from 0 – 1mg/ml. *** \(p < 0.001\), Fisher’s post-hoc test.](image-url)
3.3.2 Characterization of Gtn-HPA/PCN matrices

To determine if PCNs posed any interference with hydrogel crosslinking, the degradation profiles of Gtn-HPA/PCN matrices incorporating PCNs at mass percentage ranging from 0 – 5% were examined. Within the entire range, incorporated PCNs did not alter the hydrogels to induce any significant changes in the degradation rate of the hydrogels (Figure 3.4A & B). Incorporated PCNs also posed minimal cytotoxicity to NPCs encapsulated within the same hydrogels, which remained 91% and 97% viable on day 1 and 3 respectively (Figure 3.4C).

![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

Figure 3.4: (A) Cumulative mass loss of Gtn-HPA/PCN hydrogels degraded with 10U/ml type IV collagenase and (B) their associated degradation rate constants derived from an inverse exponential model (n=5). (C) Viability of NPCs encapsulated within Gtn-HPA/PCN matrices relative to those within blank Gtn-HPA matrices (n=6).
3.3.3 Neural Progenitor Cell Recruitment: Accumulation around Gtn-HPA/SDF-1α–PCN matrices

After 7 days in the A+C constructs, cells in the annulus accumulated at the interface with the core. Compared to blank Gtn-HPA cores, Gtn-HPA/SDF-1α-PCN cores significantly increased cell accumulation in terms of the area of increased cellularity (Figure 3.5A) and the number of cells as reflected by DAPI fluorescence integrated along the interface ($p < 0.001$, Fisher’s post-hoc test; Figure 3.5B). In contrast, Gtn-HPA/SDF-1α cores did not influence cell accumulation while Gtn-HPA/PCN cores only produced slight increases. The CXCR4 antagonist AMD3100 blocked the increase between Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN cores but did not affect the slight increase induced by Gtn-HPA/PCN cores.

![Figure 3.5: Cell accumulation along the interface between annulus and core. After 7 days, cell nuclei in the A+C constructs were stained with DAPI. (A) Regions of the annulus directly adjacent to the interface were imaged and converted into intensity plots to visualize distribution and intensity of DAPI fluorescence. Scale bars = 100μm. (B) DAPI intensities from four randomly selected regions spanning 1mm along the interface and 0.5mm from the interface were integrated and averaged for each sample ($n=5$). $^* p < 0.05$ vs. blank and SDF-1α, $^{***} p < 0.001$ vs. others, Fisher’s post-hoc test.]
3.3.4 Neural Progenitor Cell Recruitment: Migration into Gtn-HPA/SDF-1α-PCN matrices

Upon assembly of A+C constructs, cores were completely cell free (Figure 3.6). At day 7, relatively few cells were found with Gtn-HPA, Gtn-HPA/soluble-SDF-1α and Gtn-HPA/PCN cores. In contrast, a significantly higher number of cells had migrated into the Gtn-HPA/SDF-1α-PCN cores. Migration into Gtn-HPA/PCN hydrogels occurred in the form of either individual bipolar cells or chains (Figure 3.7). In the presence of AMD3100, the number of migrated cells was not affected in Gtn-HPA/PCN cores but was substantially decreased that for Gtn-HPA/SDF-1α-PCN cores.

![Image](image_url)

Figure 3.6: All cells were positioned in the annulus at day 0. Varying numbers of cells migrated into the hydrogel core by day 7. Scale bars = 100μm.

![Image](image_url)

Figure 3.7(A) Cells migrated individually through the Gtn-HPA/SDF-1α-PCN matrix core. Many cells exhibited a distinct bipolar morphology that was reflective of leading and trailing processes. (B) Cells were also observed to participate in chain migration where cells putatively migrated one following the other (20) or along one another (7). Scale bars = 50μm.
Quantification of the cells that migrated into the cores revealed that only \( \sim 1 \) and \( \sim 8 \) migrated cells per cm interface were observed within blank Gtn-HPA cores on days 4 and 7 respectively (Figure 3.8). The number of migrated cells in Gtn-HPA/SDF-1\( \alpha \) cores reached \( \sim 3 \) and \( \sim 14 \) cells per cm interface on days 4 and 7 respectively but was not significantly higher than that in blank Gtn-HPA cores. Despite the absence of SDF-1\( \alpha \), Gtn-HPA/PCN cores significantly increased the number of migrated cells by \( \sim 40 \)-fold and \( \sim 16 \)-fold over the blank Gtn-HPA cores on days 4 and 7 respectively (\( p < 0.05 \), Fisher’s post-hoc test). Gtn-HPA/SDF-1\( \alpha \)-PCN cores achieved the highest number of migrated cells which amounted to a \( \sim 81 \)-fold and \( \sim 45 \)-fold increase over the blank Gtn-HPA cores on days 4 and 7 respectively (\( p < 0.001 \), Fisher’s post-hoc test) and surpassed even the combined number from both Gtn-HPA/SDF-1\( \alpha \) and Gtn-HPA/PCN cores (\( p < 0.01 \), Student’s t test). Similar to its effects on cell accumulation, AMD3100 obliterated the increase between Gtn-HPA/PCN and Gtn-HPA/SDF-1\( \alpha \)-PCN cores but failed to completely reduce the number of migrated cells to the level seen with blank Gtn-HPA cores.

![Figure 3.8. Number of migrated cells in the core by day 4 and 7, summed over ten fields of views and normalized to the length of interface imaged (n=5).](image)

Figure 3.8. Number of migrated cells in the core by day 4 and 7, summed over ten fields of views and normalized to the length of interface imaged (n=5). ○ \( p < 0.05 \) vs. blank and SDF-1\( \alpha \) at the same time point, ◆ \( p < 0.001 \) vs. others at the same time point

Measurement of the distance each cell migrated into the hydrogel core on day 7 further revealed that the migrated cells were distributed furthest into Gtn-HPA/SDF-1\( \alpha \)-PCN cores (Figure 3.9). Cell migration changed significantly with the SDF-1\( \alpha \) PCN dose on both days 4 and
Specifically, cell migration increased significantly with the SDF-1α PCN dose in the range of 1.25 – 5μg/ml SDF-1α but remained relatively unchanged across the higher dose range of 5 – 20μg/ml SDF-1α.

Figure 3.9: Percentage of migrated cells at various distances from the interface on day 7.

Figure 3.10: Number of migrated cells in the core on (A) day 4 and (B) 7 with varying doses of soluble SDF-1α, PCNs and SDF-1α PCNs (n=4). # p < 0.05 vs. SDF-1α and PCN at the same dose, *** p < 0.001 vs. the immediate lower dose of SDF-1α PCNs, Fisher’s post-hoc test.
3.3.5 Chemotactic nature of Neural Progenitor Cell Recruitment by Gtn-HPA/SDF-1α-PCN matrices

To examine whether Gtn-HPA/SDF-1α-PCN matrices had increased cell accumulation and migration by enhancing proliferation, we measured changes in cell number in the bilayer constructs (Figure 3.11). Cell number was found to increase by days 4 and 7 for all groups. At both time points, significantly higher increases in cell number were observed in the Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices as compared to the PCN-free matrices (p < 0.05, Fisher’s post-hoc test). However, no significant differences were observed between Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices, as well as between blank Gtn-HPA and Gtn-HPA/SDF-1α matrices.

![Figure 3.11: Fold increase in cell number by day 4 and 7 (n=6). * p < 0.05, ** p < 0.001, Fisher’s post-hoc test.](image)

To further differentiate between chemokinetic and chemotactic effects, we introduced soluble SDF-1α into the medium bath for the A+C constructs to disrupt chemotactic concentration gradients of SDF-1α while preserving any chemokinetic effect that might be present. In the SDF-1α bath, the number of migrated cells in Gtn-HPA/SDF-1α-PCN cores decreased substantially on both days 4 and 7 (p < 0.01, Student’s t test; Figure 3.12A) to levels
typically seen with Gtn-HPA/PCN cores. A similar trend was also observed with the integrated DAPI fluorescence that was used to quantify cell accumulation at the interface ($p < 0.05$, Student’s $t$ test; Figure 3.12B).

![Graph A](image1)

**Figure 3.12:** Soluble SDF-1α was added into the medium bath to disrupt any SDF-1α concentration gradient arising from SDF-1α PCNs. (A) The number of migrated cells in the core ($n=4$) and (B) cell accumulation as reflected by integrated DAPI intensity along the interface ($n=4$) decreased significantly with the SDF-1α bath, evidencing the chemotactic role of SDF-1α PCNs. * $p < 0.05$, ** $p < 0.01$, Fisher’s post-hoc test.

### 3.3.6 Secondary Effects of Gtn-HPA/ SDF-1α–PCN matrices

Even without SDF-1α, PCNs mediated appreciable increases in cell migration (Figure 3.8) and proliferation (Figure 3.11). To understand this, we first used the bilayer hydrogel constructs to examine if the proliferative effects could be traced to the polyelectrolytes used to prepare PCNs. DS mediated similar increases in proliferation as PCNs while CS did not induce any significant effect relative to blank controls (Figure 3.13A). To determine if DS had achieved this effect through its heparin-like properties, we also evaluated heparin, which reproduced an increase in proliferation similar to DS and PCNs. To test the possibility that the FGF-2 accompanying the aNPCs during their incorporation into the tissue simulant was implicated in these secondary effects of PCNs, we omitted FGF-2 during the assembly of bilayer and A+C hydrogel constructs. In the absence of FGF-2, the proliferative effects of PCNs, DS and heparin disappeared (Figure 3.13B) and the number of migrated cells in Gtn-HPA/PCN core reduced.
drastically by ~81% on day 7 (Figure 3.14). Interestingly, the diminished PCN-mediated migration was expected to decrease the number of migrated cells in Gtn-HPA/SDF-1α-PCN

Figure 3.13: (A) In the presence of FGF-2, PCNs as well as DS and heparin increased proliferation when compared to blank control and CS (n=4). (B) Proliferation was minimal for all groups when FGF-2 was excluded (n=4). ** p < 0.01, *** p < 0.001 vs. control, Fisher’s post-hoc test.
Figure 3.14: Number of migrated cells under the influence of blank and SDF-1α PCNs was in the absence of FGF-2 (n=4). ** p < 0.01, *** p < 0.001 vs. -FGF, Student’s t test.

hydrogels by ~32% but we observed an overall decrease by ~77% instead. (Notwithstanding this, the number of cells recruited by Gtn-HPA/SDF-1α-PCN hydrogels remained 6.6-fold higher than that for Gtn-HPA/SDF-1α hydrogels.)

To verify that the FGF-dependent secondary effects of PCNs on cell migration could be similarly traced to the relevant polyelectrolytes, we incorporated individual polyelectrolytes within the Gtn-HPA cores of A+C constructs assembled with FGF-2 (Figure 3.15). Compared to controls, both CS and DS failed to produce any effect on the number of migrated cells even though DS had previously been observed to increase proliferation (Figure 3.13A). On the other hand, heparin and DSL, which has a low molecular weight akin to heparin, mediated increased number of migrated cells in the hydrogel core. Given the instance of DS where enhanced proliferation did not mediate an increased level of cell migration, we focused on the role of chemokinesis and/or chemotaxis by examining additional A+C hydrogel constructs that contained the individual polyelectrolytes in their medium bath. For both DSL and heparin, increases in the number of migrated cells were similar regardless of the location to which the polyelectrolytes were added. On average, these increases with DSL and heparin reached ~88% and ~60% of that with PCNs by day 4 and 7 respectively.
Figure 3.15: Number of migrated cells in the presence of FGF-2 when DS, CS, DSL and heparin were either loaded into the core or added to the medium bath (n=4). *** p <0.001 vs. control, Fisher’s post-hoc test

3.3.7 Effect of crosslinking degree on migration

Cell migration into Gtn-HPA/SDF-1α-PCN cores was significantly affected by their crosslinking degree (p < 0.001, ANOVA; Fig. 3.16). Relative to the crosslinking degree examined thus far using 0.95mM H₂O₂, a decrease in crosslinking degree to 0.85mM H₂O₂ resulted in a 34% and 65% increase in the number of migrated cells on day 4 and 7 respectively. On the other hand, an increase in crosslinking degree to 1.05mM H₂O₂ sharply decreased the number of migrated cells by 80% and 75% on day 4 and 7 respectively. Very few migrated cells were observed when the crosslinking degree was further increased to 1.20mM H₂O₂.
3.3.8 Profile of Matrix Metalloproteinases Used to Mediate Migration of Neural Progenitor Cells into Gtn-HPA/SDF-1α–PCN matrices

We examined the effects of a broad-spectrum MMP inhibitor, GM6001, to establish the role of MMPs in cell migration into Gtn-HPA/SDF-1α–PCN matrices. Compared to controls, the number of migrated cells in the presence of GM6001 was greatly diminished to 5.5% and 6.2% on days 4 and 7 respectively (Figure 3.17). The material nature of Gtn-HPA hydrogels might dictate particular types of MMPs to play more important roles than others during cell migration, prompting further examination with specific MMP inhibitors. For the two inhibitors of gelatinases, MMP-2i reduced cell migration by 41.8% while MMP-9i resulted in an even larger reduction by 54.7% (Figure 3.18). Cell migration also decreased by 30.1% when MMP-3, a type of stromelysins, was inhibited. Between the two inhibitors for collagenases, MMP8i decreased cell migration significantly by 79.6% while MMP13i exhibited no significant effect.
Figure 3.17: The number of migrated cells in the core on day 4 and 7 in relation to the presence of broad-spectrum MMP inhibitor GM6001 (n=6). *** P<0.001 vs. control, Student’s t test

Figure 3.18: Level of cell migration at day 7, expressed as a percentage of control, when specific inhibitors for MMP-2, 3, 8, 9 and 13 were applied (n=8). *** P<0.001 vs. control, Fisher’s post-hoc test.
3.3.9 Phenotypic Fate of Recruited Neural Progenitors

On day 1, the cell population in the annular tissue simulant consisted mostly of proliferating NPCs, as seen from the fact that ~91% and ~88% of the cells expressed NPC marker nestin and proliferation marker Ki67 (Figure 3.19 & 20). After 7 days in neurogenic medium, this cell population evolved into a mixture of NPCs (~35%) and TUJ1+ neurons (~45%) (Figure 3.21 & 22). The proportion of the Ki67+ cells also decreased to ~34%. Within the Gtn-HPA/SDF-1α-PCN matrix core on day 7, nestin+ and Ki67+ cells constituted ~82% and ~73% of the recruited cells which were relatively larger proportions compared to those in tissue simulant (Figure 3.23 & 24). Also, only ~15% of the recruited cells expressed TUJ1. Very few cells in either the tissue simulant or the Gtn-HPA/SDF-1α-PCN hydrogel core were GFAP+ or CNPase+. 
Figure 3.19: Immunostaining for neural progenitor marker (Nestin), neural differentiation markers (TUJ1, GFAP and CNPase) and proliferation marker (Ki67) to identify the cells in the (A) annulus on day 1. Scale bars = 50μm.

Figure 3.20: Quantification of Nestin\(^+\), TUJ1\(^+\), GFAP\(^+\), CNPase\(^+\) and Ki67\(^+\) cells in the annulus on day 1.
Figure 3.21: Immunostaining for neural progenitor marker (Nestin), neural differentiation markers (TUJ1, GFAP and CNPase) and proliferation marker (Ki67) to identify the cells in the annulus on day 7. Scale bars = 50μm.

Figure 3.22: Quantification of Nestin⁺, TUJ1⁺, GFAP⁺, CNPase⁺ and Ki67⁺ cells in the annulus on day 7.
Figure 3.23: Immunostaining for neural progenitor marker (Nestin), neural differentiation markers (TUJ1, GFAP and CNPase) and proliferation marker (Ki67) to identify the cells in the core on day 7. Scale bars = 50μm.

Figure 3.24: Quantification of Nestin+, TUJ1+, GFAP+, CNPase+ and Ki67+ cells in the core on day 7.
3.4 Discussion

A crucial step in the current work to develop NPC-recruiting biomaterial scaffolds had been to integrate injectable Gtn-HPA hydrogels with the functionality of sustained SDF-1α release. To allow independent optimization of SDF-1α release and structural support for NPCs, we adopted the strategy of devising delivery vehicles that could provide the desired release kinetics and serve as a modular addition to Gtn-HPA hydrogels. The PCNs formulated from biodegradable polymers, DS and CS, were found to fulfill the aforementioned requirements. When loaded with SDF-1α, they released 44.7% of their load gradually over a 28-day period, conceivably retaining the remaining load for release over a further prolonged period. The PCNs could also be incorporated into Gtn-HPA hydrogels without affecting the hydrogel covalent crosslinking and without being cytotoxic to aNPCs. Furthermore, SDF-1α PCNs met the practical requirements relating to their preparation and use. They offered high entrapment efficiencies (75 - 91%) that addressed the pragmatic need to minimize the loss of costly proteins. They also offered high loading efficiencies (7 - 31 wt%) that were critical in avoiding excessively high concentrations of delivery vehicles when introducing therapeutically relevant doses of SDF-1α into the small volumes associated with cavitary brain lesions.

The resulting Gtn-HPA/SDF-1α-PCN matrices were evaluated in specially developed standardized migration assays based on A+C constructs. Unlike conventional migration assays in Boyden, Dunn and Zigmond chambers, the A+C constructs did not only quantitatively assess the bioactivity and chemotactic potency of test substances, but also concurrently evaluated the compatibility of test matrices with cell migration. Cells in the A+C constructs were also maintained in three-dimensional culture, which mimic in vivo environments more closely than the monolayer cell cultures typically used in conventional migration assays [18]. Moreover, the A+C constructs were devised to distill the complexity of cavitary brain lesions into elements that were pertinent to the recruitment of NPCs. They featured a core-annular geometry to represent the hydrogel-filled cavity and the peri-lesion tissue. NPCs were encapsulated within the annular tissue simulant to correspond to endogenous NPCs within peri-lesion tissues. A one-time dose of FGF-2, which was added into the annular tissue simulant together with the NPCs to stabilize their encapsulation, served to reflect the transiently increased FGF-2 expression in peri-lesion tissues after injury [19]. Similar to implanting materials into the cavitary brain lesions where they contact
and interact with peri-lesion tissues, materials could be placed in the core where they interface with the annular tissue simulant to influence or recruit the NPCs. Finally, neurogenic cues were provided in the medium to allow NPCs to undergo neuronal differentiation, as would the endogenous NPCs in vivo during their homing towards injured tissues [9]. The A+C constructs therefore allowed us to systematically evaluate candidate materials in a rapid, yet physiologically relevant manner, as well as perform characterization/mechanistic studies (e.g. those to distinguish between chemotaxis and chemokinesis) that were otherwise difficult to implement in vivo.

In the A+C constructs, Gtn-HPA/SDF-1α-PCN matrices produced significantly larger enhancements in cell accumulation at the annulus/core interface and in the number of migrated cells in the core when compared to the effects of Gtn-HPA/SDF-1α matrices, Gtn-HPA/PCN matrices or even the sum of both. Most recruited cells migrated into Gtn-HPA/SDF-1α-PCN matrices either as individual cells exhibiting leading and trailing processes or as chains where cells were known to follow preceding cells [20] or use one another as guiding structures [7]. Both forms were highly reminiscent of the migratory behavior of endogenous NPCs observed in the injured brain [20]. The use of AMD3100 to antagonize SDF-1α receptor CXCR4 demonstrated that the enhancements by Gtn-HPA/SDF-1α-PCN matrices were largely mediated through the SDF-1α/CXCR4 pathway. The effects of Gtn-HPA/SDF-1α-PCN matrices also displayed a dose-dependent behavior, which further confirmed the involvement of SDF-1α. Apart from being a well-known chemotactic cue, SDF-1α may also stimulate proliferation [7] and chemokinesis [21]. However, SDF-1α did not increase proliferation of NPCs in our study, thus dismissing the possibility that Gtn-HPA/SDF-1α-PCN matrices drove more cells towards the interface and into the core by causing overcrowding in the tissue simulant. SDF-1α from Gtn-HPA/SDF-1α-PCN matrices also did not enhance cell accumulation and migration relative to Gtn-HPA/PCN matrices when we disrupted chemotactic SDF-1α concentration gradients and examined chemokinetic effects in isolation. This excluded the possibility that Gtn-HPA/SDF-1α-PCN matrices enhanced cell accumulation and migration simply by increasing the motility of NPCs and expediting their random dispersal from the tissue simulant. Therefore, Gtn-HPA/SDF-1α-PCN matrices had enhanced cell accumulation and migration relative to Gtn-HPA/PCN hydrogels via chemotactic recruitment of cells towards the interface and into the core. The ability of Gtn-HPA/SDF-1α-PCN hydrogels to chemotactically recruit aNPCs clearly highlighted the instrumental role of SDF-1α.
PCNs in sustaining the release of SDF-1α sufficiently for concentration gradients to be established.

Along with releasing SDF-1α, Gtn-HPA/SDF-1α-PCN matrices also provided a compatible structural support for cell migration during chemotactic recruitment. This was subsequently found to be contingent on utilizing the tunable crosslinking of Gtn-HPA matrices to maintain crosslinking degrees in the range of around 0.85 – 0.95mM H₂O₂. Above this range, the higher crosslinking degrees led to a drastic reduction or even a complete arrest in cell migration, which was a likely consequence of cells being unable to remodel/degrade highly crosslinked polymer networks. As expected from the abundance of MMP cleavage sites along gelatin polymers, migration into the Gtn-HPA matrices was almost fully dependent on MMPs and was severely impeded by broad-spectrum MMP inhibitor GM6001. It was of further interest to identify the specific MMPs involved since endogenous NPCs express MMP-2, 3 and 9 to mediate their migration through brain extracellular matrices (ECM) after brain injuries [22, 23] and will reasonably interact better with structural support that can respond readily to the MMPs they secrete. By using specific MMP inhibitors, we first noticed MMP-8 to be critically essential. This might be attributed to MMP-8 being a potent type I collagenolytic enzyme and heavily utilized to transverse the tissue simulant that was composed of type I collagen. The specific MMP inhibitors additionally revealed the involvement of MMP-2, 3 and 9. Unlike MMP-8, neither MMP-2, 3 nor 9 played a particularly dominant role relative to one another, suggesting that all three of these MMPs were used collaboratively. The MMP profile employed during cell migration through Gtn-HPA/SDF-1α-PCN matrices was therefore highly similar to that used by endogenous NPCs to migrate through the brain ECM after brain injuries. This strongly reflects the compliance of Gtn-HPA/SDF-1α-PCN matrices towards endogenous NPCs when employed within cavitary brain lesions.

Importantly, our findings demonstrated the promise of injectable Gtn-HPA/SDF-1α-PCN matrices for treating cavitary brain lesions. In the A+C hydrogel constructs, in addition to providing chemotactic cues and structural support in the otherwise structureless core, Gtn-HPA/SDF-1α-PCN matrices showcased the proof-of-concept for properly interfacing with apposing cell-laden matrices in order to achieve the crucial continuity in structural support for recruited cells to migrate across the interface. In addition, Gtn-HPA/SDF-1α-PCN matrices did
not only recruit the highest number of cells but also induced recruited cells to migrate the furthest into the core. With supportive levels of angiogenesis which could presumably be stimulated by Gtn-HPA/SDF-1α-PCN matrices given the ability of SDF-1α to recruit endothelial cells [24] and hematopoietic stem cells [25], the matrices might offer the potential to maximize the volume of the cavitary brain lesions that would be populated by recruited neurovascular cells. Furthermore, the ability of Gtn-HPA/SDF-1α-PCN matrices to enhance cell accumulation around themselves would be of value in vivo to similarly recruit and accumulate greater numbers of endogenous neural cells in the peri-lesion tissues. This could benefit the peri-lesion tissues and provide more cells for eventual recruitment into the cavitary lesion itself. Finally, Gtn-HPA/SDF-1α-PCN matrices preferentially recruited proliferative NPCs as opposed to neurons and offered the possible advantage of directing NPCs, which hold greater regenerative potential due to their proliferative capacity and multipotency, into the cavitary lesions while leaving the newly differentiated neurons and their reparative roles in the peri-lesion tissues minimally perturbed.

Consistently throughout our investigations, PCNs themselves increased proliferation and the number of migrated cells in the hydrogel core, the latter of which was unaffected by AMD3100 and occurred independently of the SDF-1α/CXCR4 pathway. These effects were subsequently found to involve PCNs inheriting heparin-like properties from DS and utilizing FGF-2 from the tissue simulant. Related polyelectrolytes, DSL and heparin, could also utilize FGF-2 and enhance cell migration, albeit to a lower extent than PCNs, through stimulating chemokinesis. It thus appeared that at least a large part of the PCN-mediated effect on cell migration might be driven by similar increases in chemokinesis. We further observed that when FGF-2 was omitted, cell migration into Gtn-HPA/SDF-1α-PCN matrices decreased substantially more than the amount expected from cell migration with Gtn-HPA/PCN matrices. This indicated that when utilizing FGF-2, PCNs did not only constitute a stand-alone mechanism that increased the chemokinetic migration of NPCs but also likely displayed synergistic effects with SDF-1α by stimulating the chemotactic responses of NPCs towards SDF-1α and/or promoting the proliferation of recruited NPCs (Figure 3.25).
Given the known effects of FGF-2 in stimulating proliferation [26], chemokinesis [27] as well as chemotaxis towards other chemokines [28], the process of utilizing FGF-2 in the above findings likely entailed PCNs stabilizing FGF-2 and/or potentiating its activity. These secondary effects of PCNs, although a serendipitous finding, were not entirely surprising because the property of PCNs that enabled the entrapment of SDF-1α had been the heparin-like property of DS. Heparin, DS and similar glycosaminoglycans have been demonstrated to bind and stabilize FGF-2 [29]. These glycosaminoglycans can further participate in the presentation of FGF-2 to its receptors and modulate the activity of the FGF-2 on cell behaviors such as mitosis and motility [30]. The secondary roles of PCNs were, however, remarkable in the following way. Unlike the DS-bearing PCNs and low molecular weight counterparts such as DSL and heparin, DS itself failed to stimulate chemokinetic migration of NPCs in the presence of FGF-2. Since the activities of growth factors can vary with the structure of binding glycosaminoglycans [31], we conjectured
that the chitosan moieties within PCNs bound to DS and altered its structural properties to approximate DSL and heparin. Nevertheless, it was evident that other than delivering SDF-1α, PCNs themselves conferred beneficial properties to Gtn-HPA/SDF-1α-PCN matrices. By being molecular aggregates that were less prone to diffuse, PCNs served as an anchored form of DS within the matrices and stably imparted the matrices with heparin-like properties, allowing them to emulate brain ECM in the aspects of sequestering and modulating the activities of heparin-binding growth factors such as FGF-2 [32]. More importantly, PCNs enabled Gtn-HPA/SDF-1α-PCN matrices to utilize FGF-2 found in situ in the tissue simulant to “self-enhance” their efficacy to draw in NPCs and in this sense, bear similar potential as biomaterials that took advantage of endogenous growth factors expressed in situ [33]. When employed within cavitary brain lesions, Gtn-HPA/SDF-1α-PCN matrices present an appealing possibility of harnessing the FGF-2 or other heparin-binding growth factors expressed in the peri-lesion tissues to synergize the recruitment of endogenous NPCs by the SDF-1α they release. This will maximize the number of endogenous NPCs recruited into the lesion and facilitate the ultimate goal of neural tissue repair/regeneration.
3.5 References


Chapter 4

Implantation and Optimization of Gtn-HPA/SDF-1α-PCN matrices in a Rat Model of Intracerebral Hemorrhagic Stroke

4.1 Introduction

Implantation of matrices can play several roles in brain lesions. They can act repository materials to deliver drugs/cytokines to drive targeted cell/tissue responses [1], maintain the structure of surrounding brain tissues [2], provide stroma for in growth of axons [3, 4] and neural cells [5] and support transplanted cells in the injury environment [6, 7]. Nevertheless, implanting matrices into brain lesions can often be a challenging endeavor. Unlike many other organs, the brain is securely housed in the rigid skull where the close fit between the brain and the skull as well as the connection of the brain to the spinal cord allows little room to manipulate the position or orientation of the brain during surgical procedures. The brain is also usually accessible only from its dorsal surface due to the presence of numerous anatomical structures around its ventral space. At the same time, much of the brain, especially the cortical region near the dorsal surface, is densely packed with axonal projections and cannot be temporarily removed or push aside lest there be injury due to stretching or shearing of the axons. On top of these, brain lesions tend to be irregularly shaped and can vary significantly from one injury to the next. When compounded together as in the case of deep-seated lesions, these circumstances pose a huge challenge for implanting matrices in brain lesions.

Numerous matrices have been developed for application in the injured brain. Several of them have displayed promising benefits for the injured brain, but their applications have been largely restricted to exposed cortical lesions [2, 5, 8] because the matrices are fabricated to a predetermined shape and size and are difficult to be implanted into deep-seated lesions for the reasons above. This has spurred the interest in identifying or developing matrices that are injectable. The property of injectability involves having the matrices be introduced into the lesion only through a syringe needle and is an attractive strategy to reach deep-seated lesions without
causing extensive damage to the overlying tissue. A few materials, such as PLGA microspheres [7], matrigel [9], platelet-rich plasma scaffolds [10] and self-assembling peptide hydrogels [3] have been employed with injectable matrices thus far, each displaying certain strengths and weaknesses. Microspheres represent miniaturized pre-fabricated matrices and allow for their material properties to be verified prior to application but may not interface seamlessly with the surrounding brain tissue if they only loosely occupy the lesion. Naturally-derived materials such as matrigel and platelet-rich plasma scaffolds are relatively easy to obtain and offer in situ gelation to conform to the irregular shape of the lesion but may have varying composition or material properties depending on their sources and collection. Self-assembling peptide hydrogels offer both well-defined chemical composition and enable robust regeneration of severed optic nerve but do not undergo covalent crosslinking and may not be adequately persistent in brain lesions that are relatively more severe.

Prior chapters in this thesis have detailed the development and characterization of matrices that comprise of Gtn-HPA hydrogels and SDF-1α-loaded polyelectrolyte complex nanoparticles (PCNs). Gtn-HPA hydrogels belong to a class of enzymatically crosslinked matrices whose crosslinking rate and degree can be tuned by varying concentration of the crosslinking reagents, horseradish peroxidase (HRP) and H₂O₂ [11]. By appropriate control of the crosslinking rate, Gtn-HPA hydrogels can be employed as an injectable matrix to circumvent the difficulties associated with implanting matrices into deep-seated brain lesions. Relative to other injectable materials that have been gainfully employed in the injured brain, Gtn-HPA hydrogels also provide for in situ gelation which is critical in ensuring conformity to irregularly shaped lesions [12] and give the additional benefit of covalent crosslinking to allow for adjustment of matrix persistence against the multitude of degradative processes in the lesion. SDF-1α PCNs were utilized for their easy incorporation into Gtn-HPA hydrogels and their ability to sustain (for up to 4 weeks) the release of SDF-1α, a well-established chemoattractant for various cell types including neural progenitors and endothelial cells. It is therefore compelling to examine the implantation of Gtn-HPA/SDF-1α-PCN matrices in brain lesions and optimize the matrices for future application in the injured brain.

In this study, we employ a rat model of intracerebral hemorrhage (ICH) to examine the implantation of Gtn-HPA/SDF-1α-PCN matrices into deep-seated lesions. In this model, ICH is induced by the injection of collagenase to cause the disruption of blood vessels, spontaneous extravasation of blood and hemotoma expansion in the rat brain. The severity of the injury, and
the subsequent size of the brain lesion, can be consistently manipulated by the amount of collagenase injected, thus avoiding huge variations that may complicate comparative analyses. More importantly, the location of the hemorrhage is also easily controlled by the injection site of collagenase to induce injury well below the dorsal surface of the brain. This allows the creation of deeply seated brain lesions to realistically evaluate the implantation of Gtn-HPA/SDF-1α-PCN matrices.

4.2 Materials and Methods

4.2.1 Preparation of Gtn-HPA conjugate and SDF-1α PCNs

Gtn-HPA conjugate and SDF-1α PCNs were prepared as previously described in Section 2.2.1 and 3.2.1.

4.2.2 Rheological Measurements of Gtn-HPA Hydrogels

Rheological measurements of Gtn-HPA hydrogels were performed with a TA instruments AR-G2 rheometer using cone geometry of 40mm diameter and 2° angle. For each measurement, 600μl of Gtn-HPA containing 0.03U/ml HRP and varying concentrations of H₂O₂ was applied to the bottom plate immediately after mixing. Solvent trap cover was placed to prevent evaporation. All measurements were taken at 37°C in the oscillation mode with a constant strain of 1% and frequency of 1 Hz.

4.2.3 Design of Animal Experiment

All experiments were performed under protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committees (IACUC, protocol 2012N000165) following the NIH Guide for Use and Care of Laboratory Animals. A total of 14 male Sprague-Dawley Rats (250 - 300g) were used in this study and were subjected to intrastratal injections of collagenase to induce ICH. Of these animals, a) 2 was sacrificed 1 day post-ICH to reveal characteristics of the hemorrhage; b) 2 was sacrificed 2 weeks post-ICH to reveal characteristics of the lesion; 3) 2 received an injection of Gtn-HPA/SDF-1α-PCN matrix at 2 weeks post-ICH.
and sacrificed immediately after to reveal characteristics of the injected matrix and; 4) 8 received injections of Gtn-HPA/SDF-1α-PCN matrices (formulated with 4, 8, 12 and 16 wt% Gtn-HPA (n=2 for each type) and SDF-1α-PCNs carrying containing 6μg of SDF-1α) at 2 weeks post-ICH and sacrificed 4 weeks post-ICH to identify the optimal formulation for further investigation (Figure 4.1).

![Diagram](image)

Figure 4.1: Time course for ICH, matrix injection and animal sacrifice for various experimental groups (n=2).

### 4.2.4 Collagenase-Induced Model of Intracerebral Hemorrhage

Rats were anesthetized using isofluorane (induction 3.5%, maintenance 1.5% to 2% in 70% N₂O and 30% O₂) and placed in a stereotaxic frame (Kopf, Tujunga, CA). Body temperature was monitored and maintained around 37°C during surgery using a rectal probe and a heating pad. A midline scalp incision was made to expose the skull. A small cranial burr hole was then drilled 0.5mm anterior and 3mm lateral to the bregma. The 25-Gauge needle of a 2μl Hamilton syringe was inserted 5mm ventral to the dural surface. 5 min after the insertion of the needle, 0.5
U Collagenase Type VII (Sigma Aldrich) in 2μl of 0.9% saline was injected into the striatum over 5 min. The needle was then left in place for 5 min and slowly withdrawn over 5 min. The burr hole was sealed with bone wax and the incision was subsequently sutured. The rats were allowed to recover from surgery in cages warmed under an incandescent bulb.

### 4.2.5 Stereotaxic Injection of Gtn-HPA/SDF-1α-PCN Matrices

Rats were placed in a stereotaxic frame using the same procedures for anesthesia and temperature monitoring/maintenance. A midline scalp incision was made to expose the cranial burr hole created in the previous surgery and bone wax removed to unseal the burr hole. 25μl of pre-gel solution of Gtn-HPA/SDF-1α-PCN matrix (containing 6μg of SDF-1α) was loaded into a 50μl Hamilton syringe. Pre-gel solution of Gtn-HPA/SDF-1α-PCN matrix was prepared immediately prior to loading by mixing Gtn-HPA solution with SDF-1α PCNs and solutions of its cross-linking reagents, horseradish peroxidase (HRP) and H2O2. The final composition of the various matrices is shown in Table 4.1. After loading of pre-gel solutions of matrices, the 22-G needle of the Hamilton syringe was promptly lowered 5mm ventral to the dural surface. The loaded pre-gel solution was injected into the ICH lesion over 3 min (Figure 4.2). After injection, the needle was left in place for 5 min and slowly withdrawn over 5 min. Similar to the previous surgery, the burr hole was sealed with bone wax, the incision sutured and the rats returned to cages warmed under an incandescent bulb.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Gtn-HPA</th>
<th>SDF-1α PCN</th>
<th>Horseradish peroxidase (HRP)</th>
<th>H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4wt% Gtn-HPA/SDF-1α-PCN matrix</td>
<td>40mg/ml</td>
<td>1.3mg/ml*</td>
<td>55.0 mU/ml</td>
<td>3.4mM</td>
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<tr>
<td>8wt% Gtn-HPA/SDF-1α-PCN matrix</td>
<td>80mg/ml</td>
<td>1.3mg/ml*</td>
<td>70.0 mU/ml</td>
<td>6.8mM</td>
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<tr>
<td>12wt% Gtn-HPA/SDF-1α-PCN matrix</td>
<td>120mg/ml</td>
<td>1.3mg/ml*</td>
<td>115.0 mU/ml</td>
<td>10.2mM</td>
</tr>
<tr>
<td>16wt% Gtn-HPA/SDF-1α-PCN matrix</td>
<td>160mg/ml</td>
<td>1.3mg/ml*</td>
<td>167.5 mU/ml</td>
<td>13.6mM</td>
</tr>
</tbody>
</table>

* corresponds to a SDF-1α concentration of 0.24 mg/ml that yields 6μg of SDF-1α in 25μl of injected matrices

Table 4.1: Final composition of Gtn-HPA/SDF-1α-PCN matrices injected into ICH lesion
Figure 4.2: Photograph showing stereotaxic injection of Gtn-HPA/SDF-1α-PCN matrices into the brain lesion 2 weeks after ICH.

4.2.6 Animal Sacrifice and Specimen Processing

Rats were deeply anesthetized using 5% isofluorane and sacrificed via transcardial perfusion with 120ml of 0.9% saline followed by 120ml 4% paraformaldehyde solution. Brains were extracted and submerged into Tissue Tek O.C.T compound contained in a tinfoil mold. The brains were then flash-frozen using isopentane cooled on liquid nitrogen and cryosectioned coronally at 30μm. The tissue sections were mounted on treated glass slides, air-dried and stored at -20°C. Prior to histological staining, the tissue sections were baked at 50°C for 1 hr to increase adhesion to the glass slides, rehydrated in PBS, permeabilized in ice-cold methanol for 10 min and washed with PBS.
4.2.7 Histology

Tissue sections were stained with hematoxylin and eosin for visualization of brain tissue and implanted matrices. The procedure included staining in Gill's hematoxylin (10 min), rinsing in water (5 min), differentiation in acid alcohol (1 s), rinsing in water (5 min), staining in eosin (1 min), dehydration in 100% alcohol (2 × 2 min), processing in xylene (2 × 3 min) and mounting of coverslip using cytoseal. Brightfield imaging was performed on the stained cryosections using an Olympus BX51 microscope. For each rat, histomorphometric analysis was performed on 7 cryosections which were taken at 1mm intervals from -3mm to 3mm anterior to bregma to span over the lesion and/or implanted matrices. Areas of both hemispheres, both ventricles, both cavitory and non-cavitary lesions resulting from ICH as well as implanted matrix were measured using ImageJ. Area of intact brain tissue in each hemisphere was calculated using Equation 4.1. Area of brain tissue loss for each hemisphere was calculated using Equation 4.2 and linearly integrated using Equation 4.3 to determine the volume of brain tissue loss.

\[
\text{Area of intact brain tissue} = \text{Area of hemisphere} - \text{Area of ventricle} - \text{Area of lesion} - \text{Area of implanted matrix} \quad \text{Equation 4.1}
\]

\[
\text{Area of brain tissue loss} = (\text{Area of intact brain tissue})_{\text{contralateral}} - (\text{Area of intact brain tissue})_{\text{ipsilateral}} \quad \text{Equation 4.2}
\]

\[
\text{Volume of brain tissue loss} = \sum_{i=-3}^{3} (\text{Area of brain tissue loss}) \times 1 \quad \text{Equation 4.3}
\]
4.3 Results

4.3.1 Storage Moduli of Gtn-HPA Hydrogels

In preparation of their use in vivo, Gtn-HPA hydrogels of varying wt% were subjected to rheological measurements to determine their storage moduli, $G'$. The measurements revealed that $G'$ of Gtn-HPA hydrogels increased significantly with increasing wt% (one-factor ANOVA, $p < 0.001$) and spanned from 8591 to 54353 Pa (Table 4.2).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>$G'$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4wt% Gtn-HPA</td>
<td>8591 ± 894</td>
</tr>
<tr>
<td>8wt% Gtn-HPA</td>
<td>22930 ± 1205</td>
</tr>
<tr>
<td>12% Gtn-HPA</td>
<td>35160 ± 8188</td>
</tr>
<tr>
<td>16wt% Gtn-HPA</td>
<td>54353 ± 13115</td>
</tr>
</tbody>
</table>

Table 4.2: $G'$ of Gtn-HPA hydrogels
4.3.2 Intracerebral Hemorrhagic Lesion 1 day and 2 weeks post-ICH

1 day after the injection of collagenase, a hemorrhagic lesion was observed, spanning over significant area of the striatum and the globus pallidus (Figure 4.3A). Within the lesion, numerous extravasated erythrocytes dissected the brain parenchyma and aggregated at multiple sites. Cell nuclei staining by hematoxylin appeared more diffuse in the lesion than in the rest of the brain. Relative to the contralateral side, the injured hemisphere underwent significant enlargement, a characteristic commonly observed to occur acutely after ICH due to the presence of extravasated blood and brain edema. Two weeks after the onset of ICH, the lesion became a well-demarcated region characterized by the presence of a hematoma, degenerating parenchyma and by the appearance of small fluid-filled cavities (Figure 4.3B). The injured hemisphere evolved to be smaller in size compared to the contralateral hemisphere and featured a considerably enlarged ventricle. Histomorphometric analysis indicated tissue loss to be 21.5 ± 6.8 mm³.

Figure 4.3: Micrographs showing the brain lesion at (A) 1 day and (B) 2 weeks after the onset of ICH.
4.3.3 Gtn-HPA/SDF-1α-PCN matrices 1 hour after Injection

25mm³ of Gtn-HPA/SDF-1α-PCN matrix, whose volume was chosen to approximately match the volume of tissue loss, was injected into the lesion in the form of a pre-gel solution shortly before the completion of its cross-linking process and its transition into a gel-based matrix. Gtn-HPA/SDF-1α-PCN matrix was successfully deposited as a continuous piece of implant around the injection site in the lesion (Figure 4.4). The implanted matrix conformed to the boundary of the lesion, enveloped the hematoma and was tightly interfaced with the host brain tissue or the hematoma. Notably, the presence of the Gtn-HPA/SDF-1α-PCN matrix appeared to compensate for the structural effects of tissue loss after ICH. Specifically, the matrix reversed the enlargement of the ipsilateral ventricle, restored the size of the injured hemisphere and re-established overall structural asymmetry between both hemispheres.

Figure 4.4: Micrograph showing Gtn-HPA/SDF-1α-PCN matrix at 1 hour after injection into a 2-week old ICH lesion.

4.3.4 Optimization of Gtn-HPA/SDF-1α-PCN matrices

4 formulations of Gtn-HPA/SDF-1α-PCN matrices, prepared with SDF-1α-PCNs and either 4, 8, 12 or 16wt% Gtn-HPA, were injected in the injured brain at 2 weeks post-ICH and evaluated 2 weeks after. At the time of evaluation, a substantial proportion of the 4wt% Gtn-HPA/SDF-1α-PCN matrix had been degraded (Figure 4.5A). Any remaining proportion of the matrix had evolved into loose cell-filled fragments that were disconnected from the surrounding host brain tissue, resulting in the emergence of small cavitory spaces within the lesion. The injured hemisphere also reverted to having an enlarged ventricle and a reduced size relative to the contralateral hemisphere. In contrast, 8wt% Gtn-HPA/SDF-1α-PCN matrix was better preserved
at 2 weeks after implantation (Figure 4.5B). The matrix continued to exist as a single piece of implant in the striatum without any cavitory spaces. The matrix retained its tight interface with the surrounding brain tissue and was heavily infiltrated with host cells and vessel-like structures. Overall, the injured hemisphere retained its structural symmetry with the contralateral hemisphere. 12 and 16 wt% Gtn-HPA/SDF-1α-PCN matrices were also well preserved at 4 weeks post-ICH (Figure 4.5C & D). However, the interface with the host brain tissue was split in several locations around the implanted matrices, resulting in the loss of physical continuity between the host brain tissue and the matrices. Compared with 8wt% Gtn-HPA/SDF-1α-PCN matrix, the denser 12 and 16 wt% Gtn-HPA/SDF-1α-PCN matrices permitted little or no cell infiltration. Structural symmetry between both hemispheres was also distorted as both of these matrices dissected into the striatum and ventricular wall and extended substantially into the ipsilateral ventricle. 8wt% Gtn-HPA was therefore selected to formulate all matrices in subsequent experiments.

Figure 4.5: Micrographs showing (A) 4wt%, (B) 8wt%, (C) 12wt% and (D) 16wt% Gtn-HPA/SDF-1α-PCN matrices at 2 weeks after injection into a 2-week old ICH lesion.
4.4 Discussion

The first part of the study focused on the implantation of Gtn-HPA/SDF-1α-PCN matrices into deeply seated brain lesions after ICH injury. The physical space needed to house the Gtn-HPA/SDF-1α-PCN matrices could be created in the acute setting by the use of procedures to aspirate the hematoma or be allowed to gradually appear in the chronic setting with focal cavitation or atrophy of the injured brain tissue. The latter scenario was chosen with the intent for this study to be informative toward other forms of brain injuries that undergo similar tissue cavitation/atrophy. The value of injectability and *in situ* crosslinking was clearly displayed in the implantation of Gtn-HPA/SDF-1α matrices into a lesion 5mm below the dorsal surface of the brain. By being injectable, a Gtn-HPA/SDF-1α matrix that was eventually as wide as ~3mm could be delivered to its target location via a needle whose diameter measured to be 0.7mm. In other words, compared to a similarly sized preformed matrix which might have to affect ~35mm³ of overlying cortical tissue¹ for its passage into the brain lesion, the implantation of Gtn-HPA/SDF-1α-PCN matrix only had a volumetric footprint of ~2mm³ on the overlying cortical tissue.² Examination of the brain right after implantation showed that the matrix had optimally filled the lesion and the overlying cortical tissue was well preserved except for the slight mechanical disruption along the needle tract. On the other hand, the ability to undergo *in situ* crosslinking evidently gave Gtn-HPA/SDF-1α matrix the benefit of being transiently fluid right after injection into the lesion. This transient fluidity persisted sufficiently long for the injected matrix fill the irregularly shaped lesion and wrap around any residual hematoma but ended soon enough for the matrix to undergo sol-gel transition into a semi-solid material that remained securely located within the lesion. *In situ* crosslinking also enabled the matrix to undergo the sol-gel transition while in direct contact with the surrounding tissue. This was presumably a key factor in establishing the seamless physical continuity observed between the matrix and host tissue, which would be critical for the inward migration of any endogenous cells.

Gtn-HPA/SDF-1α-PCN matrices are a form of biomaterial that is primarily composed of denatured collagen and can be enzymatically degraded by matrix metalloproteinases, which are known to be expressed at elevated levels in the injured brain. A crucial component of this pilot study was to identify an optimal formulation of Gtn-HPA/SDF-1α matrix for implantation into

---

¹ estimated with a matrix (with a circular cross-section of diameter 1.5mm) moving through 5mm of cortical tissue
² estimated with a needle (with a circular cross-section of diameter 0.35mm) moving through 5mm of cortical tissue
brain lesions after ICH injury. The need to identify an optimal formulation was furthermore pertinent given the opposing design criteria that stemmed from the roles that Gtn-HPA/SDF-1α matrices were expected to serve in the brain lesions. On one hand, the expectation to reside in the brain lesion and release SDF-1α over time demanded Gtn-HPA/SDF-1α matrices to have adequate macroscopic persistence against degradation after being implanted. On the other hand, the expectation to serve as a stromal framework for endogenous cells demanded Gtn-HPA/SDF-1α-PCN matrices to be amenable to cell migration by possessing sufficient susceptibility toward degradation at the microscopic scale. Given the isotropic nature of Gtn-HPA/SDF-1α-PCN matrices, one way to satisfy both the lower and upper constraints placed by the opposing design criteria was to tune the overall degradation rate of the matrices. To achieve this, we varied the wt% of Gtn-HPA (and amount of crosslinking reagents to maintain the same crosslinking degree per wt% Gtn-HPA). When we evaluated for matrix persistence and cell infiltration at 2 weeks after being implanted in the brain lesion, we successfully identified 8wt% Gtn-HPA/SDF-1α matrix as an optimal formulation satisfying both constraints.

A cross reference showed that the storage modulus of the optimal formation, 8wt% Gtn-HPA/SDF-1α matrix, was 22930 ± 1205 Pa and was considerably higher than the reported range of 400 – 3100 Pa for the brain. Given the material nature of Gtn-HPA, this high storage modulus of the matrix was a concomitant of the increase in wt% of Gtn-HPA to achieve sufficient matrix persistence in the injured brain. In our observation with 8wt% Gtn-HPA/SDF-1α matrix, we did not observe any tissue laceration, which a severe mechanical mismatch would pose a risk for. This might partly be because the storage modulus of the implanted 8wt% Gtn-HPA/SDF-1α matrix would drop significantly with degradation to approach that of the surrounding brain tissue. Although mechanical matching of matrix to host tissues has almost become a dogma in the field of regenerative medicine, our experience here argues for the cautious consideration of mechanical matching with other design criteria, especially for a degradable matrix whose mechanical properties will evolve over time. Blind pursuit of mechanical matching would have disregarded 8wt% Gtn-HPA/SDF-1α matrix as a potentially valuable formulation to investigate in an in vivo model. Nevertheless, mechanical mismatch beyond a certain point does result in detrimental outcomes. In our case, implantation of 12 and 16wt% Gtn-HPA/SDF-1α matrices, which had even higher storage moduli, lacerated the adjacent striatum and ruptured the lateral ventricle.
4.5 References


Chapter 5

Evaluation of Gtn-HPA/SDF-1α-PCN matrices in a Rat Model of Intracerebral Hemorrhagic Stroke

5.1 Introduction

Brain injuries such as ischemic strokes, intracerebral hemorrhage and trauma often cause irreversible impairments to brain parenchyma that disrupts vital neurological functions and severely impact quality of life. Depending on the severity of the injury, the mature brain with its remarkable degree of cellular specialization requires cell/tissue reconstitution for the recovery of affected neurological functions. The strategy of cell/tissue reconstitution has generally relied on the transplantation of stem cells. Over the years, numerous types of stem cells have been isolated, processed and transplanted via intravenous administration or direct injection into the injured brain. They include embryonic stem cells [1], neural stem cells [2], bone marrow-derived mesenchymal stem cells [3], adipose-derived stem cells [4] and umbilical cord blood-derived stem cells [5]. Since the discovery of induced pluripotent stem cells (iPSCs) that can be generated by reprogramming somatic cells, neural stem cells have also been derived from iPSCs and transplanted into the brain after ICH [6].

Common outcomes following the transplantation of stem cells have included: 1) differentiation of the transplanted stem cells into useful cell types such as neurons, astrocytes, oligodendrocytes and endothelial cells; 2) increased angiogenesis and; 3) improvements in neurological functions. Scaffolding matrices (e.g. polyglycolic acid scaffolds [7], poly-lactic-co-glycolic acid microspheres [8] and matrigel [9]) have further been used to improve the survival of the transplanted cells and enabling the donor and host cells to participate in reciprocal interactions which are absent otherwise. Despite all this promise demonstrated in pre-clinical models, transplantation of exogenous cells into the injured brain remains highly challenging. Isolating and
preparing the cells for transplantation are typically associated with costly processing. The number of transplanted cells has to be carefully titrated to avoid sudden introduction of cells in large numbers into the harsh environment in the injured brain [10]. Any cells from non-autologous sources need to be further weighed against possible detrimental immune complications.

Over the years, endogenous regenerative responses in the injured brain have become increasingly recognized as an alternative way to achieve cell/tissue reconstitution in the injured brain. One prominent regenerative response is injury-induced neurogenesis, which involves endogenous neural stem and progenitor cells in well-defined niches such as the subventricular zone. In the wake of a brain injury, the endogenous neural stem and progenitor cells increase their proliferation [11, 12], migrate toward the lesion persistently for several weeks after the injury [13, 14] and differentiate into neural cells that include region-specific neurons [15] and neurons that integrate successfully into the local circuitry [16]. Another regenerative response is neovascularization, which has been observed consistently across ischemic [17], hemorrhagic [18], traumatic [19] as well as surgical brain injury [20]. Neovascularization can occur in the angiogenic fashion where endothelial cells in the peri-lesion tissues proliferate, migrate and engage in tube formation to sprout new blood vessels from existing ones [21]. Circulating bone marrow-derived cells (e.g. endothelial progenitor cells) may also be recruited into the injured brain where they differentiate into endothelial cells and participate in the formation of new blood vessels [19, 20]. Both injury-induced neurogenesis and neovascularization have further been correlated with functional recovery in several pre-clinical models, demonstrating their potential to compose meaningful treatments for the injured brain.

Nevertheless, the injured brain remains far from being an adequate environment for endogenous regenerative responses to proceed favorably. One of the key inadequacies lies in the physical tissue loss commonly observed to beleaguer the injured brain. Such tissue loss can arise from: 1) clearance of necrotic tissue without any accompanying mechanism to produce a tissue substitute; 2) atrophy of localized regions due to diffuse loss of neural cells and their associated extracellular matrix or; 3) surgical removal of compromised neural tissue. The implications of tissue loss on endogenous regenerative responses are twofold. First, expression of cytokines to engage the endogenous regenerative responses is lost with the physical neural tissue. Any remaining expression is limited to a far smaller volume of tissue in the peri-lesion area and fails
to drive endogenous regenerative responses optimally, spurring several efforts to perform intracerebral infusion of cytokines to augment the responses. Second, the loss of tissue typically creates a cavity that lacks stroma to support the migration of endogenous progenitors or in-growth of blood vessels. Even with infused cytokines to augment recruitment, progenitors and blood vessels can only migrate/grow up to the surrounding tissue and fail to invade the cavitary space to initiate any form of cell/tissue reconstitution.

Toward resolving these inadequacies in the injured brain to enhance endogenous regenerative responses, we have sought to develop a matrix that can: 1) be implanted into brain lesion to occupy the physical space resulting from brain tissue loss; 2) provide sustained release of an appropriate cytokine that can engage endogenous regenerative responses and; 3) provide structural support for the infiltration of endogenous cells. Gtn-HPA/SDF-1α-PCN matrices have demonstrated their efficacy in recruiting and supporting the migration of neural progenitors in vitro. We hypothesize that upon implantation into brain lesion to occupy the physical space resulting from brain tissue loss, Gtn-HPA/SDF-1α-PCN matrix can: i) release SDF-1α to influence or enhance neurogenesis and neovascularization in the surrounding brain tissue and; ii) re-establish a physical framework to enable recruited endogenous cells to repopulate the otherwise structureless brain lesion.

These hypotheses are tested in an animal model of intracerebral hemorrhage (ICH), which is a debilitating subtype of strokes that results from extravasation of blood into the brain parenchyma. From the clinical point of view, ICH is a significant brain injury that confronts numerous people suffering from hypertension, aneurysm and brain trauma and urgently needs the development of effective treatment approaches to improve outcomes after injury. Furthermore, current clinical management of ICH already includes stereotactic needle-based surgery to aspirate hemotoma. The existence of clinical procedures to physically access the brain lesion makes ICH a highly relevant type of brain injury that can readily benefit from the current work on injecting purposefully designed matrices into brain lesions. From the experimental point of view, modeling ICH in animals can be relatively straightforward with injection of either autologous blood or collagenase. The collagenase injection model is chosen here to better mimic the disruption of blood vessels, spontaneous extravasation of blood and hemotoma expansion. The location of the hemorrhage is easily controlled by the injection site of collagenase to induce
injury well below the dorsal surface of the brain, allowing the creation of deeply seated brain lesions to realistically evaluate the implantation of Gtn-HPA/SDF-1α-PCN matrices via stereotactic injection. The severity of the injury, and the subsequent size of the brain lesion, can also be consistently manipulated by the amount of collagenase injected, thus avoiding huge variations that may complicate comparative analysis among different treatment groups. Most importantly, numerous studies have already been performed on animal models of ICH. Details of injury-induced functional behavioral deficits, macroscopic changes (e.g. tissue loss and cavity formation) as well as endogenous regenerative responses (e.g. neurogenesis and neovascularization) are well characterized and available to inform our study on the value on implanting Gtn-HPA/SDF-1α-PCN matrices in the injured brain.
5.2 Materials and Methods

5.2.1 Experimental Design

All experiments were performed under protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committees (IACUC, protocol 2012N000165) following the NIH Guide for Use and Care of Laboratory Animals. A total of 30 male Sprague-Dawley Rats (250 - 300g) were subjected to intrastriatal injections of collagenase to induce ICH. They were then randomly selected to receive injections of artificial cerebrospinal fluid (aCSF; n=8), Gtn-HPA matrices (n=7); Gtn-HPA/PCN matrices (n=7) or Gtn-HPA/SDF-1α-PCN matrices (n=8) at 2 weeks post-ICH and sacrificed at 4 weeks post-ICH (Figure 5.1). Behavior tests were performed before ICH as well as at 3 days, 1, 2, 3 and 4 weeks after ICH. 5-Bromo-2'-Deoxyuridine (BrdU) was administered every other day for 2 weeks between ICH and aCSF/matrix injection.

<table>
<thead>
<tr>
<th>Timepoints</th>
<th>ICH</th>
<th>aCSF / Matrix Injection</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Behavior Test: Pre-ICH | Pre-Injection

BrdU: ↑↑↑↑↑↑↑↑↑↑

Figure 5.1: Time course for ICH, aCSF/matrix injection, behavior test, BrdU administration and animal sacrifice for rats treated with aCSF (n=8), Gtn-HPA matrix (n=7), Gtn-HPA/PCN (n=7) and Gtn-HPA/SDF-1α-PCN matrix (n=8).

5.2.2 Preparation of Gtn-HPA conjugate and SDF-1α PCNs

Gtn-HPA conjugate and SDF-1α PCNs were prepared as described in Section 2.2.1 and 3.2.1.
5.2.3 Collagenase-Induced Model of Intracerebral Hemorrhage

Rats were anesthetized using isofluorane (induction 3.5%, maintenance 1.5% to 2% in 70% N₂O and 30% O₂) and placed in a stereotaxic frame (Kopf, Tujunga, CA). Body temperature was monitored and maintained around 37°C during surgery using a rectal probe and a heating pad. A midline scalp incision was made to expose the skull. A small cranial burr hole was then drilled 0.5mm anterior and 3mm lateral to the bregma. The 25-Gauge needle of a 2μl Hamilton syringe was inserted 5mm ventral to the dural surface. 5 min after the insertion of the needle, 0.5 U Collagenase Type VII (Sigma Aldrich) in 2μl of 0.9% saline was injected into the striatum over 5 min. The needle was then left in place for 5 min and slowly withdrawn over 5 min. The burr hole was sealed with bone wax and the incision was subsequently sutured. The rats were allowed to recover from surgery in cages warmed under an incandescent bulb. Between ICH and aCSF/matrix injection, BrdU (50mg/kg) was administered via intraperitoneal injections every other day for a total of 7 times.
5.2.4 Stereotaxic Injection of aCSF and Matrices

Rats were placed in a stereotaxic frame using the same procedures for anesthesia and temperature monitoring/maintenance. A midline scalp incision was made to expose the cranial burr hole created in the previous surgery and bone wax removed to unseal the burr hole. 25μl of aCSF, pre-gel solution of Gtn-HPA matrix, pre-gel solution of Gtn-HPA/PCN matrix or pre-gel solution of Gtn-HPA/SDF-1α-PCN matrix (containing 6μg of SDF-1α) was loaded into a 50μl Hamilton syringe. Pre-gel solution of Gtn-HPA matrix was prepared immediately prior to loading by mixing Gtn-HPA solution with solutions of its cross-linking reagents, horseradish peroxidase (HRP) and H₂O₂. Pre-gel solutions of Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices were prepared with the addition of blank PCNs and SDF-1α PCNs respectively to the Gtn-HPA solutions prior to the mixing with the cross-linking reagents. The final composition of the matrices used is shown in Table 5.1. After loading of aCSF or pre-gel solutions of matrices, the 22-G needle of the Hamilton syringe was promptly lowered 5mm ventral to the dural surface. The loaded aCSF or pre-gel solution was injected into the ICH lesion over 3 min. After injection, the needle was left in place for 5 min and slowly withdrawn over 5 min. Similar to the previous surgery, the burr hole was sealed with bone wax, the incision sutured and the rats returned to cages warmed under an incandescent bulb.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Gtn-HPA</th>
<th>SDF-1α PCN</th>
<th>Horseradish peroxidase (HRP)</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gtn-HPA matrix</td>
<td>80mg/ml</td>
<td>-</td>
<td>70.0 mU/ml</td>
<td>6.8mM</td>
</tr>
<tr>
<td>Gtn-HPA/PCN matrix</td>
<td>80mg/ml</td>
<td>1.3mg/ml</td>
<td>70.0 mU/ml</td>
<td>6.8mM</td>
</tr>
<tr>
<td>Gtn-HPA/SDF-1α-PCN matrix</td>
<td>80mg/ml</td>
<td>1.3mg/ml*</td>
<td>70.0 mU/ml</td>
<td>6.8mM</td>
</tr>
</tbody>
</table>

Table 5.1: Final composition of matrices injected into 2-week-old ICH lesions. (*) corresponds to a SDF-1α concentration of 0.24 mg/ml that yields 6μg of SDF-1α in 25μl of injected matrices

5.2.5 Behavioral Testing

Behavioral testing was performed 1 day before ICH as well as 3 days, 1, 2, 3 and 4 weeks after ICH. Behavioral testing at 2 weeks after ICH was performed prior to the injection of
aCSF or matrices. All behavior tests were conducted in an isolated room with dim lighting and by personnel blinded to the experiment design.

**Modified Neurological Severity Scores**

Each rat was evaluated in a composite of motor, sensory, reflex and balance tests (Table 5.2) [22] where points were awarded for particular test outcomes, the inability to perform the test or the absence of reflexes. The overall neurological function was reflected on a scale of 0 to 18 where higher scores would denote higher severity of neurological impairment.

<table>
<thead>
<tr>
<th>Motor Test</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raising rat by tail (Normal = 0, Maximum = 3)</td>
<td></td>
</tr>
<tr>
<td>Flexion of Forelimb</td>
<td>1</td>
</tr>
<tr>
<td>Flexion of Hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>Head moved &gt;10° within 30s</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Placing rat on floor (Normal = 0, Maximum = 3)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal walk</td>
<td>0</td>
</tr>
<tr>
<td>Inability to walk straight</td>
<td>1</td>
</tr>
<tr>
<td>Circling toward the paretic side</td>
<td>2</td>
</tr>
<tr>
<td>Fall down to the paretic side</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensory Test (Normal = 0, Maximum = 2)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placing test (visual and tactile test)</td>
<td>1</td>
</tr>
<tr>
<td>Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb)</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Beam Balance Test (Normal = 0, Maximum = 6)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balances with steady posture</td>
<td>0</td>
</tr>
<tr>
<td>Grasps side of beam</td>
<td>1</td>
</tr>
<tr>
<td>Hugs the beam and one limb falls down from the beam</td>
<td>2</td>
</tr>
<tr>
<td>Hugs the beam and two limbs fall down from the beam or spins on beam (&gt; 60s)</td>
<td>3</td>
</tr>
<tr>
<td>Attempts to balance on the beam but falls off (&gt; 40s)</td>
<td>4</td>
</tr>
<tr>
<td>Attempts to balance on the beam but falls off (&gt; 20s)</td>
<td>5</td>
</tr>
<tr>
<td>Falls off: No attempt to balance or hang on to the beam (&lt; 20s)</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reflex absence and abnormal movements (Normal = 0, Maximum = 4)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinna reflex (head shake when touching the auditory meatus)</td>
<td>1</td>
</tr>
<tr>
<td>Corneal reflex (eye blink when lightly touching the cornea with cotton)</td>
<td>1</td>
</tr>
<tr>
<td>Startle reflex (motor response to a brief noise from snapping a clipboard paper)</td>
<td>1</td>
</tr>
<tr>
<td>Seizures, myoclonus, myodystony</td>
<td>1</td>
</tr>
</tbody>
</table>

**Maximum Points** 18

Table 5.2: Modified Neurological Severity Scores.
Forelimb Placing Test

Each rat was carried with the forelimb under evaluation hanging freely and brought to approach a tabletop sideways via a maneuver that brushed the ipsilateral vibrissae. Intact rats spontaneously place the forelimb onto the tabletop while rats with a brain injury contralateral to the stimulated vibrissae may, depending on the nature and extent of the injury, fail to do so. Each rat was tested 10 times for each forelimb. The forelimb placing score reflected the percentage of trials in which the rat placed the forelimb on the tabletop upon stimulation of the vibrissae.

Corner Turn Test

Two boards (each measuring 30cm × 30cm × 1cm) were attached along their shorter edge to form a 30° corner. Each rat was placed halfway into the corner. Upon entering the corner, both sides of the vibrissae were stimulated simultaneously, prompting the rat to rear (i.e. stand on rear limbs) and turn to face the open end of the corner. Uninjured rats turn randomly to either direction while injured rats turn to the direction ipsilateral to the brain injury. For each test, 10 trials, spaced at least 30s apart from one another, were performed and the percentage of ipsilateral turns was calculated.

5.2.6 Animal Sacrifice and Specimen Processing

Rats were deeply anesthetized using 5% isofluorane and sacrificed via transcardial perfusion with 120ml of 0.9% saline followed by 120ml 4% paraformaldehyde solution. Brains were extracted and submerged into Tissue Tek O.C.T compound contained in a tinfoil mold. The brains were then flash-frozen using isopentane cooled on liquid nitrogen and cryosectioned coronally at 30μm. The tissue sections were mounted on treated glass slides, air-dried and stored at -20°C. Prior to histological and immunofluorescent staining, the tissue sections were baked at 50°C for 1 hr to increase adhesion to the glass slides, rehydrated in PBS, permeabilized in ice-cold methanol for 10 min and washed with PBS.

5.2.7 Histology

Tissue sections were stained with hematoxylin and eosin (H&E) for visualization of brain tissue and implanted matrices. The procedure included staining in Gill's hematoxylin (10
min), rinsing in water (5 min), differentiation in acid alcohol (1 s), rinsing in water (5 min), staining in eosin (1 min), dehydration in 100% alcohol (2 x 2 min), processing in xylene (2 x 3 min) and mounting of coverslip using cytoseal. Brightfield imaging was performed on the stained cryosections using an Olympus BX51 microscope. For each rat, histomorphometric analysis was performed on 7 cryosections which were taken at 1mm intervals from -3mm to 3mm anterior to bregma to span over the lesion and/or implanted matrices. Areas of both hemispheres, both ventricles, both cavitary and non-cavitary lesions resulting from ICH as well as implanted matrix were measured using ImageJ. Volume of brain tissue loss for each hemisphere was calculated using Equation 4.1 – 4.3 from the previous chapter. The area of stroma in each hemisphere, which is defined with the physical framework in both brain tissue and implanted matrices, was calculated using Equation 5.1 and linearly integrated using Equation 5.2 to determine the stromal volume.

\[
\text{Stromal area} = \text{Area of hemisphere} - \text{Area of ventricle} - \text{Area of cavitary lesion}
\]

Equation 5.1

\[
\text{Stromal volume} = \sum_{i=-3}^{3} (\text{Stroma area})_i \times 1
\]

Equation 5.2

H&E-stained sections were also analyzed for the presence of neutrophils. Neutrophils were identified as cells with multi-lobulated nuclei. The presence of neutrophils was scored using a rating scale of 0-3 where 0 denotes an absence of neutrophils while 1, 2 and 3 denote mild, moderate and significant presence respectively.

5.2.8 Fluorescence Immunohistochemical Analysis

When necessary, antigen retrieval was performed by: 1) heating tissue sections in a pressure cooker (Biocare Medical, Concord, CA); 2) cooling to room temperature over 30 min and; 3) washing with PBS. All subsequent staining procedures were performed at room temperature and all washes with PBS unless otherwise mentioned. Tissue sections were blocked with 5% donkey serum/0.3% triton-X100 in PBS for 1 hr and incubated overnight with primary antibodies at 4°C. Following 3 washes, tissue sections were incubated with secondary antibodies (dilution 1:400) for 2 hrs. Tissue sections were then washed twice, incubated with 100ng/ml 4',6-
diamidino-2-phenylindole (DAPI) (in PBS) for 5 min and washed once. Sections subjected to antigen retrieval were further incubated in 0.1% Sudan Black B (in 70% ethanol) for 30 min to reduce tissue autofluorescence and washed thoroughly. All tissue sections were eventually mounted with coverslips using Fluorescence Mounting Medium (Dako). In the particular case of detecting surface expression of CXCR4, tissue sections were not permeabilized with methanol or triton-X100 and blocked with 5% donkey serum in PBS. Details of the antibodies used and their associated antigen retrieval procedure are listed in Table 5.3. The stained sections were imaged using an epifluorescence microscope (Olympus BX60) or confocal laser scanning microscope (Zeiss LSM 510) and analyzed using ImageJ.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
<th>Secondary Antibody</th>
<th>Cells Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Polyclonal Anti-Doublecortin</td>
<td>1:2000</td>
<td>Citrate Buffer (pH 6) 100°C 20min</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Neuronal Precursors</td>
</tr>
<tr>
<td>(Abcam ab18723)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Monoclonal Anti-NeuN (Millipore MAB377)</td>
<td>1:250</td>
<td>Citrate Buffer (pH 6) 110°C 30min</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Mature Neurons</td>
</tr>
<tr>
<td>Sheep Polyclonal Anti-BrdU (Abcam ab1893)</td>
<td>1:250</td>
<td>Citrate Buffer (pH 6) 110°C 30min</td>
<td>Cy3 Donkey Anti-Sheep IgG (Jackson Immunoresearch 713-165-147)</td>
<td>Cells that proliferated during BrdU administration</td>
</tr>
<tr>
<td>Rabbit Polyclonal Anti-von Williebrand Factor (Dako A0082)</td>
<td>1:100</td>
<td>Tris-EDTA Buffer (pH 9) 100°C 20min</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Goat Polyclonal Anti-CD34 (R&amp;D Systems AF4117)</td>
<td>1:40</td>
<td>Tris-EDTA Buffer (pH 9) 100°C 20min</td>
<td>Dylight 488 Donkey Anti-Goat IgG (Jackson Immunoresearch 705-485-003)</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Rabbit Polyclonal Anti-Glial Fibrillary Acidic Protein (Dako Z0334)</td>
<td>1:1000</td>
<td>None</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>Mouse Polyclonal Anti-CD68 (Abcam ab31630)</td>
<td>1:400</td>
<td>None</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Activated macrophages and microglia</td>
</tr>
<tr>
<td>Rabbit Polyclonal Anti-CXCR4 (Abcam ab2074)</td>
<td>1:250</td>
<td>Tris-EDTA Buffer (pH 9) 110°C 20min</td>
<td>Dylight 488 Donkey Anti-Rabbit IgG (Jackson Immunoresearch 711-485-152)</td>
<td>Various cell types that express CXCR4</td>
</tr>
<tr>
<td>Mouse Monoclonal Anti-VEGF (Abcam ab1316)</td>
<td>1:100</td>
<td>Citrate Buffer (pH 6) 110°C 20min</td>
<td>Dylight 488 Donkey Anti-Mouse IgG (Jackson Immunoresearch 715-485-151)</td>
<td>Various cell types that express VEGF</td>
</tr>
</tbody>
</table>

Table 5.3: List of antibodies and their associated antigen retrieval procedures for fluorescent immunohistochemical analysis.

### 5.2.9 Statistical Analysis

Values are expressed as mean ± standard error mean (SEM). Analyses of variance (ANOVA) and Fisher’s post-hoc test were performed accordingly. Statistical significance was set at \( p < 0.05 \).
5.3 Results

5.3.1 Behavioral Outcomes

To evaluate the effects of Gtn-HPA/SDF-1α-PCN matrices on outcomes after ICH, rats were subjected to ICH and randomly grouped to receive injections of aCSF, Gtn-HPA matrix, Gtn-HPA/PCN matrix or Gtn-HPA/SDF-1α-PCN matrix. Functional behavioral outcomes of the rats were assessed by mNSS, forelimb placing test and corner turn test.

For mNSS, the scores of rats in all groups were nearly 0 pre-ICH and spiked to around 6 after ICH, indicating the appearance of neurological impairment upon the onset of injury to the brain (Figure 5.2). Over week 2 - 4 after ICH, scores were significantly different over time and across different treatments (2-factor ANOVA, \( p < 0.001 \) for both). Specifically, scores remained similarly elevated across all groups at week 2 post-ICH prior to treatment injection. In the subsequent 2 weeks, scores for the aCSF, Gtn-HPA matrix and Gtn-HPA/PCN matrix groups declined slightly in a similar fashion while scores for the Gtn-HPA/SDF-1α-PCN matrix group declined more rapidly to be significantly lower at week 3 and 4 after ICH (Fisher’s post-hoc test, \( p < 0.01 \) and \( p < 0.05 \) compared to all other groups).

Figure 5.2: Modified Neurological Severity Scores (mNSS). A decrease in the score suggests behavioral recovery. * \( p < 0.05 \), ** \( p < 0.01 \), Fisher’s post-hoc test.
A similar trend was observed with the forelimb placing test. Before ICH, all rats successfully placed their contralateral forelimb with every stimulus, thus giving a score of 100% (Figure 5.3). Forelimb placing reduced drastically to below 20% after ICH. Over week 2 - 4 after ICH, scores were significantly dependent on time and the type of treatment (2-factor ANOVA, \( p < 0.05 \) for both). Specifically, scores remained similarly low across all groups at week 2 post-ICH prior to treatment injection but improved subsequent to treatment injections. However, scores for the Gtn-HPA/SDF-1α-PCN matrix group improved more rapidly and reached the highest among all groups and time points at week 4 after ICH (Fisher’s post-hoc test, \( p < 0.05 \) compared to aCSF).

![Graph showing forelimb placing score](image)

**Figure 5.3:** Forelimb placing score. An increase in the score suggests behavioral recovery.

\* \( p < 0.05 \), Fisher’s post-hoc test.

In the corner turn test, rats randomly turned to their ipsilateral side approximately 50% of the time before ICH (Figure 5.4). Turns became primarily directed toward the ipsilateral side after ICH. Over week 2 - 4 after ICH, the percentage of turns toward the ipsilateral side was significantly dependent on time and the type of treatment (2-factor ANOVA, \( p < 0.01 \) and \( p < 0.05 \) respectively). Specifically, rats in all groups continued to turn primarily to the ipsilateral side. Rats treated with aCSF, Gtn-HPA matrix and Gtn-HPA/PCN matrix failed to recover in the subsequent weeks while rats treated with Gtn-HPA/SDF-1α-PCN matrix showed a modest recovery at week 4 after ICH (Fisher’s post-hoc test, \( p < 0.01 \) compared to both aCSF and Gtn-HPA matrix).
Figure 5.4: Percentage of turns made towards the impaired side in the corner turn test. An increase in the percentage toward 50% suggests behavioral recovery. ** \( p < 0.01 \), Fisher's post-hoc test.

5.3.2 Gross Histological Outcomes

At 4 weeks after ICH, the lesion in rats treated with aCSF presented as either a cavity or a condensed region of tissue that was intensely stained by hematoxylin (referred hereafter as cavitary and non-cavitary lesion respectively; Figure 5.5A). The cavitary lesion was completely void of physical matter under histological staining but was presumably filled with fluid prior to histological processing based on magnetic resonance images in other studies [23, 24] (Figure 5.6A). On the other hand, the non-cavitary lesion consisted of hypercellular tissue with fine trabeculae and hemosiderin-filled cells (Figure 5.6B). In both cases, the hematoma had largely resolved and the injured hemisphere shared several characteristics already observed at 2 week post-ICH, namely: 1) enlargement of the ipsilateral ventricle; 2) reduction in hemispheric size and; 3) overall asymmetry with the contralateral side.
Figure 5.5: Micrographs showing H&E-stained sections with (A) aCSF-treated (left) cavitary and (right) non-cavitary lesions, (B) Gtn-HPA matrix-treated lesions, (C) Gtn-HPA/PCN matrix-treated lesions and (D) Gtn-HPA/SDF-1α-PCN matrix-treated lesions. Bar: 1mm.
Hematoma had also mostly resolved in the injured hemispheres of rats treated with Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices (Figure 5.5B-D). The injured hemispheres were, however, notably distinct from those of rats treated with aCSF in several other aspects. Instead of empty space or necrotic-looking tissue, the lesion was substantially filled with an implant material that was distinguishable from the surrounding host tissue. In addition, the injured hemispheres mostly maintained the overall structural symmetry that had been achieved with the injection of matrices at 2 week post-ICH and did not suffer the same ventricular enlargement or hemispheric size reduction as those treated with aCSF. All the implanted Gtn-HPA-based matrices shared similar characteristics of being a physical structure weakly stained by eosin and featuring a porous morphology that had likely resulted from histological processing (Figure 5.6C-E). The matrices were also physically continuous with apposing host tissues and had
evidently maintained the tight interface achieved right after injection of matrix at 2 week post-ICH. In several locations within the striatum, the implanted matrices could be seen to directly appose host tissues with distinct white and gray matter structures. Implanted Gtn-HPA matrices were mostly void of cells. In contrast, the implanted Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices contained numerous host cells. The latter was also found to contain several vessel-like structures.

The volume of stroma was measured to assess the physical framework present in the host brain tissue (minus empty spaces such as ventricles and cavitary lesion) and implanted matrices. When treated with aCSF alone, the volume of stroma in the injured hemispheres of rats was reduced to $92.0 \pm 0.5\%$ as compared to the contralateral side (Figure 5.7A). Injection of Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices substantially diminished this reduction and significantly increased the volume of stroma to $96.2 \pm 0.6\%$, $95.8 \pm 0.8\%$ and $98.2 \pm 0.9\%$ respectively relative to the contralateral side (Fisher’s post-hoc test, $p < 0.01$).

The volume of intact brain tissue (defined to exclude empty spaces, necrotic-looking tissue or implanted matrices) was also measured to determine brain tissue loss, which has been known to occur progressively over time after ICH [23]. Brain tissue loss in rats treated with aCSF was found to reach $32.7 \pm 2.7 \text{ mm}^3$ at 4 weeks post-ICH (Figure 5.7B). Injection of Gtn-HPA and Gtn-HPA/PCN matrices did not produce any significant effect on brain tissue loss. In contrast, injection of Gtn-HPA/SDF-1α-PCN matrix significantly reduced brain tissue loss by 37.7% to $20.4 \pm 3.7 \text{ mm}^3$ (Fisher’s post-hoc test, $p < 0.05$).
Figure 5.7: (A) Relative volume of stroma as a percentage of contralateral hemisphere. (B) Tissue loss in the injured hemisphere. *p < 0.05, **p < 0.01, ***p < 0.001, Fisher’s post hoc test.

5.3.3 Endogenous Neurogenesis in Host Tissue

Doublecortin was used as the marker to identify neuronal precursors (i.e. neuronal-restricted progenitors and immature neurons). In the contralateral (i.e. uninjured) hemispheres of rats across all groups, the DCX+ cells in the subventricular zone (SVZ) existed as a thin lining along most of the lateral border of the ventricle and as a small wedge-shaped aggregation of contiguous cells at the dorsal tip of the ventricle (Figure 5.8). Very few DCX+ cells were observed outside the subventricular zone. ICH injury resulted in marked changes to the population of DCX+ in the ipsilateral hemisphere. As seen in rats treated with aCSF, the overall population of DCX+ cells had increased substantially.
Figure 5.8: Fluorescence micrographs showing DCX immunoreactivity in the contralateral and ipsilateral hemispheres of 2 representative rats in the (A) aCSF, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix. v: ventricle. Bar: 500μm.

This increase manifested as a thicker lining of DCX+ cells in certain regions of SVZ and a significantly larger aggregation at the dorsal tip of the ventricle. Several more DCX+ cells were in the striatum although the majority of the DCX+ cells still remained in the SVZ. Such injury-induced changes to the population of DCX+ cells were also observed in rats treated with Gtn-HPA and Gtn-HPA/PCN matrices. An outstanding difference was, however, noted in rats injected with Gtn-HPA/SDF-1α-PCN matrices. In the aCSF, Gtn-HPA matrix and Gtn-HPA/PCN groups, most DCX+ cells remained as relatively rounded cells in a contiguous lining or aggregation in the SVZ.
However, the majority of the DCX$^+$ cell population in ipsilateral hemispheres injected with the Gtn-HPA/SDF-1α-PCN matrix was observed as discrete cells scattered across the injured striatum (Figure 5.8D). Unlike the contiguous cells within the SVZ, these discrete cells were mostly elongated with long processes (Figure 5.9D).

![Figure 5.9](image-url)

Figure 5.9: Fluorescence micrographs showing DCX$^+$ neuronal precursors as contiguous cells in the SVZ of the (A) aCSF, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix groups and as discrete elongated cells in the striatum of the (D) Gtn-HPA/SDF-1α-PCN matrix. Bar: 50μm.

The area and distribution of the DCX$^+$ cell population in the SVZ and striatum were analyzed as illustrated by the schematic in Figure 5.10A. The area occupied by DCX$^+$ cells in the contralateral hemispheres was similar across all four treatment groups at ~ 0.17 mm$^2$ (Figure 5.10B). In comparison, the total area occupied by DCX$^+$ cells in the ipsilateral hemispheres of rats was also similar across all treatment groups but had increased significantly by ~5-fold to ~ 0.78 mm$^2$ (Fisher’s post-hoc test, $p < 0.01$). In terms of distribution, the proportion of DCX$^+$ area in the contralateral striatum was measured to be ~ 9 -11% and was similar across all treatment groups (Figure 5.10C). In contrast, the proportion of DCX$^+$ area in the ipsilateral striatum had increased significantly in all four treatment groups.

The increase in both the aCSF, Gtn-HPA and Gtn-HPA/PCN matrix groups was similar, bringing the proportion of DCX$^+$ area in the ipsilateral striatum to ~ 36 - 45%. On the other hand, the increase in Gtn-HPA/SDF-1α-PCN matrix group was notably larger. Proportion of DCX$^+$ area in the ipsilateral striatum of the Gtn-HPA/SDF-1α-PCN matrix group reached 64%, which was significantly higher than that for the other treatment groups (Fisher’s post-hoc test, $p < 0.05$). Despite the distribution shift of DCX$^+$ neuronal progenitors into the striatum, the vast majority of them were observed up to approximately the midpoint between the SVZ and the lesion/implanted matrix in all treatment groups.
Figure 5.10: (A) Schematic depicting regions in the SVZ and striatum where areas of DCX immunoreactivity are analyzed. (B) Total area covered by DCX⁺ cells (expressed in mm²). (C) Percentage of DCX⁺ area observed in the striatum. * p < 0.05, ** p < 0.01, *** p < 0.001, Fisher’s post hoc test.
Double immunohistochemical staining for BrdU and NeuN was performed to identify newly generated mature neurons in the striatum. The number of BrdU⁺/NeuN⁺ cells was relatively few and only observed in striatal regions near the SVZ (Figure 5.11). Quantification showed the density to be around 10 BrdU⁺/NeuN⁺ cells per mm² across all groups (Figure 5.12).

Figure 5.11: Micrographs showing NeuN (green) and BrdU (red) immunoreactivity in the striatum of rats in the (A) aCSF, (B) Gtn-HPA, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix groups. Arrows indicates cells that are immunoreactive for both BrdU and NeuN. Bar: 50µm.
Figure 5.12. Number of BrdU+/NeuN+ cells per mm² in the striatum near the SVZ.

5.3.4 Neovascularization in Host Tissue

Regardless of the treatment group, all the contralateral hemispheres were generally marked with thin blood vessels that were uniformly all scattered throughout the brain parenchyma (Figure 5.13). In contrast, blood vessels in the ipsilateral hemispheres were thicker and appeared to be of greater numbers, especially in areas near the lesion or implanted matrix. Quantitative analysis revealed that the length density of blood vessels in the contralateral hemispheres were ~ 2 mm per mm² across all the treatment groups (Figure 5.14). Relative to this, the length densities of blood vessels in the ipsilateral hemispheres across the various treatment groups were all significantly higher (Fisher’s post-hoc test, \( p < 0.05 \) or \( p < 0.001 \)). Specifically, the length densities of blood vessels in the ipsilateral hemispheres of the aCSF, Gtn-HPA matrix and Gtn-HPA/PCN matrix groups had increased significantly to 3.5, 3.7 and 3.3 mm per mm² respectively (Fisher’s post-hoc test, \( p < 0.05 \) and \( p < 0.001 \) respectively relative to contralateral hemispheres) but were statistically indistinct from each other. On the other hand, the length density of blood vessels in the ipsilateral hemispheres of the Gtn-HPA/SDF-1α-PCN matrix increased to reach 4.9 mm per mm² (Fisher’s post-hoc test, \( p < 0.001 \) relative to contralateral hemispheres), which was also significantly higher than length density of blood vessels in the ipsilateral hemispheres in the other groups (Fisher’s post-hoc test, \( p < 0.01 \) respectively).
Figure 5.13. Micrographs showing vWF+ vessels around (*) lesion/implanted matrices in the ipsilateral hemisphere and corresponding regions in the contralateral hemisphere of rats treated with (A) aCSF, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Bar: 100μm.
Figure 5.14. Length density of vWF⁺ blood vessels in brain tissue around the lesion/implanted matrices.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Fisher’s post hoc test.
5.3.5 Astrocytic Response in Host Tissue

Numerous GFAP$^+$ astrocytes were observed around all lesions/implanted matrices (Figure 5.15). Within approximately 200-300 µm of the lesion/matrix boundary, density of GFAP$^+$ astrocytes was so high that GFAP$^+$ reactivity was largely continuous and individual astrocytes could not be easily demarcated. In the host tissue outside this dense band, GFAP$^+$ astrocytes mostly existed as scattered cells. Some were in the form of enlarged, rounded cells with short, thick processes while others appeared as elongated cells with long, thin processes. Quantitative analysis of the area covered by GFAP$^+$ astrocytes indicated no significant difference among the different treatment groups (Figure 5.16).

Figure 5.15: Micrographs showing GFAP immunoreactivity around (*) lesion/implanted matrices in the ipsilateral hemisphere of rats treated with (A) aCSF, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Bar: 200µm.
5.3.6 Neutrophils and Activated Macrophages/Microglia in Host Tissue

The host tissue was assessed any acute and chronic inflammatory cells in response to the ICH injury and/or the implanted matrices. Across all treatment groups, the presence of neutrophils around the lesion and implanted matrices was very mild (Figure 5.17). Specifically, ~0-2 neutrophils were observed for the aCSF and Gtn-HPA matrix groups in a typical field of view (using a 40x objective) while ~3-5 neutrophils were observed for the Gtn-HPA/SDF-1α-PCN matrix group. These were consistent with the absence of necrotic cells/tissues in the host tissue, which are common triggers of acute inflammation. The identified neutrophils were largely found within 50 μm of the lesion/matrix boundary. Overall, the various groups were scored as either 0 or 1 (Figure 5.18)
Figure 5.17: Micrographs of H&E-stained sections showing the brain tissue in contact with (A) aCSF-treated lesion, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Arrows denote the neutrophils identified. Bar: 50µm.

Figure 5.18: Rating for the presence of neutrophils in brain tissue around the lesion/implanted matrices.
On the other hand, immunostaining for CD68 showed a strong presence of activated macrophages/microglia around the lesion and/or implanted matrices (Figure 5.19). Around both cavitary and non-cavitary lesions in the aCSF group, the activated macrophages/microglia were predominantly enlarged, spherical/spheroid cells without any processes. Numerous macrophages/microglia were found to contain hemosiderin or to localize around extracellular deposits of hemosiderin, which was easily visualized with its intense autofluorescence. In contrast, activated macrophages/microglia in the host tissue around implanted Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices possessed a small soma with a few ramified processes. Quantitative analysis showed that the area fraction occupied by activated macrophages/microglia was similar across all the treatment groups (Figure 5.20).

Figure 5.19: Micrographs showing CD68 immunoreactivity around (*) lesion/implanted matrices in the ipsilateral hemisphere of rats treated with (A) aCSF, (B) Gtn-HPA matrix, (C) Gtn-HPA/PCN matrix and (D) Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Bar: 200μm.
5.3.7 Endogenous Neuronal Precursors in Lesion/Implanted Matrices

Both cavitary and non-cavitary lesions in the aCSF group as well as the implanted matrices in the Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrix groups were examined for any infiltration by DCX\(^+\) cells. Cavitary lesions were void of any physical framework and did not contain any cells (Figure 5.21A). No DCX\(^+\) cell was also observed in the non-cavitary lesion, Gtn-HPA matrix and Gtn-HPA/PCN matrix (Figure 5.21B-D). On the other hand, DCX\(^+\) cells could be found in the Gtn-HPA/SDF-1α-PCN matrices near the interface with the host brain tissue for 4 out of 8 rats in the group (Figure 5.21D). Their presence was generally meager, consisting of only 1 - 5 cells per brain tissue section from each rat.
Figure 5.21: Fluorescent micrographs showing (green) DCX immunoreactivity, (blue) DAPI nuclei staining and their merger for (A) aCSF-treated cavitary lesion, (B) aCSF-treated non-cavitary lesion, (C) lesion treated with Gtn-HPA matrix, (D) lesion treated with Gtn-HPA/PCN matrix and (E) lesion treated with Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Arrows denote DCX+ cells that had successfully migrated into the Gtn-HPA/SDF-1α-PCN matrix. Bar: 100μm.
5.3.8 Blood vessels in Lesion/Implanted Matrices

Cavitary lesions did not contain any blood vessel (Figure 5.22A) while several blood vessels were found to persist in the tissue within non-cavitary lesions (Figure 5.22B). For the implanted matrices, Gtn-HPA and Gtn-HPA/PCN matrices remained void of blood vessels (Figure 5.22C) while an appreciable number of blood vessels had grown into Gtn-HPA/SDF-1α-PCN matrices (Figure 5.22D). Quantitative analysis revealed that the vessel length of blood vessels per mm² were statistically comparable between the non-cavitary lesions and the Gtn-HPA/SDF-1α-PCN matrices (Figure 5.23).

Figure 5.22: Micrographs showing vWF⁺ blood vessels within (A) aCSF-treated cavitary lesion, (B) aCSF-treated non-cavitary lesion, (C) lesion treated with Gtn-HPA matrix, (D) lesion treated with Gtn-HPA/PCN matrix and (E) lesion treated with Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Bar: 200μm.
Figure 5.23: Length density of vWF⁺ blood vessels within lesions/implanted matrices.
5.3.9 Astrocytes in Lesion/Implanted Matrices

Immunostaining for GFAP revealed the absence of astrocytes within the lesion or implanted matrices (Figure 5.24).

Figure 5.24: Micrographs showing absence of GFAP⁺ immunoreactivity within (A) aCSF-treated cavitary lesion, (B) aCSF-treated non-cavitary lesion, (C) lesion treated with Gtn-HPA matrix, (D) lesion treated with Gtn-HPA/PCN matrix and (E) lesion treated with Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. Bar: 200μm.
5.3.10 Neutrophils and Activated Macrophages/Microglia in Lesion/Implanted Matrices

No neutrophil was observed in the cavitary lesion (Figure 5.25A) while a trace number of them was found throughout the non-cavitary lesion in the midst of hemosiderin-laden macrophages (Figure 5.25B). A trace number of neutrophils was also noted within implanted Gtn-HPA matrices but was mostly restricted within 50 μm of the interface between the host tissue and matrix (Figure 5.25C). In contrast, significant numbers of neutrophils were observed throughout the implanted and Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices (Figure 5.25D & E). They occurred as either scattered cells near the tissue/matrix interface, clusters that were closely associated with in-grown blood vessels (Figure 5.26A) or dense pools of cells that were typically located away from the tissue matrix interface (Figure 5.26B). When immunostaining for CXCR4, the receptor for SDF-1α, was performed without methanol- or detergent-mediated permeabilization in order to evaluate surface expression, many neutrophils were stained positively for CXCR4 (Figure 5.27A). CXCR4 staining was also observed to be punctate and localized to the boundary of the cells (Figure 5.27B). Many neutrophils were also stained positive for vascular endothelial growth factor (VEGF; Figure 5.28). Overall, the presence of neutrophils in lesions treated with aCSF and Gtn-HPA matrix was rated as 0-1 while that in Gtn-HPA/SDF-1α-PCN matrix was mostly rated as 3 (Figure 5.32).
Figure 5.25: Micrographs of H&E-stained sections showing (A) aCSF-treated cavitary lesion, (B) aCSF-treated cavitary lesion, (C) lesion treated with Gtn-HPA matrix, (D) lesion treated with Gtn-HPA/PCN matrix and (E) lesion treated Gtn-HPA/SDF-1α-PCN matrix. Neutrophils with multi-lobulated nuclei were found to be a common cell type within Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices. Bar: 50μm.

Figure 5.26: Micrograph showing neutrophils (A) closely associated with newly formed vasculature and (B) in clusters within Gtn-HPA/SDF-1α-PCN matrix. Bar: 50μm.
Figure 5.27: Immunostaining for CXCR4 was performed without cell permeabilization. (A) Micrograph showing CXCR4 immunoreactivity on a cluster of neutrophils within Gtn-HPA/SDF-1α-PCN matrix. Bar: 20μm. (B) Confocal 3D reconstruction of neutrophils showing CXCR4 immunoreactivity. Reconstructed orthogonal images are presented as viewed in the x-z (top) and y-z (right) planes.

Figure 5.28: Micrograph showing VEGF immunoreactivity on a cluster of neutrophils within Gtn-HPA/SDF-1α-PCN matrix. Bar: 100μm.
Activated macrophages/microglia were present in non-cavitary lesions and Gtn-HPA/SDF-1α-PCN matrices (Figure 5.30). Activated macrophages/microglia were noticeably more prevalent in non-cavitary lesions than in Gtn-HPA/SDF-1α-PCN matrices. Quantitative analysis revealed that area fraction occupied by the activated macrophages/microglia was 30.5 ± 8.9 % in the non-cavitary lesions but was significantly lower at 4.2 ± 1.0 % in the Gtn-HPA/SDF-1α-PCN matrices (p <0.001, Fisher’s post-hoc test; Figure 5.31). Within the Gtn-HPA/SDF-1α-PCN matrices, activated macrophages/microglia were observed to be scattered cells (Figure 5.30E) or closely associated with neutrophil clusters (Figure 5.32).
Figure 5.30: Micrographs showing absence of CD68⁺ immunoreactivity within (A) aCSF-treated cavitary lesion, (B) aCSF-treated non-cavitary lesion, (C) lesion treated with Gtn-HPA matrix, (D) lesion treated with Gtn-HPA/PCN matrix and (E) lesion treated with Gtn-HPA/SDF-1α-PCN matrix. Dotted line denotes interface between host tissue and lesion/implanted matrices. CD68⁺ macrophages/microglia were mostly observed in aCSF-treated non-cavitary lesion as well as both Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices. Bar: 200μm.
Figure 5.31: Percentage of area covered by CD68+ macrophages/microglia within the lesions/implanted matrices. *** p < 0.001, Fisher's post-hoc test.

Figure 5.32: Micrograph showing some CD68+ macrophages/microglia to be closely associated with cell clusters that were previously identified to be neutrophils. Bar: 200μm
5.4 Discussion

In this chapter, we implanted Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices into brain lesions resulting from ICH injury and evaluated their effects alongside with lesions that were only treated with aCSF. The foremost assessment we conducted was the evaluation of behavior recovery, given that the most tangible and important goal of any strategy to treat brain injury is to reduce neurological impairments and their devastating effects on quality of life. We had selected a battery of three tests, namely mNSS, forelimb placing test and corner turn test for various reasons. mNSS covers a spectrum of sensory functions, motor skills, balance and reflexes and provides the means to obtain a perspective of the overall neurological well being of the animal. Forelimb placing test is a relatively more focused evaluation that requires the animal to display its ability to integrate sensory and motor functions to complete the task of sensing a table edge and placing its limb on the surface. Corner turn test requires the animal to approach and sense the corner, execute a turn based on its postural symmetry and emerge from the corner. By examining the ability of the animal to perform a multi-step task, it is highly sensitive and can detect long-term impairment [25]. In our study, animals with the control treatment of aCSF were on the trajectory of behavior recovery after ICH injury in terms of the tasks in mNSS and forelimb placing test. Such spontaneous behavior recovery is commonly observed across various forms of brain injury and has been attributed to the return of functions to neural cells that suffer but survive the initial injurious event and to neuro-plasticity that allows certain functional behaviors to be re-wired to alternative neural transmission pathways. We also found that the impairment determined by corner turn test failed to resolve to any degree, suggesting that within a certain time frame, spontaneous recovery might be either inadequate or incapable of providing noticeable improvements for relatively more challenging behaviors. Our data showed that implantation of Gtn-HPA/SDF-1α-PCN matrices accelerated the spontaneous recovery observed in mNSS and forelimb placing test and initiated improvements in the corner turn test. SDF-1α PCNs had appeared to have an integral role in these effects, given that Gtn-HPA and Gtn-HPA/PCN matrices failed to elicit the same benefits. These findings are a definitive statement that Gtn-HPA/SDF-1α-PCN matrices are beneficial to the injured brain after ICH. They also add to gathering evidence that appropriate intervention can produce functional improvements even when applied in a delayed setting after brain injury. The observed enhancement in behavioral recovery might have been orchestrated by the multiple effects of the
Gtn-HPA/SDF-1α-PCN matrices, some of which we have observed in our study and will discuss below. However, the exact mechanism by which Gtn-HPA/SDF-1α-PCN matrices enhanced behavioral recovery is difficult to pinpoint, given the highly sophisticated and largely unknown processes underlying functional behavior.

At the gross histological level, lesions resulting from ICH injury were found to present either a cavitary or non-cavitary appearance. The non-cavitary appearance could be due to incomplete liquefaction of the dying brain tissue, structural collapse of surrounding brain tissue into a cavitary lesion or a combination of both. In either case, the benefit of implanting Gtn-HPA-based matrices was apparent; such matrices served as a persistent physical framework in the injured brain and restored its stromal volume. Importantly, the matrices remained tightly interfaced with the host brain tissue, where the absence of necrotic cells/tissues as well as the preservation of distinct white and gray matter structures demonstrated that the matrices could reside in the brain without causing any deleterious effect. The value of implanting matrices to provide stroma is significant given that: 1) the mature brain typically undergoes loss of neural tissue and its associated stroma after injury and; 2) a compensatory physical framework usually does not materialize spontaneously or in response to stimulation by drugs/growth factors (unless the lesion is physically connected with the meninges and can be readily infiltrated with matrix-producing fibroblasts). While this restoration of stromal volume does not necessarily equate recovery of lost tissues, the provision of a physical framework is a pre-requisite for enabling spontaneous or stimulated tissue remodeling and is of potential benefit to the injured brain. On top of restoring stromal volume, Gtn-HPA/SDF-1α-PCN matrices, but not Gtn-HPA or Gtn-HPA/PCN matrices, were found to have the additional benefit of dampening the progressive loss of tissue after the initial ICH injury and resulting in a reduced volume of tissue loss by the time of our evaluation at 4 weeks after ICH. Their effects on tissue loss could have occurred through reported neuroprotective effects of SDF-1α and/or the spectrum of regenerative effects to be discussed below.

One of our hypotheses in this study was that Gtn-HPA/SDF-1α-PCN matrix, when implanted into brain lesions, could enhance the endogenous neurogenic response and neovascularization in the surrounding brain tissue. Similar to numerous reports on neurogenesis in the injured brain, ICH triggered an endogenous neurogenic response in our study. Using DCX+
area as indicator for the presence of endogenous neuronal precursors [26], the endogenous neurogenic response in the injured hemisphere of aCSF-treated animals was found to mount a ~5-fold increase in the presence of neuronal progenitors over the baseline detected in the contralateral hemisphere. This robust increase was similar to numerous reports on endogenous neurogenesis in the injured brain and was likely mediated through injury-induced expression of a plethora of mitogens such as FGF [27], SCF [28] and erythropoietin [29]. The endogenous neurogenic response also included a second dimension where a higher proportion of DCX+ neuronal precursors exited the SVZ and migrated into the injured striatum, although the majority of the newly generated DCX+ neuronal precursors still remained as contiguous cells in the SVZ. Gtn-HPA, Gtn-HPA/PCN and Gtn-HPA/SDF-1α-PCN matrices did not increase the total pool of DCX+ neuronal precursors. In the case of Gtn-HPA/SDF-1α-PCN matrices, this observation was in line with the reportedly weak mitogenic effect of SDF-1α [30] as well as our in vitro finding that SDF-1α did not increase the proliferation of adult neural progenitor cells beyond the level achieved with a strong mitogen such as FGF-2 (Section 3.3.5). On the other hand, Gtn-HPA/SDF-1α-PCN matrices profoundly influenced the endogenous neurogenic response in terms of the distribution of DCX+ neuronal precursors between the SVZ and striatum. In the presence of Gtn-HPA/SDF-1α-PCN matrices, a substantially higher proportion of DCX+ neuronal precursors were dispersed over a wide area in the striatum with stereotypic migratory morphology [31], strongly suggesting that they had been actively recruited out of the SVZ. Such a distribution shift was absent with the implantation of Gtn-HPA and Gtn-HPA/PCN matrices. This ruled out the possibility that the physical presence of Gtn-HPA matrices and PCNs in the striatum might elevate endogenous expression of chemokines that could attract neuronal precursors and pointed to SDF-1α PCNs as the primary driver of the observed effect.

Our observation that Gtn-HPA/SDF-1α matrices induced a distribution shift of DCX+ neuronal precursors into the striatum is in accordance with numerous reports on SDF-1α being one of the key mediators for recruiting endogenous neuronal progenitors toward the lesion. Our finding also relates particularly well with one study where intraventricular infusion of AMD3100, the small molecular antagonist of SDF-1α receptor CXCR4, was performed after ischemic stroke injury [13]. In this event of disrupted SDF-1α/CXCR4 signaling, more DCX+ neuronal precursors stayed in the SVZ and less of them migrated into the striatum. The distribution shift of DCX+ neuronal progenitors toward the striatum has previously been achieved by other strategies. One
strategy involved the systemic administration of SDF-1β with the notion that SDF-1β would permeate through the leaky blood-brain-barrier of newly formed vessels in the peri-lesion tissue and recruit DCX⁺ neuronal progenitors [32]. Another strategy involved the injection of hepatocyte growth factor-loaded gelatin microspheres into the peri-lesion tissue [33]. Our study demonstrates that using Gtn-HPA/SDF-1α-PCN matrices to fill brain lesions can also be a comparably effective strategy to shift the distribution of DCX⁺ neuronal precursors toward the striatum.

By far, findings from multiple studies have been strongly indicative of DCX⁺ neuronal precursors being beneficial after brain injury. Their differentiation can be specific to the type of neurons found in the striatum. The newly generated neurons can further form functional synapses with neighboring neurons and integrate functionally into local circuit. They have also been associated repeatedly with improvements in functional behavior. Highly specific ablation of DCX⁺ neuronal precursors via transgenic manipulation is further shown to reduce spontaneous behavioral recovery after ischemic stroke [34] and traumatic brain injury [35]. Channeling a higher proportion of DCX⁺ neuronal precursors into the injured striatum with the use of Gtn-HPA/SDF-1α matrices may therefore be a way to optimize the benefits of neurogenic responses after every brain injury.

Despite the observed distribution shift, the presence of the DCX⁺ neuronal precursors in the striatum did not extend significantly within close proximity of the implanted Gtn-HPA/SDF-1α-PCN matrices. We also found very few BrdU⁺/NeuN⁺ mature neurons in the striatum near the SVZ and within the implanted Gtn-HPA/SDF-1α-PCN matrices. Collectively, these observations suggest that even with Gtn-HPA/SDF-1α-PCN matrices to augment SDF-1α release, the endogenous neurogenic response remains constrained by several other limitations. These limitations may include inefficient differentiation of the DCX⁺ neuronal progenitors into NeuN-expressing mature neurons, high death rate and limited motility within the injured brain.

Gtn-HPA/SDF-1α-PCN matrices were also found to exert a prominent effect on neovascularization around lesion. In our study, the vasculature in all the uninjured contralateral hemispheres measured to be ~ 2 mm per mm², which put every cell to be within 250μm from a blood vessel and was reasonably consistent with the oxygen diffusion limit of 200μm in living
tissues [36]. In agreement with previous reports on injury-triggered neovascularization in the brain, we found vessel length density to 3.4 mm per mm² around the lesion after ICH injury. Gtn-HPA/SDF-1α-PCN matrices significantly amplified this injury-triggered neovascularization, the vessel length density around the lesion further to 4.9 mm per mm². Gtn-HPA and Gtn-HPA/PCN matrices did not produce such an amplificatory effect, attesting that the observed amplification of injury-triggered neovascularization with Gtn-HPA/SDF-1α-PCN matrices was primarily driven by SDF-1α PCNs and not due to the physical effect of an implanted matrix or the bioactivity of Gtn-HPA leading to expression of angiogenic or vasculogenic factors in the peri-lesion tissue. The ability of SDF-1α PCNs to potently stimulate neovascularization in our study was likely derived from the known effects of SDF-1α on angiogenesis and vasculogenesis. SDF-1α has been reported to boost the proliferation of endothelial cells [37], increase their survival, guide their migration via chemoattraction [38] and regulate their ECM-dependent branching morphogenesis [39], thus orchestrating an overall enhancement in angiogenesis. On the other hand, SDF-1α also actively recruits endothelial progenitor cells [40] that has been known to circulate in the peripheral blood in greater numbers after brain injuries [41], attenuates their apoptosis [42] and helps to drive vasculogenesis in the injured brain [43]. Neovascularization has been regarded to be critically important in injured brain tissues. In ischemic injuries, neovascularization plays the vital role of restoring perfusion to match physiological requirements and salvaging underperfused brain tissues. In the non-ischemic context as was the case in the current study, neovascularization has been postulated to serve the role of improving metabolite delivery to injured neurons [19] as well as follow the “clean-up” hypothesis of facilitating macrophage infiltration for the removal of necrotic brain tissue.

The above findings validate our hypotheses that implanted Gtn-HPA/SDF-1α-PCN matrices can enhance endogenous regenerative responses in the brain tissue surrounding the lesion. A recurring theme has been that endogenous regenerative responses (along with behavioral recovery) occurred spontaneously after brain injury but intensified/accelerated with in the presence of Gtn-HPA/SDF-1α-PCN matrices. This verifies the presumption underlying our hypotheses that endogenous regenerative processes usually unfold in a sub-optimal manner due to various barriers and typically fail to reach their full potential without intervention. The implication of this is that more emphasis should be placed to identify barriers that curtail endogenous regenerative responses and to develop tools to address these barriers as part of an
overall strategy to maximize the self-healing capacity of the brain. Several other plausible barriers have already been noted over our assessment of the endogenous neurogenic responses with Gtn-HPA/SDF-1α-PCN matrices. They include: 1) failure of endogenous neuronal precursors to give rise to significant numbers of new mature neurons as a possible consequence of inefficient differentiation or high death rate and; 2) failure of endogenous neuronal precursors to migrate sufficiently far into the injured striatum prior to their disappearance. To further optimize the endogenous neurogenic response, it is of great interest to retrofit Gtn-HPA/SDF-1α-PCN matrices with additional proteins/drugs to address these deficiencies. The proteins/drugs may include: 1) mitogens such as transforming growth factor-alpha (TGF-α) to enlarge the pool of endogenous neuronal progenitors [44, 45]; 2) caspase inhibitors to reduce their apoptosis and increase their survival [13] and; 3) neurotrophins such as brain-derived neutrophilic factor (BDNF) to enhance their differentiation [46] and motility [47].

Another recurring theme is that although Gtn-HPA and Gtn-HPA/PCN matrices were on par with Gtn-HPA/SDF-1α-PCN matrices in serving the role as a physical structure that interfaced tightly with the surrounding host tissue and restored the stromal volume of the injured brain, they did not yield any appreciable improvement in all other measured outcomes. This is a significant departure from the experience in other tissues (e.g. skin and bone) where implanted matrices themselves can induce appreciable regenerative effects, and is expectedly so given that all processes are more strictly regulated in the brain. This is also in line with reports where matrices without instructive cues remained as relatively inert physical structures in the injured brain [48]. Instructive cues, such as SDF-1α PCNs in this case, are therefore indispensable and should be appropriately included in the design of matrices for the injured brain.

The other hypothesis of this study is that Gtn-HPA/SDF-1α-PCN matrices, when implanted into brain lesions, can re-establish a physical framework to enable recruited endogenous cells to migrate into the brain lesion. To associate with our findings on endogenous regenerative responses in the surrounding brain tissue, we first examined for the presence of DCX⁺ neuronal precursors within the lesion/implanted matrices. In our observation, a very small number of DCX⁺ neuronal precursors successfully migrated into the implanted Gtn-HPA/SDF-1α-PCN matrices when none did in the case of cavitary lesion, non-cavitary lesion as well as Gtn-HPA and Gtn-HPA/PCN matrices, presumably because of the absence of structure, the presence
of an unfavorable environment and lack of instructive cues respectively. Although this is a promising proof-of-concept that the lesion can be restructured to promote the infiltration of neuronal progenitors, it is clear that Gtn-HPA/SDF-1α-PCN matrices must be combined with other strategies to sufficiently amplify this migration in order to achieve meaningful repopulation of brain lesions with endogenous neuronal precursors.

In addition to DCX+ neuronal progenitors, we observed the presence of vasculature into brain lesions filled with Gtn-HPA/SDF-1α-PCN matrices. The newly constructed vasculature was an evident improvement over the absence of blood vessels in lesions that were cavitary in nature or filled with Gtn-HPA and Gtn-HPA/PCN matrices. Despite being less developed than vasculature in non-cavitary lesion in terms of vessel length density, Gtn-HPA/SDF-1α-PCN matrices were completely acellular and avascularized at the point of implantation and were clearly engaging in an active process of vascular construction over time. In general, revascularization does not occur spontaneously in brain lesions (except those which are physically connected to the meninges and can be filled with a fibrotic scar tissue after meningeal fibroblasts intrude the lesion and secrete ECM [49]) after the clearance of necrotic tissues and the associated blood vessels. Yet, blood vessels constitute a fundamental pre-requisite for regeneration of neural tissue in the lesions, since the oxygen and metabolic demands of endogenous or transplanted cells have to be met for them to survive and amount to any regenerative effect. Blood vessels may also be necessary promoters of neuroregeneration given their roles as conduits for blood-borne progenitors to enter the lesion and cellular scaffolds to guide migration of neural cells [32, 50-52]. With these in mind, the use of Gtn-HPA/SDF-1α-PCN matrices to reconstruct vasculature of brain lesion is therefore a significant advancement toward cell/tissue reconstitution in brain lesions. The reconstruction of vasculature in a biomaterial-filled brain lesion has previously been demonstrated with a preformed collagen scaffold carrying soluble Nogo receptor [53] and in unpublished work by a laboratory in Clemson University and. The trauma-induced lesions in these contexts were either open wounds or cortical surface wounds that remained in close contact with the meninges whose fibroblasts and vasculature might facilitate the growth of blood vessels into the implanted biomaterial. In this study, we demonstrated that vasculature could also be constructed in a deeply seated brain lesion completely surrounded by neural tissues with the use of Gtn-HPA/SDF-1α-PCN matrices.
One major finding from our examination of the implanted Gtn-HPA/SDF-1α-PCN matrices is the significant presence of neutrophils. As short-lived cells that last 5 – 6 days, neutrophils typically participate a rapid inflammatory response toward any injury or introduction of foreign material and undergo spontaneous apoptosis [54]. Their existence in the Gtn-HPA/SDF-1α-PCN matrices 2 weeks after matrix implantation or 4 weeks after the ICH injury, along with their absence in aCSF-treated lesions, therefore indicated the presence of an underlying stimulus that continuously recruited neutrophils. One possible stimulus could be persisting necrosis in the host tissue as a result of deleterious effects arising from the implanted Gtn-HPA/SDF-1α-PCN matrices. Our examination, however, revealed that the surrounding host tissue remained tightly interfaced with the implanted matrices and showed no signs of cell/tissue necrosis. The presence of neutrophils was also predominantly localized within the Gtn-HPA/SDF-1α-PCN matrices instead of spreading into the surrounding host tissue. On this note, the stimulus was likely to be PCNs given that: 1) implanted Gtn-HPA matrices in our study did not elicit similar neutrophil response and; 2) Gtn-HPA/PCN elicited a highly similar neutrophilic response as Gtn-HPA/SDF-1α-PCN matrices. In our study, the infiltrated neutrophils were stained positively for CXCR4 even in the absence of cell permeabilization. The CXCR4 staining also displayed a punctate pattern around the perimeter of each positively stained cell and was characteristic of receptors expressed on cell surface. In addition, they were vastly positive for the angiogenic factor, VEGF.

The presence of neutrophils has been classically understood to be detrimental for the injured brain. Upon being recruited into a site of inflammation, neutrophils release reactive oxygen species, proteases and pro-inflammatory cytokines, all of which either adversely affect the neural cells and blood-brain-barrier or further intensify the inflammatory process [55]. Several studies also show that neutrophil depletion after brain injury can be beneficial in terms of reducing tissue loss [56] and behavioral impairments [57]. On the other hand, there has emerged data that shows neutrophils to exhibit different phenotypes and functions instead of being a homogenous population devoted only to driving deleterious acute inflammation. For instance, neutrophils are the only cell type to secrete MMP-9 without its endogenous inhibitor, tissue inhibitor of metalloproteinases-1 [58]. Through the use of MMP-9 to remodel ECM and release sequestered angiogenic factors, neutrophils have been noted to mediate VEGF-induced angiogenesis in the injured brain [59]. In another example, CD11b+/Gr-1+/CXCR4hi neutrophils
played the critical role of secreting MMP-9 to enable proper revascularization of transplanted pancreatic islets in a mouse model [60]. Our study, together with several others [61, 62], also found neutrophils to express VEGF. Such VEGF expression has been found to be vital in orchestrating physiological angiogenesis in endometrial tissue [63], pathological angiogenesis in tumors [64] as well as nerve regeneration in the cornea [65]. Finally, neutrophils infiltrating the eye after optic nerve injury expressed high levels of oncomodulin and were found to represent an essential innate immune response to reverse axonal growth inhibition in the CNS and allow lengthy axons to regenerate through the optic nerve [66].

The observed neutrophil accumulation has several implications on the use of Gtn-HPA/SDF-1α-PCN matrices in the injured brain. It is of interest to investigate the neutrophils as an endogenous response that may contribute toward neuroregeneration and neovascularization, akin to what has been observed in other studies [63, 66]. In our study, the neutrophil accumulation alone in rats treated with Gtn-HPA/PCN matrices did not appear to bear any positive influence on the injured brain at 2 weeks after treatment but should be monitored over a longer period of time to fully determine the effects of the neutrophils. It is also pertinent to characterize the cytokine profile of the neutrophils. If the expression of pro-inflammatory cytokines such as TNF-α and IFN-γ remains persistently high, Gtn-HPA/SDF-1α-PCN matrices should be retooled to deliver SDF-1α without the use of PCNs.

Apart from those discussed above, Gtn-HPA/SDF-1α-PCN matrices do not address other aspects of the complex environment in the injured brain by nature of their intent and design. One such aspect is the presence of growth inhibitors such as Nogo-A, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, ephrin B3 and semaphorin 4D. Although not specifically measured in this study, these growth inhibitors might be presumed to be prevalent around the lesion given their robust production after brain injury [67]. The growth inhibitors might attribute to the absence of axonal projections from host neurons into the implanted Gtn-HPA/SDF-1α-PCN matrices. They might also adversely affect the survival and functions of the recruited neuronal progenitors and the newly differentiated neurons.

Gtn-HPA/SDF-1α matrices also do not address astrogliosis around the brain lesion. As a defense mechanism against brain injury, astrogliosis involves massive proliferation of astrocytes followed
by their activation into the reactive state. The reactive astrocytes then participate in the formation of a glial scar where their endfeet is stacked against the edge of the lesion and tightly woven with gap and tight junctions to form a highly impermeable barrier around the lesion [68]. This cellular barrier serves the beneficial role of reducing the spread of excitotoxicity, oxidative stress and inflammation [69] but is inhibitory in terms of axonal regeneration [67]. In our study, Gtn-HPA/SDF-1α-PCN matrices did not intensify or dampen the presence of reactive astrocytes in the peri-lesion tissues. Neither did Gtn-HPA/SDF-1α-PCN matrices induce the migration of the reactive astrocytes into the lesion to result in some disruption or disentanglement of the tightly woven cellular barrier. Although migration of cells across the glial scar is not completely ruled out (given reports of transplanted cells crossing the boundary of a chronic lesion into the host tissue [70, 71] and our observation of a few endogenous neuronal progenitors crossing the lesion boundary and migrating into the implanted Gtn-HPA/SDF-1α-PCN matrices), it is reasonable to expect that the glial scar is highly obstructive toward migrating cells and has to be appropriately tackled to facilitate substantial in-growth of neural cells into the lesion.

The third aspect is the accumulation of macrophages/microglia. In the event of brain injury, blood-borne monocytes infiltrate the lesion and differentiate into macrophages while microglia, the resident macrophages of the brain, migrate into the lesion. Being highly phagocytotic cells, they serve the critical role of clearing cell/tissue debris to reduce secondary damage and prime the injured brain for repair/regeneration. Given that monocytes/macrophages/microglia share the SDF-1α/CXCR4 signaling pathway [72, 73], Gtn-HPA/SDF-1α-PCN matrices were poised to chemoattract them despite it not being the primary intent of the matrices in this study. In our observations, Gtn-HPA/SDF-1α-PCN matrices did not increase the accumulation of macrophages/microglia in the peri-lesion tissues beyond the level induced by ICH injury, presumably because the injury-induced accumulation was already sufficiently robust. Lesions filled with Gtn-HPA/SDF-1α-PCN matrices did, however, contain more macrophages/microglia than cavitary lesions and lesions filled with Gtn-HPA matrices, although they did not approach the level seen in the non-cavitary lesions where the necrotic-like tissue was densely packed with macrophages/microglia. To appreciate the significance of these cells, the key issue, as recent studies of the role of macrophage/microglia in the CNS have pointed out, is less about the number but more about the polarization state of these cells. Macrophages/microglia of the M1 state are pro-inflammatory and drive inflammation with their
secretion of TNF-α and IL-12 while those of the M2 state are immunoregulatory or pro-regenerative and can secrete beneficial cytokines such as IL-10, VEGF and FGF [74]. With a study showing effects of SDF-1α of M2 polarization [75], it is of interest to examine in the future if Gtn-HPA/SDF-1α-PCN matrices, in their current form or with additional cytokines/agents, can be a tool to drive M2 polarization of macrophages/microglia to achieve additional regenerative effects after brain injury.

In summation, implantation of Gtn-HPA/SDF-1α-PCN matrices within brain lesions was an effective strategy to increase migration of endogenous neuronal progenitors into the injured striatum and boost neovascularization in the surrounding tissues. The use of Gtn-HPA/SDF-1α-PCN matrices also led to the reconstruction of vasculature within brain lesions and heavy infiltration of neutrophils. While the newly constructed vasculature was valuable for potential cell/tissue reconstitution of the brain lesion, the neutrophils did not seem to impose detrimental inflammatory effects and compelled further investigation to verify their role in neuroregeneration. Most importantly, Gtn-HPA/SDF-1α-PCN matrices proved to be a tangibly useful treatment for the injured brain in terms of reducing tissue loss and enhancing recovery in functional behaviors that has relevance to quality of life after brain injury.
5.5 References


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Chapter 6

Conclusions and Future Work

In this thesis, we developed a lesion-filling matrix to address the loss of chemokine expression and the disappearance of stroma within brain lesions. The underlying hypotheses were that when implanted within brain lesions, the matrix would: 1) provide chemokine release and enhance endogenous regenerative responses in the surrounding brain tissues and; 2) re-establish a physical framework to enable recruited endogenous cells to migrate into the brain lesions. The developed matrix was eventually evaluated in a deep-seated brain lesion in a rat model of collagenase-induced ICH.

Development of the matrix began with the \textit{in vitro} characterization of Gtn-HPA hydrogels. These hydrogels were selected for their unique crosslinking chemistry that allowed them to be injectable, covalently crosslinked \textit{in situ} and thus excellent candidate materials for implantation into brain lesions. Adult neural progenitor cells (NPCs) were cultured within the Gtn-HPA hydrogels as an approximation of the context where endogenous neural stem and progenitor cells entered the Gtn-HPA hydrogels that were used to fill the brain lesions. In our findings, the adult NPCs within the hydrogels retained their ability to proliferate in response to the mitogen FGF-2, undergo migration to different positions and differentiate into neurons and astrocytes when stimulated with the differentiation cues. The importance of proliferation, migration and differentiation in regeneration and the demonstrated ability of Gtn-HPA hydrogels to provide favorable environments for these processes collectively supported the use of Gtn-HPA hydrogels as the building block for providing appropriate stroma.

The next phase of matrix development centered on the synthesis of DS/CS polyelectrolyte complex nanoparticles (PCNs) to achieve the functionality of SDF-1α release. SDF-1α was the chemokine of choice due to its widely reported involvement in driving numerous endogenous regenerative responses. PCNs displayed great compatibility with Gtn-HPA hydrogels and could be incorporated readily without compromising the integrity of the hydrogels. At the DS:CS:SDF-1α ratio of 1:0.33:0.2, encapsulation efficiency was over 80%, demonstrating PCNs to be a practical delivery vehicle that minimized the loss of the costly chemokine. Loading efficiency also reached ~ 20 wt%, indicating that the mass of PCNs needed to deliver a
therapeutic dose of SDF-1α could remain within 5 times of the mass of SDF-1α. Most importantly, SDF-1α PCNs sustained the release of SDF-1α over 4 weeks and proved effective as the second building block for providing chemokine release.

The resulting Gtn-HPA/SDF-1α-PCN matrix was evaluated in an in vitro three-dimensional migration assay where it was used to fill a core region that was surrounded by a NPC-laden hydrogel construct (akin to its eventual in vivo application of filling a brain lesion that was surrounded by brain tissue containing endogenous cells). In this assay, Gtn-HPA/SDF-1α-PCN matrix significantly enhanced accumulation of NPCs around the core region as well as migration of NPCs into the core region when compared to Gtn-HPA and Gtn-HPA/PCN matrices, therefore demonstrating its ability to achieve the objectives of providing: i) release of bioactive SDF-1α to recruit cells and; ii) appropriate stroma to support migration of recruited cells. Specifically for the latter, Gtn-HPA/SDF-1α-PCN matrix displayed the capacity to maintain physical continuity with the apposing hydrogel construct and enabled recruited cells to successfully migrate across the interface. Gtn-HPA/SDF-1α-PCN matrix also greatly enhanced the accumulation and migration of NPCs relative to Gtn-HPA/soluble SDF-1α, thus proving to be the necessary formulation for releasing SDF-1α. The use of CXCR4 inhibitor and soluble SDF-1α to disrupt concentration gradients showed the Gtn-HPA/SDF-1α-PCN matrix to act specifically on the SDF-1α/CXCR4 pathway and to actively recruit NPCs through the chemotactic effects of SDF-1α as opposed to merely increasing the chemokinesis of the surrounding NPCs. On the other hand, the use of specific MMP inhibitors established that Gtn-HPA/SDF-1α-PCN matrix supported MMP-2 and MMP-9-mediated migration and was poised to be a highly compatible stroma in the injured brain where endogenous cells commonly employ MMP-2 and MMP-9 to migrate.

In the transition to in vivo experimentation, the emphasis for the developed matrix to be injectable had proven to be particularly valuable. Gtn-HPA/SDF-1α-PCN matrix was successfully implanted into a deep-seated brain lesion in an ICH model with the use of a needle-based injection procedure that caused minimal morbidity on the overlying cortical tissue. The in situ crosslinking was also useful in enabling Gtn-HPA/SDF-1α-PCN matrix to conform to the irregularly shaped lesion and establish tight interface with the surrounding brain tissue. When optimized to contain 8wt% Gtn-HPA, the lesion-filling Gtn-HPA/SDF-1α-PCN matrix persisted for at least 2 weeks after implantation, maintained the tight interface with surrounding host tissue and was capable of support infiltration of endogenous cells.
In the *in vivo* study where our hypotheses were evaluated in a rat ICH model, Gtn-HPA/SDF-1α-PCN matrix did not increase the overall scale of injury-induced neurogenesis as compared to aCSF and Gtn-HPA matrix, but enhanced the response by shifting the distribution of endogenous neuronal progenitors/immature neurons toward the injured striatum. Gtn-HPA/SDF-1α-PCN matrix also amplified injury-induced neovascularization around the lesion and led to the construction of a new vasculature within the lesion. These verified our hypotheses that by re-establishing chemokine release and stroma within the lesion, the lesion-filling matrix could enhance endogenous regenerative responses and enable migration of endogenous cells into the lesion. Interestingly, the endogenous cells in Gtn-HPA/SDF-1α-PCN matrix also included a sizeable population of neutrophils. If found to have similar angiogenic roles as neutrophils in other tissues, they could represent a novel endogenous response that would be useful for reconstructing vasculature in matrix-filled brain lesions. Amidst these significant effects, Gtn-HPA/SDF-1α-PCN matrix did not have any influence on the number of newly generated mature neurons in the striatum, the injury-induced gliosis and accumulation of activated macrophages/microglia. Finally, Gtn-HPA/SDF-1α-PCN matrix resulted in behavioral improvements and reduced tissue loss. This marked Gtn-HPA/SDF-1α-PCN matrix as a potentially beneficial treatment for the injured brain after ICH injury.

Overall, this thesis has followed the development of an injectable brain lesion-filling matrix, the *in vitro* demonstration of its capability to provide chemokine release and stromal support and the *in vivo* validation of its ability to enhance endogenous regenerative responses. Even with these advancements, the work on developing highly effective lesion-filling matrices, maximizing the potential of endogenous regenerative responses and inducing neuroregeneration in general in the injured brain is clearly far from over. The course of pursuing this thesis has revealed several specific directions that may be considered in the future.
Future work related to the development and use of lesion-filling matrices

- The ideal brain lesion-filling matrix is expected to be macroscopically persistent to prevent collapse of the surrounding brain tissue, stably anchor delivery vehicles (such as nanoparticles) and importantly, remain as a physical framework until new stroma is produced. It is also expected to permit microscopic cell-mediated degradation that usually precedes cell migration. For a homogeneous material such as Gtn-HPA/SDF-1\(\alpha\)-PCN matrix, these requirements create a sandwiching constraint where only a narrow range of formulations may be applicable. This constraint may be removed if the modular design approach extends to different roles of the lesion-filling matrix i.e. developing a heterogeneous matrix where different components address the macroscopic and microscopic roles separately. Gtn-HPA hydrogels may be employed with injectable macroscopic structures such as cryogels [1] or short electrospun fibers [2] to achieve the desirable scenario where Gtn-HPA hydrogels provides the modality to conform to irregularly shaped lesion and establish tight interface with surrounding brain tissue while the macroscopic structures provide the macroscopic persistence.

- The volume and location of lesions are consistent in the collagenase-induced induced model and allow for the injection of Gtn-HPA/SDF-1\(\alpha\)-PCN matrix to be sufficiently guided by previous histological findings and the stereotaxic coordinates of the prior collagenase injection. For other forms of brain injury (e.g. ischemic strokes in a sub-cortical region) where the lesion size and location are relatively more variable, an imaging modality such as magnetic resonance imaging (MRI) will be necessary to ensure an appropriate volume of matrix and accurate deposition of matrix within the lesion.

- For greater relevance to current clinical management of ICH, it is pertinent to combine the implantation of Gtn-HPA/SDF-1\(\alpha\)-PCN matrix with a preceding procedure to aspirate the hematoma. The aspiration of hematoma can be performed with or without clot-lysing drugs such as streptokinase [3, 4] and can enable Gtn-HPA/SDF-1\(\alpha\)-PCN matrix to be implanted acutely after the ICH injury.
Future work related to maximizing endogenous regenerative responses in the injured brain

- The number, motility, differentiation and survival of neuronal progenitors and immature neurons are other aspects of injury-induced neurogenesis that need to be dealt with. As mentioned previously, Gtn-HPA/SDF-1α-PCN matrix can be further exploited to deliver potent mitogens (e.g. EGF, EPO and TGF-α), motility-enhancing factors (e.g. BDNF [5]), differentiating cues (ephrin-B2 [6] and retinoic acid [7]) and pro-survival factors (e.g. [8]) to increase the robustness of injury-induced neurogenesis.

- The presence of neutrophils within the implanted Gtn-HPA/SDF-1α-PCN matrix compels further investigation. First, neutrophil depletion should be performed to examine if the infiltrating neutrophils contribute to any enhancements in endogenous regenerative responses in the surrounding brain tissue. Second, the implanted Gtn-HPA/SDF-1α-PCN matrix can be retrieved and digested to isolate the neutrophil infiltrates and analyze their cytokine expression profiles using RT-PCR and FACS. This will determine whether the neutrophils display a pro-inflammatory or pro-regenerative phenotype. Third, the Gtn-HPA/SDF-1α-PCN matrix should be evaluated at a later time point (e.g. 4 and 6 weeks) to examine if the presence of neutrophil is transient or persistent. These future experiments will elucidate the roles and consequences of the neutrophil infiltrates and clarify whether the neutrophil infiltrate should be pursued as another beneficial endogenous regenerative response.
Future work related to inducing neuroregeneration in the injured brain

- Along the course of characterizing Gtn-HPA hydrogels as an appropriate matrix for adult neural progenitors, Gtn-HPA hydrogels were found to increase the oxidative stress resistance of encapsulated neural progenitors. Further investigation is needed to determine the exact pathway/defense mechanism involved in the heightened oxidative stress resistance, and to verify if gene and protein expression profiles of the encapsulated neural progenitors remain comparable to their native profiles.

- Gtn-HPA hydrogels exhibit high cytocompatibility with neural progenitors and are appealing injectable matrices that can be implanted into brain lesions in a minimally invasive manner. It is of interest to employ Gtn-HPA hydrogels as a carrier matrix for transplanting neural progenitors into brain lesions. Investigation should focus on whether the Gtn-HPA hydrogels provide a supporting framework for the transplanted neural progenitors to occupy the lesion and if they increase the survival of the transplanted cells, either by preventing anoikis or by boosting their resistance against the highly oxidative environment in the injured brain.
References


